Gene Expression Analysis in Neurons throughout Late-Onset Alzheimer’s Disease Pathological Progression

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Abstract

Over 20 susceptibility loci for late-onset Alzheimer’s disease (LOAD) have been identified in large-scale genome-wide association studies (GWAS), and several past studies have found differences in gene expression between normal and AD brain tissue. Several limitations exist in this previous research, including the use of whole brain tissue and comparing control brain tissue to AD brain tissue, which provides minimal knowledge about which genes play a critical role in the early stages of the disease. To overcome these limitations, the aims of this study were: (1) to develop and optimize a method to isolate single cells from frozen brain tissue while preserving RNA for downstream gene expression analysis and (2) to utilize this method to analyze gene expression in neurons over the course of LOAD pathological progression. We combined immunohistochemistry with laser capture microdissection (LCM) to collect single neurons from normal, mild-cognitive impairment (MCI), mild AD, and severe AD frozen human temporal cortex tissues. Gene expression was determined using the NanoString nCounter Single Cell gene expression assay. Analyzed samples showed at least 10-fold neuronal enrichment, validating the collection of homogenous pools of neurons. Our results confirm that for many LOAD-associated genes, mRNA levels indeed vary throughout disease progression. Notably, APOE mRNA levels were found to increase throughout LOAD pathological progression, while APP mRNA levels were elevated in the AD samples but not in the MCI samples. These results suggest that regulation of APOE may contribute to the development of LOAD while APP regulation may become altered only once the disease has progressed. This endeavor diverges from past studies by examining a single cell type (neurons) as opposed to whole brain tissue, allowing us to gain accuracy and specificity in identifying target genes. Moreover, findings in MCI tissue implicate the expression regulation of critical genes in playing a part in the early stages of disease, suggesting a role in causing LOAD.
**Introduction**

Alzheimer’s disease (AD) is the most common form of dementia and is the sixth leading cause of death in the United States (Alzheimer’s Association, 2017). As of 2017, approximately 5.5 million Americans suffer from the disease with this number projected to triple by 2050 without improved treatment. The high prevalence of AD is especially alarming when considering the particularly demoralizing symptoms of the disease, which include memory loss, confusion about place and time, the inability to perform simple tasks or responsibilities, difficulty solving problems, a change in personality, withdrawal from social activities, and anxiety. Unfortunately, the devastating effects of AD also reach beyond the individual to impact family, friends, and society as whole. In 2016, approximately 18.2 billion hours of unpaid care valued at $230.1 billion were given to AD and dementia patients by friends or family members, and many private and public dollars are spent each year helping those with AD, with Alzheimer’s and other forms of dementia costing the nation as much as $259 billion in 2017 and Medicare and Medicaid covering approximately 67% of that cost. Despite its significant burden on individuals and society, much is still unknown about the etiology of Alzheimer’s disease, and a cure is yet to be found. (Alzheimer’s Association, 2017)

Alzheimer’s disease is named after Dr. Alois Alzheimer, who treated and studied a female patient named Auguste D. from 1901-1906 for symptoms that included progressive loss of memory, confusion, paranoia, and personality changes (Hippius & Neundörfer, 2003). Auguste D. died in 1906, and in examining her brain, Dr. Alzheimer discovered two hallmark aberrant features of AD that are now known as extracellular amyloid beta (Aβ) plaques and intracellular neurofibrillary tangles (NFTs; consisting of hyperphosphorylated tau) (Hippius & Neundörfer, 2003). AD is also characterized neuropathologically by degeneration and loss of synaptic connections that originate in the medial temporal lobe, specifically the hippocampus and entorhinal cortex, before spreading throughout parietal areas and finally the frontal cortex, culminating in extreme overall atrophy of the brain (Korolev, 2014). Clinically, probable AD dementia is diagnosed by considering medical history and neuropsychological examinations, and although AD is fundamentally a clinical diagnosis, cerebrospinal fluid biomarkers and neuroimaging can increase certainty of diagnosis or help exclude other causes of disease (Korolev, 2014; McKhann et al., 2011). AD is definitively diagnosed with post-mortem identification of Aβ plaques and NFTs following a hierarchical manner (Thal & Braak, 2005).
The Genetics of Early-Onset Familial AD

Family history is the second most important risk factor for developing AD, after age (Chouraki & Seshadri, 2014). For example, researchers found in an examination of 1,694 families that risk of disease was 39% for first-degree relatives of AD patients and thus approximately twice the incidence rate of the general population (Lautenschlager et al., 1996). These and other findings reinforced the heritable nature of AD and consequently led researchers to conclude that genetics plays a critical role in the disease.

In the early 1980s, a connection was made between Down syndrome (DS) and AD following the realization that both AD patients and persons with DS share in common abnormal accumulations of Aβ peptides within the brain (Glenner & Wong, 1984). Moreover, people with DS are much more likely to develop early-onset Alzheimer’s disease (EOAD), and it is estimated that the disease arises in approximately two-thirds of people with DS by the age of 60 (McCarron, 2014). Drawing on the knowledge that DS occurs as a result of trisomy of chromosome 21, researchers hypothesized that a genetic defect localized to chromosome 21 could be at least partially responsible for both AD and DS (Glenner & Wong, 1984). This hypothesis was confirmed by applying genetic linkage analysis to pedigrees suffering from familial EOAD to find a linkage peak on chromosome 21 (St George-Hyslop et al., 1987) and was further bolstered when the gene encoding the amyloid peptide was localized to chromosome 21 using in-situ chromosome hybridization (Robakis et al., 1987). By screening families afflicted with EOAD, a point mutation was eventually found on chromosome 21 in APP (amyloid precursor protein) (Goate et al., 1991), the gene encoding the protein that is cleaved to generate β-amyloid, the primary component of the plaques associated with AD (Wiseman et al., 2015). From these discoveries, it was hypothesized that the triplication of APP is a key component of the development of EOAD in those with DS, a postulation that was augmented by experiments showing that adding an additional copy of the APP gene in the absence of trisomy of the rest of chromosome 21 was sufficient for EOAD to occur in mice (Wiseman et al., 2015).

Although a strong connection was formed between AD and APP by studying DS, it was noted that not all families suffering from EOAD showed linkage to the chromosome 21 marker, and thus EOAD was determined to be a genetically heterogeneous disease (Goate et al., 1991). Additional linkage studies of multi-generational pedigrees led to the discovery of two additional genomic loci associated with EOAD: PSEN1 (presenilin 1) and PSEN2 (presenilin 2) (Bertram,
Lill, & Tanzi, 2010). These two genes are highly associated with APP; APP is cleaved by β- and γ – secretases to produce β-amyloid, and the catalytic site of γ – secretase is encoded by PSEN1 and PSEN2 (Cole & Vassar, 2008). Rare mutations in these three genes, APP, PSEN1, and PSEN2, confer a high risk for developing AD, with PSEN1 accounting for the largest proportion of EOAD (Chouraki & Seshadri, 2014).

**The Genetics of Late-Onset AD**

*Apolipoprotein E (APOE)*

The genetic causes of late-onset Alzheimer’s disease (LOAD) are much more elusive and difficult to study than those of EOAD. Progress first arrived in 1991 when APOE immunoreactivity was discovered in amyloid plaques in AD brains (Namba, Tomonaga, Kawasaki, Otomo, & Ikeda, 1991). *APOE* encodes a lipoprotein involved in lipid metabolism in the central nervous system (Yu, Tan, & Hardy, 2014). In 1993, an association was found between the *APOE* ε4 allele and both late-onset familial AD and sporadic AD (Saunders et al., 1993), and moreover, *APOE* ε4 allele dose was found to be a major risk for LOAD, with homozygosity being sufficient to result in developing AD before the age of 80 (Corder et al., 1993). Studies and replications since 1993 have maintained *APOE* ε4 as being the most important risk factor for LOAD, however, about half of AD sufferers lack the *APOE* ε4 allele, and thus *APOE* ε4 is not necessary to cause LOAD nor is it sufficient to cause the disease; it is merely a risk factor (Chouraki & Seshadri, 2014). Thus, LOAD was acknowledged as a genetically complex disease, and the focus of research turned to identifying other genes associated with LOAD.

**Candidate Gene Approaches for LOAD**

The candidate gene approach is based on prior knowledge about the genetics of a disease and is used to determine whether or not an allele of a gene suspected in being involved with a disease is seen more frequently in diseased subjects than in non-diseased subjects (Kwon & Goate, 2000). These studies are thus hypothesis-based and are used to identify markers that are either causal variants or are in linkage disequilibrium with a causal variant (Chouraki & Seshadri, 2014). Rogaeva et al. used this approach to study variants in genes involved in endocytic pathways, which are implicated in APP re-uptake, recycling, and further processing. As a result, they found SNPs in *SORL1* that showed signification association with AD (Rogaeva
et al., 2007). Although the candidate gene approach led to the discovery of SORL1 risk variants, many variants identified could not be replicated, and such studies were not inherently useful for studying novel pathways since candidate genes were chosen based on well-known pathways or other sources of justification (Chouraki & Seshadri, 2014).

### Identifying LOAD-risk Variants Using Genome-Wide Association Studies

In recent years, the rise of the genome-wide association study (GWAS) has enabled identification of other genes associated with LOAD. GWAS allows for analysis of the entire genome, thus eliminating the need for identifying candidate genes and opening the door for novel discoveries not based on prior hypotheses. GWAS also detects common variants that may have small effect sizes, thus giving a broader view of all the genes involved in a disease.

Beginning in 2009, several studies were able to detect novel variants outside of APOE that were associated with AD, although the effect sizes of these genes were small compared to that of APOE (Harold et al., 2009; Hollingworth et al., 2011; Lambert et al., 2009; Lambert et al., 2013; Naj et al., 2011; Seshadri et al., 2010). Then, in 2013, a large-scale meta-analysis of previous GWA studies was conducted and successfully identified a set of 19 loci (20 with the addition of APOE) associated with LOAD, nine of which were corroborated by previous findings and 11 of which were newly identified (Table 1) (Lambert et al., 2013).

