AWARD NUMBER: W81XWH-12-1-0302

TITLE: White Matter Glial Pathology in Autism

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REPORT DATE: November 2015

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| 1. REPORT DATE | 2. REPORT TYPE | 3. DATES COVERED |
|---|---|---|
| November 2015 | Final | 1 Sep 2012 - 31 Aug 2015 |

**4. TITLE AND SUBTITLE**

White Matter Glial Pathology in Autism

**5a. CONTRACT NUMBER**

**5b. GRANT NUMBER**
**W81XWH-12-1-0302**

**5c. PROGRAM ELEMENT NUMBER**

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**5d. PROJECT NUMBER**

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
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807 University Pkwy
Johnson City, TN 37614-6500

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSOR/MONITOR'S ACRONYM(S)**

**11. SPONSOR/MONITOR'S REPORT NUMBER(S)**

**12. DISTRIBUTION / AVAILABILITY STATEMENT**

Approved for Public Release; Distribution Unlimited

**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**

Methods used to directly study the autism brain include brain imaging in living patients and pathology studies using postmortem brain tissues from deceased autism spectrum disorder (ASD) donors. These methods typically focus on brain regions as a whole with little regard to the underlying cellular complexity. While informative, these approaches do not provide information about the specific brain cells affected and also have not been successful in revealing the underlying cause of autism. This project uses innovative methods and a novel approach to investigate the pathology of the brain in autism spectrum disorder (ASD). This research employed laser capture microdissection to isolate specific cell populations from carefully defined and specific brain regions from ASD and typically developing control brains. These cells were used to interrogate gene expression abnormalities that may underlie biological mechanisms that contribute behavioral abnormalities of ASD. By examining the ASD brain at the level of its most basic component, the cell, we seek to reveal a potentially unifying cellular pathology of the ASD brain that could be used for the development of therapeutic alternatives for ASD patients.

**15. SUBJECT TERMS**
Laser capture microdissection, transcriptional analysis, RNA-Seq, autism spectrum disorder, postmortem human brain tissue

| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON |
|---|---|---|---|---|---|
| a. REPORT | b. ABSTRACT | c. THIS PAGE | | | USAMRMC |
| Unclassified | Unclassified | Unclassified | Unclassified | 15 | 19b. TELEPHONE NUMBER *(include area code)* |

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# INTRODUCTION

Within the field of research on autism spectrum disorder (ASD), molecular pathology studies to date have used brain samples containing multiple cell types. The results of these studies have been insufficient to formulate a theoretical etiology of the disease. By using an approach targeted at molecular pathology within an enriched population of a single cell type, a common cellular dysfunction might be found that could unify our conceptualization of ASD brain pathology throughout the spectrum. Identification of key cellular abnormalities could result in the development of novel targeted treatments for ASD. In this project, laser capture microdissection (LCM) was used to obtain clusters of multiple cell types and separately, selected cell populations. Using these samples, we attempted to develop experimental protocols to permit gene expression profiling using RNA-Seq technology. For this approach to be useful for future studies, the number of cells captured by LCM had to be reasonably low because of the time and expense of LCM. Ultimately, we hoped to identify unique gene expression abnormalities in specific cell populations to further our understanding of ASD pathology. This type of analysis has the potential to bring light to unanswered questions of ASD pathology, and also to establish a powerful method to investigate the contributory roles of different cell types of the brain in neurological diseases.

# KEYWORDS

Laser capture microdissection, transcriptional analysis, postmortem human brain tissue, RNA-Seq, autism spectrum disorder (ASD)

# ACCOMPLISHMENTS

**What were the major goals of the project?**

The overall goal of this project was to use transcriptional analysis of single cell populations to gain a better understanding of ASD brain pathology. This goal was divided into two tasks briefly described below.

- Task 1 involved the laser capture and preparation of postmortem brain tissue samples from the anterior cingulate cortex for analysis using RNA-Seq. This analysis included both sequencing and bioinformatics to determine genes/pathways of interest. Gene expression changes found to be different between control and ASD samples would be confirmed using PCR methods.
- Task 2 further was to examine the RNA-Seq finding by determining if the gene expression changes found in the anterior cingulate cortex were also present in the prefrontal cortex. This task involved the laser capture of brain tissue samples for PCR analysis for the genes chosen in Task 1.

**What was accomplished under these goals?**

*Amplification Optimization*

Despite delays in laser capture early in the project (discussed below), we were able to use that time to ensure that sample preparation steps were adequate for downstream applications. This included verifying tissue staining methods and confirming that RNA isolation provided adequate amounts of high quality RNA. These experiments were necessary since high RNA integrity is of the upmost importance for the analysis outline in this grant. Most of these experiments went into validating our RNA amplification protocol. The RNA amplification step in sample preparation is a sensitive process that could introduce experimental artifact