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*Genes located ± 100 kb of the top SNP (Lambert et al., 2013)
Identifying Rare LOAD-risk Variants

GWAS, a huge advancement in AD genetics research, enabled the identification of frequent variants associated with AD. Next, the study of complex diseases was furthered by the advent of whole exome sequencing (WES), a technological advancement that sequences exonic DNA to identify rarer, potentially highly penetrant variants. Using WES, studies have found rare mutations associated with AD in ADAM10, the gene encoding α-secretase which cleaves APP to prevent Aβ creation (Tanzi, 2013), MAPT, the gene encoding for tau (Coppola et al., 2012), and PLD3, a phospholipase gene (Cruchaga et al., 2014). Using WES combined with genotypic imputation, the rs75932628-T variant of the TREM2 gene, encoding triggering receptor expressed on myeloid cells 2, was also found to confer a higher risk of developing Alzheimer’s disease (Jonsson et al., 2013).

Expression GWAS and Functional Variants

The identification of common and rare LOAD-associated variants begs the question of how exactly these risk loci are involved in the etiology or pathogenesis of LOAD. The observation that multiple neurodegenerative diseases such as Parkinson’s disease and Alzheimer’s disease appear to develop from the over-deposition of proteins that are quantitatively abnormal but not qualitatively abnormal prompted the question of whether or not the underlying mechanisms of these diseases involve abnormal gene regulation (Singleton, Myers, & Hardy, 2004). To uncover the genuine risk genes at the loci identified in GWAS studies and to make inferences on their disease mechanisms, it is imperative to identify functional variants, or variants associated with both disease risk and modulation of gene expression, within these genes of interest. To that end, several expression-GWAS (eGWAS) studies have focused on expression-SNPS (eSNPs), single nucleotide polymorphisms that influence gene expression, and whether or not those eSNPs are associated with LOAD (Zou et al., 2012). For example, variants in the LOAD-associated genes CLU, MS4A4A, ABCA7 were found to correlate with increased expression of those genes in the temporal cortex (Allen et al., 2012). The authors of the study thus concluded that these variants might influence disease risk and pathophysiology through changes in expression. Furthermore, the length of an intronic poly-T region within the TOMM40 gene, encoding a channel-forming subunit of a translocase of the mitochondrial outer membrane, was found to modulate APOE and TOMM40 mRNA expression (Linnertz et al., 2014). VL homozygotes (VL=very long and refers to the number of T residues)
exhibited higher levels of APOE and TOMM40 mRNA. Finally, carriers of the minor alleles at SNPs rs7945931 and rs2298525 within SORL1 exhibited a 2-fold increase in SORL1- mRNA level within the temporal cortex of neurologically normal subjects (McCarthy et al., 2012). Variants that modulate gene expression levels thus exist within several LOAD-associated genes, suggesting that regulation and modulation of the expression of certain genes contributes to LOAD susceptibility.

**Gene Expression Analysis in Diseased and Control Brains**

As a continuation of the study of functional variants, several studies have analyzed differential gene expression between non-AD and AD individuals. For example, the expression of both APOE and TOMM40 were found to be significantly increased with disease (Linnertz et al., 2014), and researchers also found increased APP mRNA levels in AD brain samples compared to normal brain samples (Matsui et al., 2007). An analysis of genes related to neuroinflammation led to the discovery that CX3CL1 and TREM2 are expressed at different levels between control, mild AD, and severe AD stages. Finally, several studies have found differences in gene expression between different regions of the brain (McCarthy et al., 2012; Puthiyedth, Riveros, Berrett a, & Moscato, 2016; Strobel et al., 2015), which may provide insight into why certain regions of the brain are more susceptible to AD pathology.

Previous studies analyzing differential gene expression in control and AD brains offer important knowledge about how gene expression regulation relates to disease mechanism. However, several important limitations exist with these studies. One limitation of past studies is the absence of analysis of gene expression in brain tissue representing the intermediate stage between normal and AD states. Consequently, one pathological stage that is of great importance is mild cognitive impairment (MCI), which has been recently defined as the symptomatic pre-dementia stage between normal cognitive functioning and AD (Albert et al., 2011). Analyzing how gene expression levels are altered between normal and MCI brains can give insight into the early stages of the disease. Several studies have given merit to the MCI diagnosis. Besser et al, 2016, found that mild cognitive impairment leading to Parkinson’s disease with dementia (PD-MCI) is clinically distinct from mild cognitive impairment due to Alzheimer’s disease. Specifically, fewer PD-MCI patients developed dementia over one year as well as performed better than AD-MCI patients over one year in cognitive measure tests such as language, attention, and memory tests(Besser et al., 2016). We can therefore examine AD-MCI tissue as a
transition step between normal cognitive functioning and AD.

Another critical limitation of past studies is the use of whole brain tissue. Analyzing gene expression in heterogeneous populations of cells obscures important information about different gene regulatory patterns in dissimilar cell-types. Moreover, the importance of studying gene expression in specific cell-types is compounded by the fact that pathology in neurodegenerative diseases affects cell types in different ways and to varying degrees (Saxena & Caroni, 2011). Another reason for studying individual cell types is that many of the genes with LOAD-associated variants are found to be generally associated with at least one of three pathways: inflammatory, lipid metabolism and endocytosis (Rosenthal & Kamboh, 2014). Hence, one key approach to understanding potential pathways involved with LOAD pathology is to look at the expression profiles of genes at a cell-specific level, since different types of cells perform different tasks and are involved in different pathways.

To overcome the formerly discussed challenges, this study was designed with two aims: (1) to develop and optimize a method to isolate single cells from human frozen brain tissue while preserving RNA quality for gene expression analysis, and (2) to determine and characterize changes in LOAD-associated gene expression profiles across LOAD pathological stage. To accomplish these objectives and to facilitate a cell-specific approach to gene expression analysis, laser capture microdissection (LCM) was utilized. LCM is a technology that allows collection of discrete pieces of tissue and, importantly for this project, homogenous populations of cells. It can be combined with immunohistochemical staining to allow one to stain specific cell types and then cut and collect individual cells from a piece of tissue (Waller et al., 2012). In this project, neurons were collected utilizing the LCM approach in order to examine neuronal gene expression profiles specifically.

Gene expression analysis for this project was conducted using the nCounter single cell gene expression technology (NanoString). The nCounter gene expression is a digital based approach to measure mRNA expression and represents a recent advancement with many benefits (Veldman-Jones et al., 2015). The automated nCounter platform hybridizes fluorescently labeled probes, which are custom-designed and target-specific to genes of interest, directly to specific RNA sequences. The probes are then counted individually such that the exact number of transcripts for a specific gene in a sample is known. There are many benefits to single-count technology, such as the ability to assess many different targets from one sample. Moreover, this
technology negates the need for amplification by PCR and thus avoids bias. This allows for sensitive measurements for even degraded RNA. Studies have also shown that the sensitivity of target detection remains impressive even at very low input RNA amounts (Veldman-Jones, 2015).

Here, we incorporate the above-mentioned techniques of rapid immunohistochemical staining, laser capture microdissection, and nCounter single cell gene expression technology (NanoString) to analyze gene expression in neurons. We also use the novel approach of studying gene expression in MCI brain tissue in order to determine if the regulation of certain genes plays potentially causative roles in LOAD etiology. Thus, we will analyze expression levels of LOAD-associated genes in neurons from normal, MCI, mild AD, and severe AD samples. We hope that this will advance our understanding of how gene expression relates to molecular mechanisms involved in late-onset Alzheimer’s disease.
**Materials and Methods**

We optimized a technique to successfully isolate single cells from human frozen brain tissues utilizing immunohistochemistry and laser capture microdissection. This method allows us to obtain RNA of quality suitable for mRNA expression analysis. The principles and validation of the method were published in *Frontiers in Molecular Neuroscience* (Tagliafierro, Bonawitz, Glenn, & Chiba-Falek, 2016). Below, I describe these methods as they are applied to my project.

**Initial Sample Selection**

This project was approved by the Duke Institutional Review Board (IRB). Rapidly autopsied, frozen human temporal cortex tissues (small pieces of approximately 2 cm x 2 cm x 5 cm) were obtained from the Joseph and Kathleen Bryan Brain Bank (Duke University). From a database of all temporal cortex tissue available, 17 samples from cognitively normal donors, 17 samples from donors diagnosed with mild AD (Braak and Braak (B&B) scores of 3 or 4), and 17 samples from donors diagnosed with severe AD (B&B scores of 5 or 6) were selected. Samples in this initial sample cohort had post-mortem intervals of less than 16 hours, and all donors were Caucasian. All samples were *APOE* 33. Samples were matched by age and sex across pathological category. Additionally, 13 MCI samples were selected for an initial sample cohort for the ensuing quality control step. These samples were not selected based on any criteria, as there existed a limited amount of available MCI tissue.

**Sample Slide Preparation**

Microscope slides (VWR Micro Slides Superfrost® Plus White, USA, 48311-703) were cleaned with RNase zap (Ambion, USA, AM9780) and 100% ethanol (VWR, USA, 89125-188), decontaminated under UV light for 30 min, and then chilled at −20°C until usage. Brain tissue samples were broken into smaller sections (≤0.5 cm³) using a clean mortar and pestle chilled in dry ice. Next, the brain pieces were embedded within Optimal Cutting Temperature Compound (Sakura Finetek, CA, 4583) in disposable vinyl specimen cryomolds over dry ice. Embedded tissues were then held at −80°C. After 24 h, tissues from the molded blocks were sliced and mounted onto the pre-cleaned microscope slides. Samples were sliced into 8 µm sections using the Microm HM 505 N Cryostat at −20°C utilizing High Profile Microtome Blades (Leica, USA, 1115454). Slides with brain tissue slices were stored at −80°C. All steps were performed under RNase-free conditions.
RNA Extraction and RIN Analysis

A whole-tissue collection step was performed to assess whether high quality RNA can be preserved from each brain tissue sample post-slide preparation. RNA samples were extracted from the contents of the entire slide (one slice of brain tissue). Each tissue section on the slide was dissolved using 100 µL of lysis buffer from the Arcturus PicoPure RNA Isolation Kit (Applied Biosystems, USA, 12204-01) and gently scraped from the slide surface. RNA was extracted following the manufacturer's protocol along with the addition of DNase I treatment for 15 minutes at room temperature (Qiagen, DE, 79254). The quantity of RNA was analyzed using a NanoDrop 8000 UV-Vis spectrophotometer (Thermoscientific, UK, ND-8000). The quality of the RNA was assessed on a 2100 Bioanalyzer (Agilent, CA, G2943CA) using the RNA 6000 Pico Chip (Agilent, USA, 5067-1513). Samples above the upper limit of the qualitative range for the Agilent RNA 6000 Pico Chip (50–5000 pg/µl), as determined by the NanoDrop results, were diluted 20-fold with elution buffer before RNA analysis.

Quality Control and Final Sample Cohort Selection

Forty-three samples were selected for the final sample cohort. 12 cognitively normal, 12 mild AD, and 12 severe AD samples were chosen, with 6 females and 6 males in each pathological category. All samples within these three categories had PMIs of less than 12.2 hours (except one sample with a PMI of 15.75 hours) and had RIN values >4.8, post-mounting and slicing. Samples were matched by age and sex across pathological stage. Five female MCI samples and two male MCI samples were chosen for the final cohort. Due to the limited amount of MCI samples to choose from, MCI selection criteria was less strict. All MCI samples had RIN values of at least 4.6.

Rapid Immunohistochemical Staining

Slides were held in place using a pre-chilled slide holder block. The holder block was submerged in ice throughout the staining process to maintain the cool temperature of the slide with the tissue section. Staining was performed rapidly using the Vectastain ABC Kit peroxidase enzyme system (Vector Labs, CA, PK-4000). Prior to conducting the staining protocol we prepared a 10X working solution by applying 50 µL of avidin/biotinylated enzyme complex (ABC) into 500 µL of cold 1X Tris Buffered Saline (TBS; Corning, 46-012-CM) and incubating for ~30 min. The staining procedure was then performed. First, the tissue was hydrated onto the slide using a series of cold (~20°C) fresh ethanol dilutions: 100% (1 min) to 95% (15 s) to 75%
(15 s). Next, the tissue was blocked using a 2% normal goat serum in 1X TBS for 3 min. The primary and secondary antibodies were diluted in the blocking solution. Anti-neurofilament primary rabbit IgG antibody (ABCAM) was used to stain for neurons (dilution 1:200 2% blocking solution). The primary antibody was applied for 3 min and then rinsed twice with 1X TBS. Tissue was incubated with anti-rabbit IgG secondary antibody (5%) from the ABC kit for 3 min and then washed twice with 1X TBS. Next, the tissue was incubated with the ABC solution for 3 min. During ABC incubation, a dilution of the enzyme substrate was freshly prepared avoiding light exposure, using the DAB (3,3 diaminobenzidine) Peroxidase Substrate Kit (Vector Laboratories, CA, SK-4100). After ABC incubation, the tissue was washed again with 1X TBS. The DAB solution was gently added to the tissue slice. After ~10 s, the slide was rinsed with RNase-free laboratory grade water to halt the enzymatic reaction. The slide was then immediately rinsed in the ethanol series for 15 s each from 75% to 95% to 100%. Tissue section was then cleared in xylene for 5 min and air dried for 5 min. Lastly; slides were taken immediately to the LCM facility.

**Laser Capture Microdissection (LCM)**

LCM was performed using the ZEISS PALM Microbeam 4.2 Microscope system. Parameters were as follows: Exposure time, 50–120 ms; Cut Focus, 40; Cut Energy, 40%; Delta, 2, 12; Laser Speed, 80%; Objective, 63X. The procedure was performed in RNase-free conditions. For visualization, the slide was fitted onto the Axio Observer Z1 microscope stand and an adhesive cap (500 Opaque version, ZEISS, DE, 415190) was attached onto the collector stage. The corresponding PALM Robomover Z software was used to position the cap over the desired tissue section on the slide. Cells were visualized on the computer interface and manually selected using the CenterRoboLPC function. Subsequently, the microscope laser cut the tissue and catapulted the cells into the above adhesive cap. From each used slide 60–200 cells were collected. After microdissection, the adhesive caps were steriley removed from the plastic tube and placed into 100 µl aliquots of lysis buffer (Ambion, LT, 8540G12). The cap and lysis buffer were vortexed and incubated at 42°C for 30 min before being stored at −80°C until RNA extraction.

**RNA Extraction and Pre-Nanostring Technologies® Processing**

For each sample, collected cells were combined in aliquots of a total of 200 cells. RNA was extracted from each aliquot of 200 cells using the Ambion RNAqueous-Micro Total RNA
Isolation kit following the manufacturer's instructions. Samples were incubated with DNase I (Qiagen, DE, 79254) for 15 min at room temperature to control for DNA contamination. RNA was eluted in 8 µl of Elution Solution and stored at −80°C.

Six microliters of RNA were used for reverse-transcription (RT) into complementary DNA (cDNA) using 2 µL of SuperScript VILO Master Mix (Invitrogen, 11755050), under the following conditions: 10 min at 25°C, 120 min at 42°C, and 5 min at 85°C. Next, Multiple Target Enrichment (MTE) was performed using specific primers (Table 2). Briefly, 1 µL of the pooled MTE primers and 7 µL of PreAmp TaqMan (Applied Biosystem, CA, 4384266) were added to the cDNA. MTE amplification was carried out for 12 cycles under the following conditions: 10 min at 94°C, 15 s at 94°C, 4 min at 60°C. The Pre-NanoString Technologies® processing was performed the day before the analysis and samples were kept at 4°C overnight.

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</tr>
<tr>
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<td>TCACATGGTTACACGGCG</td>
</tr>
<tr>
<td>SDHA</td>
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<td>CTCTCTACCCCAAGGGCC</td>
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<tr>
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<td>GTGTTGCTAGTTTGCTATG</td>
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<tr>
<td>CYC1</td>
<td>CTTGCCGGGTTGCTTGT</td>
<td>TCACAGCCGAATGCAGAG</td>
</tr>
<tr>
<td>EIF4A2</td>
<td>ACTGCAAGACGCAACA</td>
<td>CCAATGCGAGCATGACAA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CCGCATCTCTTTTGCGT</td>
<td>TTTGCGATGGTTGAATC</td>
</tr>
<tr>
<td>YWHAZ</td>
<td>CCTGCTTTTGATCC</td>
<td>TGCTGAGTAAATAGGAAGG</td>
</tr>
</tbody>
</table>

ENO2, Enolase 2; SYP, Synaptophysin; NFLH, Neurofilament; GFAP, Glial Fibrillary Acid Protein; B2M, Beta-2-Microglobulin; SDHA, Succinate Dehydrogenase Complex, Subunit A; LDHA, Lactate Dehydrogenase A; CYC1, Cytochrome C-1; EIF4A2, Eukaryotic Translation Initiation Factor 4A2; GAPDH, Glyceraldehyde-3-Phosphate Dehydrogenase; YWHAZ, Tyrosine 3-Monoxygenase/Tryptophan 5-Monoxygenase Activation Protein, Zeta.

Source: Tagliafierro et al., 2016
nCounter Single Cell Gene Expression Assay (NanoString Technologies®)

Gene expression was quantified using a digital approach by nCounter Single Cell Gene Expression Assay (NanoString Technologies® http://www.nanostring.com/products/single_cell). Each sample was hybridized in a strip tube by adding buffer and a CodeSet of target-specific reporter and capture probes (listed in Table 3) and the samples underwent solution phase hybridization overnight at 65°C. After hybridization, the strip tubes containing the samples were loaded onto the nCounter Prep Station. The reagents in the nCounter Master Kit plates were added and automated purification began to remove the excess probes. The targeted probe complexes were then aligned and immobilized in the nCounter cartridge. The cartridge was removed from the Prep Station and transferred onto the nCounter Digital Analyzer. Digital quantitation was done by the assigned colored barcodes on the surface of the cartridge, which were counted for each desired target molecule. Finally, a digital report of the expression quantity of each target gene was given. Data was analyzed using the nSolver Analysis Software.
Results

Quality Control and Final Sample Cohort

The PMI, age, B&B score, and RIN number for each normal, mild AD, and severe AD sample in the initial sample set can be found in Table 3 (females) and Table 4 (males). The mean age, PMI, and RIN number for all samples in each pathological category were calculated (Table 5). Within this initial sample group, several samples demonstrate large amounts of RNA degradation, as indicated by low RIN values, and thus were deemed unfit for the final sample cohort. Data for the initial MCI sample set can be seen in Table 6. RNA degradation is especially evident for several of the MCI samples. Additionally, some MCI samples originate from patients suffering from Parkinson’s disease, and thus were excluded from the final sample cohort.

Table 3
Initial Sample Group Statistics for Normal, Mild AD, and Severe AD Samples: Females

<table>
<thead>
<tr>
<th>ID</th>
<th>PMI</th>
<th>Age</th>
<th>RIN</th>
<th>ID</th>
<th>PMI</th>
<th>Age</th>
<th>B&amp;B</th>
<th>RIN</th>
<th>ID</th>
<th>PMI</th>
<th>AGE</th>
<th>B&amp;B</th>
<th>RIN</th>
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<tbody>
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<td>80</td>
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<td>459</td>
<td>4.83</td>
<td>79</td>
<td>3</td>
<td>6.6</td>
<td>632</td>
<td>12.6</td>
<td>69</td>
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<td>4.3</td>
</tr>
<tr>
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<td>82</td>
<td>7.8</td>
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<td>80</td>
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<td>5.7</td>
<td>766</td>
<td>4.08</td>
<td>77</td>
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<td>7.5</td>
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<td>83</td>
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<td>79</td>
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<td>7.2</td>
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<td>1600</td>
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<td>85</td>
<td>2.9</td>
<td>444</td>
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<td>86</td>
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<td>7.8</td>
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<td>909</td>
<td>10.6</td>
<td>90</td>
<td>7.1</td>
<td>1078</td>
<td>7.17</td>
<td>86</td>
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</tbody>
</table>

Table 4
Initial Sample Group Statistics for Normal, Mild AD, and Severe AD Samples: Males

<table>
<thead>
<tr>
<th>ID</th>
<th>PMI</th>
<th>Age</th>
<th>RIN</th>
<th>ID</th>
<th>PMI</th>
<th>Age</th>
<th>B&amp;B</th>
<th>RIN</th>
<th>ID</th>
<th>PMI</th>
<th>AGE</th>
<th>B&amp;B</th>
<th>RIN</th>
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</thead>
<tbody>
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<td>66</td>
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<td>5.9</td>
<td>778</td>
<td>2.00</td>
<td>60</td>
<td>5</td>
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<td>8</td>
<td>651</td>
<td>12.4</td>
<td>67</td>
<td>5</td>
<td>3.6</td>
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<tr>
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<td>544</td>
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<td>78</td>
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<td>4.4</td>
<td>510</td>
<td>5.92</td>
<td>70</td>
<td>5</td>
<td>6.6</td>
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<td>85</td>
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<td>0.60</td>
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<td>7.0</td>
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<td>6</td>
<td>5.9</td>
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<td>11.1</td>
<td>80</td>
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<td>6.6</td>
<td>1558</td>
<td>10.7</td>
<td>77</td>
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<td>5.4</td>
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<td>863</td>
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<td>4.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Applying the selection criteria outlined in the methods section, six females and six males each were chosen for the normal, mild AD, and severe AD sample sets, with samples matched across pathological category by sex and approximate age, as demonstrated in Table 7 (females) and Table 8 (males). To create the final sample cohort, samples were selected to minimize average PMI and maximize average RIN (Table 9). Samples were also chosen such that each B&B score (3, 4, 5, and 6) was represented in the final cohort in order to create a sample set that
represents a full range of disease progression (Figure 1). Finally, as shown in Table 10 and Figure 2, *TOMM40* poly-T genotype was considered such that each of the three allele groups (S, L, VL) were represented as evenly as possible in the final sample cohort for normal, mild AD, and severe AD samples (*TOMM40* genotype was unavailable for the MCI samples).

Table 7

**Final Sample Cohort Statistics for Normal, Mild AD, and Severe AD Samples: Females**

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Mild AD</th>
<th>Severe AD</th>
</tr>
</thead>
<tbody>
<tr>
<td>ID</td>
<td>PMI</td>
<td>Age</td>
<td>RIN</td>
</tr>
<tr>
<td>913</td>
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</tr>
<tr>
<td>963</td>
<td>8.12</td>
<td>82</td>
<td>7.8</td>
</tr>
<tr>
<td>1690</td>
<td>4.42</td>
<td>84</td>
<td>7.6</td>
</tr>
<tr>
<td>247</td>
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</tr>
<tr>
<td>909</td>
<td>10.61</td>
<td>90</td>
<td>7.1</td>
</tr>
</tbody>
</table>

Table 8

**Final Sample Cohort Statistics for Normal, Mild AD, and Severe AD Samples: Males**

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Mild AD</th>
<th>Severe AD</th>
</tr>
</thead>
<tbody>
<tr>
<td>ID</td>
<td>PMI</td>
<td>Age</td>
<td>RIN</td>
</tr>
<tr>
<td>676</td>
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<td>60</td>
<td>7</td>
</tr>
<tr>
<td>707</td>
<td>4.25</td>
<td>80</td>
<td>4.8</td>
</tr>
<tr>
<td>542</td>
<td>3.25</td>
<td>82</td>
<td>7.4</td>
</tr>
<tr>
<td>99</td>
<td>2.00</td>
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</tr>
<tr>
<td>893</td>
<td>5.50</td>
<td>90</td>
<td>6.8</td>
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</tbody>
</table>

Table 9

**Total Final Cohort Statistics for Normal, Mild AD, and Severe AD Samples**

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Mild</th>
<th>Severe</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age ± Std</td>
<td>81.3 ± 9.17</td>
<td>79.4 ± 4.36</td>
<td>73.3 ± 7.96</td>
<td>76.7 ± 8.61</td>
</tr>
<tr>
<td>Mean PMI ± Std</td>
<td>6.38 ± 4.18</td>
<td>5.15 ± 4.08</td>
<td>5.63 ± 3.35</td>
<td>4.96 ± 3.75</td>
</tr>
<tr>
<td>Mean RIN ± Std</td>
<td>6.93 ± 1.12</td>
<td>6.92 ± 1.04</td>
<td>6.65 ± 1.16</td>
<td>7.07 ± 0.94</td>
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</table>
Table 10

<table>
<thead>
<tr>
<th>TOMM40 Genotype</th>
<th>S/S</th>
<th>S/VL</th>
<th>VL/VL</th>
</tr>
</thead>
<tbody>
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<td>3</td>
</tr>
<tr>
<td>Mild</td>
<td>3</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Severe</td>
<td>2</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>5</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>Females</td>
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<td></td>
<td></td>
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<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Mild</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Severe</td>
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<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
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<td>7</td>
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</tr>
<tr>
<td>Mild</td>
<td>6</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Severe</td>
<td>4</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
<td>14</td>
<td>8</td>
</tr>
</tbody>
</table>

Figure 1. Number of samples with each Braak and Braak score.
Seven samples were ultimately chosen for the MCI cohort. All samples came from subjects with a diagnosis of only MCI at time of death, without cognitive impairment due to other diseases. Samples with low PMI’s and high RIN numbers were chosen for the final sample cohort. MCI statistics are presented in Table 11.

Table 11

<table>
<thead>
<tr>
<th>ID</th>
<th>Sex</th>
<th>PMI</th>
<th>Age</th>
<th>RIN</th>
<th>APOE Genotype</th>
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<td>&gt;89</td>
<td>8.1</td>
<td>33</td>
</tr>
<tr>
<td>931</td>
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<td>76</td>
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<tr>
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<td>4.33</td>
<td>86</td>
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<td>17.70</td>
<td>&gt;89</td>
<td>6.7</td>
<td>33</td>
</tr>
</tbody>
</table>

Mean ± Std - 13.40 ± 9.41 81.50 ± 5.26 6.31 ± 1.17 -

Figure 2. Distribution of TOMM40 genotypes among samples. We attempted to include each genotype within all pathological categories.
Figure 3 demonstrates that the average RIN numbers for each pathological category increased after final sample cohort selection, because samples with low RIN numbers were not included. The average RIN number for all samples increased by 0.92. Total average PMI decreased after final sample cohort selection, with an average decrease for all samples by 0.23 hours. This difference is small due to the fact that in choosing the initial sample set, low PMI was a selection criterion. Figure 4 depicts the correlation between PMI and RIN number for all samples in the initial sample set and demonstrates that the two variables have a negative linear relationship such that a longer PMI indicates a smaller RIN number. The coefficient of determination, $r^2 = 0.1998$, indicates that approximately 20% of the variation in RIN number can be explained by PMI. Thus, PMI has a minor effect on RIN, and the correlation is significant enough that PMI was minimized to ensure high RNA quality.

![Initial vs. Final RIN](image)

*Figure 3. Comparison of average RIN number for each pathological category and for all samples before and after final sample cohort selection.*
Method Optimization: The immune-LCM technique to isolate single neurons from frozen human brain

*Rapid Immunostaining Effects on RNA Quality*

In order to assess the effect of rapid immunostaining on RNA quality, RNA from frozen slides with samples from the same subject was analyzed. Sample 3 was stained using the protocol outlined in the methods section. Sample 4 was not stained. RNA was extracted from both tissues and RNA quality was assessed using a 2100 Bioanalyzer (procedure outlined in methods section). Figure 5 shows the electropherogram and the gel of the Sample 4 (unstained) while Figure 6 shows the electropherogram and gel of the stained slide (Sample 3). Figure 7 is a comparison of the two electropherograms and shows a slight decrease in RNA quality from the unstained sample to the stained sample. The RIN number dropped 1.2 points from 4.8 to 3.6. Thus, the negative impact of staining on RNA quality is minor and therefore does not preclude downstream gene expression analysis.
Figure 5. Electropherogram and gel of unstained sample. Clearer peaks indicated higher quality RNA. RIN is 4.8.

Figure 6. Electropherogram and gel of stained sample. Peaks are less defined. RIN is 3.6.

Figure 7. Comparison of electropherograms for the stained (Sample 3, blue) and unstained (Sample 4, red) samples. The plots are similar, indicating that RNA quality decreased by only a small amount.
**Laser Capture Microdissection Enables Single Cell Collection of Neurons**

Rapid immunohistochemistry enabled the identification of neurons in normal, MCI, mild AD, and severe AD samples. Neurons were identified by neurofilament immunoreactivity, i.e., positive cells presented with a golden-brown color that provided contrast against the underlying background tissue. Immunopositive cells were visualized, cleanly isolated from surrounding tissue by LCM, and collected into an adhesive cap (Figure 8). Isolating single cells left the surrounding tissue intact, validating LCM as a proper method to selectively collect single cells without collecting adjacent tissue. The range of neurons collected from each slide was 60-200, with an average of two slides used to collect 200 neurons per tissue sample. The PalmRobo software counted the amount of neurons throughout the LCM procedure. Slides from which only a small number of neurons (<60) could be identified were discarded.

**Figure 8. Laser capture microdissection of AD neurons.** Eight-micrometer brain tissue slices were stained with anti-neurofilament antibody. Immunopositive cells presented with a golden-brown color. Neurons were first visualized (A). Neurons were then manually selected (B) and catapulted into an adhesive cap, leaving surrounding tissue unaffected (C). Red arrows indicate neurons before (A, B) and after (C) collection. 200 neurons were collected from each sample. Size bar: 30 μm. Magnification: 63X.

**Impact of Durations of Immunostaining and LCM Protocol Steps on RNA Quality**

Using data generated by the nCounter Single Cell Gene Expression Assay from an initial submission of 22 samples (Appendix B, Table B1), we evaluated four parameters relating to the immune-LCM procedure that potentially affect successful RNA quality maintenance: (1) duration of staining (Figure 9), (2), duration of LCM (Figure 10), and (3) days elapsed between LCM and RNA extraction (Figure 11). We found that the probability of success, defined as samples that generated an average count per gene of 4 or more counts and thus had good enough
RNA quality for downstream gene expression analysis, decreased with increasing duration of these parameters. These results are likely due to the effect of time-elapsed on RNA stability. In accordance with these results, to increase the probability of success in obtaining digital expression data, we added the following conditions to the immune-LCM protocol: LCM duration should be minimized and a maximum time of 120 min to complete LCM should be adhered to, and days elapsed between cell collection and RNA extraction should be kept to 7–10 days. Table 12 gives the average durations of protocol steps for submission 1 and submission 2 and demonstrates the precautions taken during sample collection for submission 2, taking into account the data from submission 1, to minimize the duration of each step.

![Probability of Success vs. Average Time Spent Staining](image)

**Figure 9.** Probability of success vs. average duration of staining (minutes). A general additive model. A negative correlation was found between probability of obtaining good counts and duration of staining. Samples had the best probability of success when staining took less than an hour.
Figure 10. Probability of success vs. duration LCM. A general additive model. Success decreases drastically after long amounts of time are spent at the LCM.

Figure 11. Probability of success vs. days elapsed between cell collection and RNA extraction. A general additive model. The probability of obtaining counts decreases with increasing amount of days between cell collection and RNA extraction.
Table 12

<table>
<thead>
<tr>
<th>Submission</th>
<th>Average days slides stored at -80°C after slicing</th>
<th>Average duration of staining (min)</th>
<th>Average duration of LCM (min)</th>
<th>Average days elapsed between LCM and RNA extraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25.06</td>
<td>51.4</td>
<td>124.2</td>
<td>79.7</td>
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<td>2</td>
<td>11.48</td>
<td>42.988</td>
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<tr>
<td>Percent Change</td>
<td>-54.19%</td>
<td>-16.43%</td>
<td>16.89%</td>
<td>-90.64%</td>
</tr>
</tbody>
</table>

Validating Collection of Homogenous Cell Populations: Enrichment in Neurons

Several neuronal genes (NFLH, ENO2, SYP, Synphilin; Appendix A, Table A3) were included in the NanoString gene panel as potential markers to evaluate neuronal enrichment and assess the homogeneity of the collected cells. GFAP and S100B (Appendix A, Table A3), two astrocyte marker genes, were also included as markers to check for astrocyte contamination in collected populations of neurons. Using the data generated by nCounter Single Cell Gene Expression Assay (Nanostring Technologies®), it was found that RNA obtained from neurons displayed higher levels of specific neuronal marker transcripts relative to a calibrator. The calibrator sample consisted of RNA extracted from a whole section of normal brain tissue (heterogeneous cell populations). The enrichment of neurons was evaluated using the neuronal specific probe SYP2 (probe for the SYP gene) as the primary indicator of neuronal enrichment. This marker was selected as the primary indicator of neuronal enrichment because it demonstrated the greatest amount of efficiency and consistency. Counts for the SYP2 neuronal marker were obtained for all samples and were normalized relative to the geometric mean of the housekeeping gene counts for each sample, respectively. The housekeeping genes used in this study were B2M and YWHAZ. The selection of these housekeeping genes was based on the counts obtained: we selected genes with high and medium counts (YWHAZ>B2M). The GFAP and S100B astrocyte markers counts were also normalized to the housekeeping genes, and the normalized SYP2/S100B and SYP2/GFAP ratios were obtained for each sample. Then, for each sample, the SYP2/S100B and SYP2/GFAP ratios were normalized relative to the SYP2/S100B ratio and SYP2/GFAP ratio, respectively, of the average counts for the four calibrators. Samples that showed either an increase in SYP2/S100B ratio of >10-fold compared to the calibrators or an increase in SYP2/GFAP ratio of >10-fold compared to the calibrators were considered to be enriched for neurons. Samples with fold enrichment <10 for both GFAP and S100B were
accordingly excluded from further analysis. In summary, the isolation of enriched neuronal cells- aliquots by the immuno-LCM method was validated.

**Gene Expression Analysis**

*Analyzing Raw Data and Sample Exclusion*

Three submissions of samples were made to NanoString at three different dates (Appendix B). Submission 1 was comprised of 22 samples, submission 2 was comprised of 24 neuronal samples, and submission 3 included 11 neuronal samples. Samples for which data was analyzed for gene expression come from submissions 1 and 2, as submission 3 resulted in poor counts due to RNA degradation. Thus, post-NanoString exclusion criteria were applied to the 46 samples from submission 1 and 2, some of which were duplicates.

Gene expression analysis was ultimately performed for fifteen samples after exclusion criteria was applied: 3 normal samples (2 female, 1 male), 4 MCI samples (3 female, 1 male), 6 mild AD samples (1 female, 5 male), and 2 severe AD samples (2 female). The steps taken to compute relative expression levels are as follows: (1). The background threshold was determined for each individual sample by taking the geometric mean + 2 standard deviations of the counts from four negative control probes (NEG_C, NEG_D, NEG_G, NEG_H). Any counts less than or equal to the calculated value for each sample were considered to be equal to zero. Next, counts were normalized to positive markers to control for platform-associated sources of variation, (e.g. hybridization, automated purification). To find the positive normalization factor for each sample, the geometric mean of the positive probes was calculated for each sample, and the average of these geometric means was determined (across submissions). Next, each sample’s unique positive normalization factor was computed by dividing the average of all geomeans by each sample’s individual positive geomean. Any sample with a positive normalization factor outside the range of 0.3-3.0 was discarded (2 samples). All counts for each sample were multiplied by the sample’s unique positive normalization factor. Next, counts underwent housekeeping gene normalization. As stated earlier, the housekeeping genes chosen for analysis were *YWHAZ* and *B2M*. The selection of these housekeeping genes was partially based on the counts obtained: we selected genes with high and medium counts (*YWHAZ>*B2M). These two housekeeping genes had identical probes between the two submissions, and showed consistent behavior within the calibrators between the two submissions (Figure 12). One calibrator showed poor counts in the first submission and was not included in this housekeeping gene analysis. For
each sample, the geomean of the counts for these two genes was calculated, and each positive-normalized count for a sample was divided by that sample’s housekeeping gene geomean. Samples were included in further analysis only if both $B2M$ and $YWHAZ$ displayed counts above background threshold. The final criterion for sample inclusion in gene expression analysis was neuronal enrichment. Neuronal enrichment calculation and inclusion criteria were discussed in detail in the preceding section. Table 13 details the enrichment data for the final sample set included in gene expression analysis.

![Housekeeping Gene Analysis](image)

**Figure 12.** Analysis of housekeeping gene efficiency and consistency within and across samples. $B2M$ and $YWHAZ$ were chosen due to consistent trends seen between submission 1 (Cal A) and submission 2 (Cal B, Cal C, Cal D). It's important to note that the GAPDH probes targeted different regions within the gene between the two submissions. The GAPDH trend changed between the two submissions, thus confirming that using different probes gives different results and leading us to decide to not use GAPDH as a reference gene for gene expression analysis.
Analysis of Gene Expression Profiles

Using the samples from submission 1 and submission 2 that met the criteria of showing reliable counts (average per gene >4), having counts for B2M and YWHAZ, and showing neuronal enrichment, the expression profiles of various genes (Appendix A, table A1) were determined. For each gene, the average normalized expression for each pathological stage was calculated. Outliers were determined for each gene within each pathological category and excluded (any sample for a gene where the normalized expression level was outside the range of (1st quartile -1.5 * interquartile range - 3rd quartile +1.5*interquartile range) for each pathological category).

The APOE expression profile demonstrated expression levels that increased across disease progression (Figure 13). Expression levels remained unchanged between normal and MCI stages for APP with a subsequent increase in Mild AD samples followed by a decrease in severe AD samples (Figure 14). BIN1 (Figure 15), PICALM (Figure 16), SORL1 (Figure 17), and APOE share in common a role in cholesterol metabolism and trafficking and endocytosis. An upward trend in expression level across disease progression is seen for each gene except PICALM, which has no definite trend. Figure 18 shows all genes for comparison. CLU is also

<table>
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<tr>
<th>Sample ID and Pathology</th>
<th>*SYP2/GFAP</th>
<th>**SYP2/S100B</th>
</tr>
</thead>
<tbody>
<tr>
<td>99 Normal</td>
<td>215.8</td>
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</tr>
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<td>673 Normal</td>
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<td>1574 MCI</td>
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<td>1682 MCI</td>
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<tr>
<td>75 Mild AD</td>
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</tr>
<tr>
<td>111 Mild AD</td>
<td>39.3</td>
<td>90.1</td>
</tr>
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</tr>
<tr>
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<td>402.1</td>
</tr>
<tr>
<td>1133 Severe AD</td>
<td>1.08</td>
<td>60.5</td>
</tr>
<tr>
<td>766 Severe AD</td>
<td>3.24</td>
<td>33.2</td>
</tr>
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</table>

*This value represents the ratio SYP2 counts/GFAP counts relative to the calibrator.
**This value represents the ratio SYP2 counts/S100B counts relative to the calibrator.
highly implicated in lipid metabolism. The probe for *CLU* was different between the two submissions, thus data from the two samples could not be compared, but Figure 19 shows the *CLU* expression profile using only samples from submission 2. Normal samples contained the highest expression levels for *CLU*; however from MCI to severe AD the same trend as *BIN1* and *SORL1* is seen.

* TOMM40 showed an increase in expression level from normal to mild AD, followed by a slight decrease in severe AD samples (Figure 20). Expression levels were shown to increase across pathological stage for *MAP1B* and *MAPT*, genes that encode microtubule-associated protein (Figure 21). *MAPT* encodes the tau protein, which is found in an abnormally hyperphosphorylated state in neurofibrillary tangles, a pathological hallmark of AD.

Figure 22 depicts the expression profile of *SYP*, the gene encoding synaptophysin, a synaptic vesicle protein and a neuronal marker, as well as *ENO2*, the gene encoding enolase 2, another neuron marker. The expression pattern for these two genes is interesting as expression is decreased in MCI and severe AD samples but remains elevated in mild AD samples. Expression levels of *GFAP* and *S100B*, the two astrocyte markers used in our analysis, are shown in Figure 23. Interestingly, *GFAP* expression increases with disease severity, while *S100B* does not appear to be associated with pathological stage. This result justifies the use of both markers to determine neuronal enrichment, as all samples included in final analysis showed neuronal enrichment when using *S100B* as an astrocyte marker, while several AD samples did not show enrichment when *GFAP* was used. The decreasing probability of seeing enrichment for neurons in severe AD samples when using *GFAP* as an astrocyte marker for analysis thus also corroborates the expression profile results for *GFAP*.

Figure 24 depicts the expression profiles for three immune response genes: INPP5D, CR1, and HLA-DRB5. Expression levels are greatly elevated in mild AD samples. Figure 25 depicts the expression levels of four genes implicated in cytoskeletal function and axonal transport pathways: *CASS4*, *FERMT2*, *MS4A6A*, and *CELF1*. Finally, Figure 26 includes the expression profiles for the remaining genes included in the gene expression assay. These genes are all involved in a variety of different pathways and show various expression trends.
Figure 13. 

APOE expression levels by pathological stage. Bars represent standard error of the mean (SEM).

Figure 14. 

APP normalized expression levels by pathological stage. Bars represent SEM.
LOAD GENE EXPRESSION IN NEURONS

Figure 15. *BIN1* normalized expression levels by pathological stage. Bars represent SEM.

Figure 16. *PICALM* normalized expression levels by pathological stage. Bars represent SEM.
Figure 17. SORL1 normalized expression levels by pathological stage. Bars represent SEM.

Figure 18. Normalized expression levels by pathological stage for genes involved in cholesterol metabolism and trafficking. Bars represent SEM.
Figure 19. CLU normalized expression levels by pathological stage. Bars represent SEM. Due to each submission having different target regions for the CLU probe, this data comes exclusively from submission 2.

Figure 20. TOMM40 normalized expression levels by pathological stage. Bars represent SEM. Expression levels increase and then decrease in severe AD samples.
Figure 21. Normalized expression levels by pathological stage for MAP1B and MAPT, genes that encode microtubule associated protein. Bars represent SEM. MAPT encodes the tau protein, which is found in an abnormally hyperphosphorylated state in neurofibrillary tangles, a pathological hallmark of AD. Expression levels increase for both genes (however are decreased for MAPT in severe AD samples).

Figure 22. SYP and ENO2 normalized expression levels by pathological stage. Bars represent SEM. Expression levels are decreased in cognitively impaired brains, more notably in MCI and severe AD samples.
Figure 23. Expression profiles for astrocyte marker genes GFAP (left) and S100B (right). Bars represent SEM. GFAP expression levels increase throughout disease progression, while S100B levels appear to be unrelated to disease severity. This result justifies the use of both markers to determine neuronal enrichment, as all samples included in final analysis showed neuronal enrichment when using S100B as an astrocyte marker, while several AD samples did not show enrichment when GFAP was used.

Figure 24. Expression profiles for INPP5D, CR1, and HLA-DRB5. Bars represent SEM. These three genes are involved in immune response pathways. Expression levels are greatly elevated in mild AD samples.
Figure 25. Expression profiles for CASS4, FERMT2, MS4A6A, and CELF1 genes (starting at the upper left corner and moving clockwise). Bars represent SEM. These genes are all implicated in cytoskeletal function and axonal transport pathways. No general expression trend can be discerned connecting these genes.
Figure 26. Expression profiles for APOC1, ATP5A1, GRIA3, ABCA7, PTK2B, and RNASET2 genes. Bars represent SEM. Genes are involved in a variety of pathways (Appendix A).
Discussion

Previous studies have found changes in gene expression in AD patients relative to controls using brain tissue homogenates that contain heterogeneous cell populations (Liang et al., 2008; Linnertz et al., 2014; Strobel et al., 2015). The use of whole brain tissue however obscures important information about different gene regulatory patterns in dissimilar cell-types. Moreover, the importance of studying gene expression in specific cell-types is compounded by the fact that pathology in neurodegenerative diseases affects cell types in diverse ways and to varying degrees (Saxena & Caroni, 2011). Consequently, the first aim of this project was to develop and optimize a method to isolate cells from human frozen brain tissue without compromising RNA quality in order to obtain reliable gene expression data. To this end we optimized a method of rapid immunostaining on ice under RNASE-free conditions followed by laser capture microdissection (LCM) collection of neurons. We examined factors that affect RNA stability throughout the duration of the protocol and determined that duration of LCM and days elapsed between cell collection and RNA extraction should be kept minimal. We also validated enrichment for neurons in our collected samples using the neuronal-specific marker SYP and the astrocyte-specific marker S100B. In summary, we have demonstrated that collecting 200 cells per sample using our optimized rapid immune-LCM technique is sufficient for obtaining reliable gene expression data with the nCounter Single Cell Gene Expression assay (NanoString Technologies®).

Our second aim was to determine and characterize changes in LOAD-gene expression profiles related to pathological stage. Our novel approach utilized samples from cognitively normal control donors as well as three different LOAD pathological stages: mild cognitive impairment (MCI), mild AD, and severe AD. The use of MCI samples was especially important as this stage of cognitive impairment may contain information about gene regulatory patterns that play a causative role in AD etiology. Our results found that gene expression profiles do change across disease progression, and moreover, many large changes can be see between the normal and MCI stages, justifying the use of tissue with this diagnosis in gene expression studies.

One gene of particular interest is APOE due to the APOEε4 allele being the most important genetic risk factor associated with AD (Allen D. Roses, 1996). APOE is thought to take up lipids generated after neuronal degeneration and redistribute them to cells for
proliferation, membrane repair, and remyelination of new axons. APOE receptors also effect APP trafficking and amyloid-beta production. Moreover, APOEε3 is thought to facilitate the degradation and clearance of amyloid-beta through a variety of proposed mechanisms such as facilitation of clearance through the blood-brain barrier (BBB), facilitation of uptake and degradation by astrocytes and microglia, and facilitation of extracellular proteolytic degradation (Yu et al., 2014).

APOE has long been known to be expressed in glial cells, particularly in astrocytes, yet the presence and expression of APOE in neurons has historically been debated (Han et al., 1994). However, cortical neurons have been found to show APOE-immunoreactivity using immunoelectron microscopy on human temporal lobe specimens, leading to the postulation that APOE taken up by cortical neurons effects neuronal metabolism (Han et al., 1994). It was then demonstrated that not only is APOE taken up by neurons, but it is synthesized in neurons as well. In situ hybridization on paraffin-embedded and frozen human brain sections with antisense APOE cRNA probes verified that APOE mRNA is transcribed and expressed in neurons in the frontal cortex and hippocampus (P.-T. Xu et al., 1999). Our results, which found an increase in expression level of APOE across disease progression, establish further that APOE is expressed within neurons.

Additionally, new insights into the function of APOE have led to increased interest in the role it plays within neurons. A recent study has shown for the first time that APOEε4 binds double-stranded DNA within the nuclei of cultured neural cells and acts as a transcription factor (Theendakara et al., 2016). Moreover, the binding sites of this factor were found to include approximately 1700 gene promoter regions, and the genes associated with these promoters are associated with a wide range of functions relating to AD pathogenesis including trophic support, programmed cell death, synaptic function, aging, and insulin resistance. Moreover, our results showing an increase in APOE expression at the onset of disease is consistent with the proposed model that up-regulated function of APOE contributes to LOAD etiology (Gottschalk, Mihovilovic, Roses, & Chiba-Falek, 2016). This proposed model hypothesizes that an increase in APOE activity, caused by elevated gene expression and/or amplified protein activity, leads to increased amyloid-beta plaque accumulation, misfolded proteins, and neurotoxicity. Ultimately, these changes would then result in Alzheimer’s disease. Our results are consistent with this hypothesis.
We also demonstrated an increase in \textit{APP} expression limited to AD brain samples, with no change between normal and MCI samples. This result agrees with results reported in literature that \textit{APP} appears to be up-regulated in AD whole brain samples, with increases in both mRNA transcripts and protein levels (Matsui et al., 2007). Our findings thus demonstrate the importance of including MCI samples and suggest that overexpression of \textit{APP} possibly does not play a causative role in AD etiology and may only play a role in later stages of disease. Hence, the accumulation of amyloid-beta may be caused by problems with APP metabolism, Aβ aggregation, or APP clearance, but perhaps not with early up-regulation of the gene. Although the role of APP under normal physiological conditions is poorly understood (O'Brien & Wong, 2011), it has been postulated to have a positive effect on cell growth, as shown in a study where overexpression of \textit{APP} in mice resulted in enlarged neurons (Oh et al., 2009). Moreover, recent findings have introduced the hypothesis that Aβ oligomerization activates antimicrobial properties in the protein that protect against infection (fungal and bacterial) in mouse, cell, and nematode AD models (Kumar et al., 2016). This hypothesis substantiates our finding that \textit{APP} mRNA levels are higher in later disease stages but not early stages, suggesting that Aβ accumulation is a reaction to changes in the diseased brain and not a cause of disease itself.

We also found an increase in \textit{TOMM40} expression across pathological stage, which upholds previous studies that have reported a significant increase in \textit{TOMM40} expression in AD brain homogenate (Linnertz et al., 2012). \textit{TOMM40} encodes Tom40, the translocase of the outer mitochondrial membrane pore subunit. \textit{TOMM40} is an important gene to study in AD due to multiple reasons, one being that it is in linkage disequilibrium with \textit{APOE}, and another being that mitochondrial dysfunction occurs early in LOAD and is related to cell death (A. D. Roses et al., 2010). APP has also been shown to amass in mitochondrial channels, causing dysfunction (A. D. Roses et al., 2010). Our data shows increased \textit{TOMM40} expression early in disease, and thus our data supports the theory that up-regulation of \textit{TOMM40} might play a causative role in mitochondrial dysfunction contributing to LOAD pathogenesis.

A comparison between expression trends in neurons and protein levels and expression trends in blood, plasma, and cerebrospinal fluid (CSF) is warranted due to the enormous benefits that would accompany finding a reliable biomarker to predict onset of AD. Interestingly, we find that many of the trends previously seen in blood, plasma, and CSF directly contrast those seen with our neuronal expression data. For example, while we see an increase in \textit{APOE}
expression corresponding with increasing severity of LOAD pathology, previous studies have found that both plasma protein levels and CSF protein levels decrease in association with APOE genotype, such that patients with APOEε4 homozygosity have on average lower APOE levels (Cruchaga et al., 2012). In the same vein, amounts of Aβ in the brain are negatively correlated with Aβ42 levels in CSF, meaning that increasing disease severity leads to lower levels of Aβ in CSF (Tapiola et al., 2009). This trend opposes our results that show that APP expression is increased in mild AD and severe AD samples. Finally, our findings showing that TOMM40 expression increase with pathological stage contrast studies of TOMM40 expression levels in blood. Previously, downregulation of TOMM40 expression within the blood of AD patients compared to control patients was found (J. Lee et al., 2012; T. S. Lee et al., 2012), and tracking TOMM40 mRNA in the blood of AD patients over a 2-year period revealed a steady decrease (Goh et al., 2015). These finding might indicate that TOMM40 expression in brain works in opposite directions of TOMM40 levels in blood. The opposing trends between brain expression and protein and expression levels found in blood, plasma, and CSF deserve further study to determine the biological reason behind these patterns and to advance research on biomarkers.

Interestingly, we found that GFAP expression increases in neurons throughout disease progression, and thus the legitimacy of using GFAP as an astrocyte-specific marker is not upheld by our findings. This corroborates the results of a study that used in situ hybridization methods to find that GFAP protein was present in the neurons of hippocampal tissue from AD brain samples (Hol et al., 2003). These results suggest that the expression of certain glial proteins increases with disease progression, and these findings warrant further investigation.

SORL1 encodes a neuronal intracellular sorting receptor called sorting-related receptor. The rs1699102 polymorphism has also been found to be associated with normal age-related cognitive decline and gray matter volume reduction of the right middle temporal pole in Han Chinese subjects (Li et al., 2017). Previous research has found that SORL1 co-localizes with APP \textit{in vitro} and in living cells (Andersen et al., 2005). Moreover, the same study found that a decrease in Aβ and an accumulation of APP in the Golgi could be induced by overexpressing SORL1 in SH-SY5Y cell lines, with a complementary discovery that Aβ production is increased in SORL1-deficient mice (Andersen et al., 2005). The authors thus theorized that SORL1 plays a role in processing and intracellular trafficking of APP (Andersen, Rudolph, & Willnow, 2016). McCarthy et al. (2012) demonstrated that individual SORL1 genetic variants show different
expression patterns in different regions of the brain, with delta-2-\textit{SORL1} (an alternative splice variant lacking exon 2) mRNA levels being higher in the frontal cortex (FC) relative to the temporal cortex (TC) of neurologically normal subjects (McCarthy et al., 2012). Moreover, the TC cortex of minor allele carriers at the rs7945931 and rs2298525 SNPs, compared with non-carriers, showed a 2-fold increase in \textit{SORL1}-mRNA levels (McCarthy et al., 2012). These studies are important because the TC is highly susceptible to AD pathology, and thus higher levels of \textit{SORL1} in the FC may suggest a protective effect. Past studies have also reported a down-regulation in \textit{SORL1} expression within AD brains compared to control brains (Liang et al., 2008; Scherzer et al., 2004). In our study, we observed the opposite: an increase in \textit{SORL1} expression, which corroborated our lab’s previous finding that \textit{SORL1} shows increased expression in severe AD whole brain tissue (unpublished data). Thus, there is good evidence that genetic regulation of the expression of \textit{SORL1} plays a role in disease risk, and one possible explanation for increased expression could be that up-regulation is a mechanism to attempt to clear A\textbeta.

After \textit{APOE}, bridging integrator 1 (\textit{BIN1}) has been implicated as the second most significantly associated susceptibility locus for LOAD (Harold et al., 2009; Holler et al., 2014; Lambert et al., 2013). It has been found that smaller isoforms of \textit{BIN1} are increased in several brain regions of AD samples, while protein levels of the longest isoform (isoform 1) are decreased (Holler et al., 2014). Interestingly, the same study reported that the amount of \textit{BIN1} isoform 9 (a short isoform) was found to be correlated with amount of neurofibrillary tangles but was not significantly correlated with either A\textbeta 40 or A\textbeta 42. \textit{BIN1} has also been found to co-localize with Tau in mouse brain and human neuroblastoma cells, and decreased expression of a \textit{BIN1} ortholog in \textit{Drosophila} corresponded with lower levels of Tau toxicity (Chapuis et al., 2013). We found in our study an increase in \textit{BIN1} across LOAD pathological progression, which fits with the association with abnormal Tau accumulation.

Clusterin (\textit{CLU}) is a lipoprotein thought to assist in transportation of A\textbeta across the BBB and thus facilitate clearance (Miners, Clarke, & Love, 2016). \textit{CLU} levels have been found to be elevated in AD brain samples relative to control AD samples, and furthermore, correlated with amounts of both A\textbeta 40 and A\textbeta 42 (Miners et al., 2016). However, despite the increase in clusterin levels, the molar ratio of clusterin: A\textbeta 42 was decreased in AD brains within regions that are particularly susceptible to A\textbeta pathology. Another study found that the \textit{CLU} rs11136000
SNP altered cerebrospinal fluid (CSF) levels of Tau in AD patients (Zhou et al., 2014). Clusterin has also been implicated in playing a role in early cognitive decline. In a study examining both cognitively normal and MCI type 2 diabetes mellitus (T2DM) patients (a disease which is associated with AD (Vacinova et al., 2017)), the rs11136000 SNP TT genotype (a SNP associated with AD), was correlated with reduced risk for MCI, and additionally, higher plasma clusterin levels were found in the MCI patients (Cai et al., 2016). We found an increase in CLU expression from MCI to severe AD, therefore replicating past studies.

PICALM encodes the phosphatidylinositol-binding clathrin assembly protein (Tebar, Bohlander, & Sorkin, 1999) and has been shown to have an association with AD by several GWAS studies (Harold et al., 2009; Lambert et al., 2013; Seshadri et al., 2010). PICALM plays a role in endocytosis, trafficking of endocytic proteins, internalization of receptors, iron homeostasis, immune pathways, lipid metabolism, synaptic transmission, and clearance of apoptotic cells (Baig et al., 2010; W. Xu, Tan, & Yu, 2015). PICALM is expressed predominantly within microvessels in endothelial cells (Baig et al., 2010; Parikh, Fardo, & Estus, 2014). The AD-associated polymorphism rs3851179, a protective allele, is associated with higher levels of PICALM mRNA, suggesting that increased levels of PICALM provide a defense against AD risk (Parikh et al., 2014). In severe AD brains (Braak V-VI), PICALM levels have been found at higher levels within neurons via immunostaining (Zhao et al., 2015). Importantly, in the same study PICALM endothelial levels were found to inversely correlate with disease severity (Braak stage and clinical dementia rating) as well as Aβ load, and in Picalm knockdown mice, diminished Aβ clearance across the BBB was observed. Moreover, to corroborate the finding that PICALM mediates Aβ clearance, inducible pluripotent stem cell–derived human endothelial cells with the rs3851179 protective allele not only showed augmented Aβ clearance but also demonstrated elevated levels of PICALM (Wu et al., 2015). The putative roles of PICALM can be roughly divided into two groups: Aβ–dependent and Aβ–independent. The latter group consists of pathways such as lipid metabolism immunity, iron homeostasis, and tauopathy (W. Xu et al., 2015). Here, we find that PICALM levels are lower in MCI and severe AD neurons, a result that opposes the Zhao et al. (2015) finding that PICALM levels are elevated in neurons of severe AD brains. Perhaps the lower levels of PICALM in MCI brains play a causative role by diminishing the protective effect of PICALM.

Several of the previously discussed genes (APOE, BIN1, CLU, PICALM, and SORL1) are
related due to their involvement in cholesterol metabolism and trafficking (Dong, Gim, Yeo, & Kim, 2017; Giri, Zhang, & Lu, 2016; Van Cauwenberghe, Van Broeckhoven, & Sleegers, 2016). It’s imperative that the function of these genes be studied independently and in relation to their interactions with each other. *BIN1* and *CLU* have been found independently to associate with NFT pathology (Chapuis et al., 2013; Holler et al., 2014; Wang et al., 2016; Zhou et al., 2014). Additional studies have identified interactions between BIN1 and CLU, with the discovery that the two proteins interact via coiled-coil motifs (Zhou et al., 2014). Even more importantly, co-immnoprecipitation experiments have demonstrated an association between CLU, BIN1, and Tau in neurons of AD human brain tissue (Zhou et al., 2014). *APOE, PICALM,* and *BIN1* have an interesting relationship in which the *PICALM* polymorphism rs541458 and the *BIN1* polymorphism rs744373 were associated with LOAD, but only in subjects without the *APOE* ε4 allele (Gharesouran, Rezazadeh, Khorrami, Ghojazadeh, & Talebi, 2014). Thus, the roles of these genes should be studied in tandem in order to elucidate a clearer picture of disease mechanism.

Finally, the gene expression profiles of several immune-related genes: *CR1, INPP5D,* and *HLA-DRB5* showed an interesting trend in which expression in mild AD neurons was much higher than in neurons from the other pathological stages. These genes are expressed primarily in microglia and play roles in clearance of immune complexes, cell-debris, and Aβ (Villegas-Llerena, Phillips, Garcia-Reitboeck, Hardy, & Pocock, 2016). The results of this study are accordingly surprising in that these genes appear to be expressed in neurons during mild stages of AD. Further study of these genes is warranted within microglia and astrocytes.

In conclusion, we have developed and optimized a method using immunohistochemical staining combined with laser capture microdissection to collect single neurons from frozen human brain tissue such that RNA quality is preserved for gene expression analysis. Using normal, mild-cognitive impairment, mild AD, and severe AD tissue, we have utilized our optimized technique to analyze gene expression in neurons across the full progression of LOAD pathology. This approach gives us a better idea of how gene regulation impacts molecular mechanisms involved in the pathophysiological changes in late onset Alzheimer’s disease. One limitation of this study is the lack of ability to compare gene expression in normal brains that precede and do not precede AD. Expression levels in our normal samples thus cannot be interpreted as directly preceding those seen in the MCI samples. Another major limitation is the
ability to obtain large amounts of intact RNA due to the fact that single cells are collected and RNA stability is especially tenuous in frozen human tissue. However, to account for this limitation we have optimized the technique to preserve RNA as much as possible, and we have been extremely stringent and conservative with our data, using samples in analysis only if they show >10 fold enrichment in neurons and display high mRNA transcript counts. Future directions include analyzing gene expression in microglia and astrocytes to give a better picture of how individual cell types are involved in LOAD.
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C. M. (2015). Changes in the expression of genes related to neuroinflammation over the
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## Appendix A

**Genes Used in NanoString Panels**

### Table A1

*Genes with the same probes in both submissions*

<table>
<thead>
<tr>
<th>Gene</th>
<th>Position</th>
<th>Pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCA7 (ATP-binding cassette transporter A)</td>
<td>4965-5064</td>
<td>Lipid metabolism and immune response</td>
</tr>
<tr>
<td>APOC1</td>
<td>271-370</td>
<td></td>
</tr>
<tr>
<td>APOC2</td>
<td>557-656</td>
<td></td>
</tr>
<tr>
<td>APOE (apolipoprotein E)</td>
<td>97-196</td>
<td>Cholesterol and lipid metabolism</td>
</tr>
<tr>
<td>APP</td>
<td>1726-1825</td>
<td></td>
</tr>
<tr>
<td>ATP5A1</td>
<td>311-410</td>
<td></td>
</tr>
<tr>
<td>BIN1 (bridging integrator 1)</td>
<td>1836-1935</td>
<td>Endosomal vesicle cycling</td>
</tr>
<tr>
<td>CASS4 (Cas scaffolding protein family member 4)</td>
<td>79-178</td>
<td>Cytoskeletal function and axonal transport</td>
</tr>
<tr>
<td>CD2AP (CD2-associated protein)</td>
<td>1896-1995</td>
<td>Endosomal vesicle cycling</td>
</tr>
<tr>
<td>CELF1 (CUGBP, Elav-like family member 1)</td>
<td>221-320</td>
<td>Cytoskeletal function and axonal transport</td>
</tr>
<tr>
<td>CR1 (complement component (3b/4b) receptor 1)</td>
<td>40-139</td>
<td>Immune response</td>
</tr>
<tr>
<td>DSG2</td>
<td>236-335</td>
<td>Cell-cell adhesion</td>
</tr>
<tr>
<td>EPHA1 (EPH receptor A1)</td>
<td>2076-2175</td>
<td>Endosomal vesicle and cycling immune system</td>
</tr>
<tr>
<td>FERMT2 (fermitin family member 2)</td>
<td>921-1020</td>
<td>Cytoskeletal function and axonal transport</td>
</tr>
<tr>
<td>GRIA3</td>
<td>427-526</td>
<td></td>
</tr>
<tr>
<td>HLA-DRB5 (major histocompatibility complex, class II, DR beta 5)</td>
<td>132-231</td>
<td>Immune response</td>
</tr>
<tr>
<td>INPP5D (inositol polyphosphate-5-phosphatase)</td>
<td>4076-4175</td>
<td>Immune response</td>
</tr>
<tr>
<td>MAP1B</td>
<td>8666-8765</td>
<td></td>
</tr>
<tr>
<td>MAPT</td>
<td>1206-1305</td>
<td></td>
</tr>
<tr>
<td>MEF2C (myocyte enhancer factor 2C)</td>
<td>2446-2545</td>
<td>Immune response, neural development, synaptic function</td>
</tr>
<tr>
<td>MS4A6A (membrane-spanning 4-domains, subfamily A)</td>
<td>516-615</td>
<td>Cytoskeletal function and axonal transport</td>
</tr>
<tr>
<td>PICALM (phosphatidylinositol-binding clathrin assembly protein)</td>
<td>1866-1965</td>
<td>Clathrin-mediated endocytosis</td>
</tr>
<tr>
<td>PPARG</td>
<td>1036-1135</td>
<td></td>
</tr>
<tr>
<td>PPARGC1A</td>
<td>1506-1605</td>
<td></td>
</tr>
<tr>
<td>PTK2B (protein tyrosine kinase 2 beta)</td>
<td>736-835</td>
<td>Hippocampal synaptic function; cell migration</td>
</tr>
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<td>PVRL2</td>
<td>1338-1437</td>
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</tr>
<tr>
<td>RNASET2</td>
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<tr>
<td>SNCA</td>
<td>292-391</td>
<td></td>
</tr>
<tr>
<td>SORL1 (sortilin-related receptor-1)</td>
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<td>Endosomal vesicle cycling</td>
</tr>
<tr>
<td>TOMM5</td>
<td>61-160</td>
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<tr>
<td>TOMM6</td>
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<td>TOMM7</td>
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</tr>
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<td>TOMM20</td>
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</tr>
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<td>TOMM22</td>
<td>261-360</td>
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</tr>
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<td>TOMM40</td>
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</tr>
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<td>TOMM70A</td>
<td>1696-1795</td>
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</tr>
<tr>
<td>ZCWPW1 (zinc finger, CW type with PWWP domain 1)</td>
<td>921-1020</td>
<td>Epigenetic regulation</td>
</tr>
</tbody>
</table>

*The position indicates the target region within the gene.*

*(Giri, Zhang, & Lu, 2016; Van Cauwenberghe, Van Broeckhoven, & Sleegers, 2016)*
Table A2

*Genes with different probes in each submission*

<table>
<thead>
<tr>
<th>Gene</th>
<th>Submission 1 Position</th>
<th>Submission 2 Position</th>
<th>Pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD33 (CD33 molecule)</td>
<td>731-830</td>
<td>355-454</td>
<td>Immune system</td>
</tr>
<tr>
<td>CLU (clusterin)</td>
<td>2461-2560</td>
<td>919-1018</td>
<td>Cholesterol and lipid metabolism</td>
</tr>
<tr>
<td>NME8 (NME family member 8)</td>
<td>481-580</td>
<td>1727-1826</td>
<td>Cytoskeletal function</td>
</tr>
<tr>
<td>SLC24A4 (solute carrier family 24)</td>
<td>1201-1300</td>
<td>856-955</td>
<td>Possible cardiovascular link</td>
</tr>
<tr>
<td>TREM2 (triggering receptor expressed on myeloid cells 2)</td>
<td>612-711</td>
<td>870-969</td>
<td>Immune response</td>
</tr>
</tbody>
</table>

*The position indicates the target region within the gene.*

Table A3

*Genes used for normalization and enrichment validation*

<table>
<thead>
<tr>
<th>Gene</th>
<th>Position</th>
<th>Specificity</th>
<th>Note</th>
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<tr>
<td>Enolase2</td>
<td>1856-1955</td>
<td>Neuron</td>
<td>Not used for enrichment validation</td>
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<tr>
<td>Neurofilament</td>
<td>1351-1450</td>
<td>Neuron</td>
<td>Not used for enrichment validation</td>
</tr>
<tr>
<td>Synphilin</td>
<td>423-522</td>
<td>Neuron</td>
<td>Not used for enrichment validation</td>
</tr>
<tr>
<td>SYP (synaptophysin)</td>
<td>2266-2365</td>
<td>Neuron</td>
<td>USED for enrichment validation</td>
</tr>
<tr>
<td>GFAP (glial fibrillary acidic protein)</td>
<td>590-689</td>
<td>Astrocyte</td>
<td>USED for enrichment validation</td>
</tr>
<tr>
<td>S100B (S100 calcium-binding protein)</td>
<td>305-404</td>
<td>Astrocyte</td>
<td>USED for enrichment validation</td>
</tr>
<tr>
<td>B2M (beta-2 microglobulin)</td>
<td>236-335</td>
<td></td>
<td>USED for analysis</td>
</tr>
<tr>
<td>EIF4A2 (eukaryotic translation initiation factor 4A2)</td>
<td>311-410</td>
<td>Housekeeping</td>
<td>Not used for analysis</td>
</tr>
<tr>
<td>GAPDH (glyceraldehyde-3-phostate dehydrogenase)</td>
<td>105-204 (1) 973-1072 (2)*</td>
<td>Housekeeping</td>
<td>Not used for analysis</td>
</tr>
<tr>
<td>LDHA (lactate dehydrogenase A)</td>
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<td>Housekeeping</td>
<td>Not used for analysis</td>
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<tr>
<td>SDHA (succinate dehydrogenase complex flavoprotein subunitA)</td>
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<td>Housekeeping</td>
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<tr>
<td>YWHAZ (tyrosine-3-monoxygenase/tryptophan 5-monoxygenase activation protein zeta)</td>
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<td>Housekeeping</td>
<td>USED for analysis</td>
</tr>
</tbody>
</table>

* Probe designs for submission 1 (1) and submission 2 (2)
Appendix B
Samples Submitted to NanoString for Gene Expression Analysis

Table B1
Data for samples sent in the first NanoString submission, April 2016

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<th>Sample ID</th>
<th>Sample Type</th>
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<th>Average duration of LCM (min)</th>
<th>Average days elapsed between LCM and RNA extraction</th>
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<tr>
<td>99</td>
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<td>58.5</td>
<td>55</td>
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<td>126.5</td>
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<tr>
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<td>130</td>
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<tr>
<td>542</td>
<td>Normal</td>
<td>36</td>
<td>45</td>
<td>90</td>
<td>149</td>
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<tr>
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<td>75</td>
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<td>162.5</td>
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<td>50</td>
<td>100</td>
<td>151</td>
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<td>Normal</td>
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<td>70</td>
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<td>Normal</td>
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<td>75</td>
<td>154</td>
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<td>90</td>
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<td>45</td>
<td>110</td>
<td>141</td>
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<td>MCI</td>
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<td>130</td>
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<td>45</td>
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<td>4</td>
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<td>MCI</td>
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<td>110</td>
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<td>Mild AD</td>
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<td>70</td>
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<td>Severe AD</td>
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<td>AVERAGE</td>
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</table>
Table B2

Data for neuron samples sent in the second NanoString submission, September 2016

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<th>Average duration of LCM (min)</th>
<th>Average days elapsed between LCM and RNA extraction</th>
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</thead>
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<td>120</td>
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<td>180</td>
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<td>23</td>
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AVERAGE  11.48  42.988  145.2  7.46
Table B3

*Data for samples sent in the third NanoString submission, March 2017*

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*Samples not used for analysis do to low counts from RNA degradation*
### Appendix C

**Gene Expression Values**

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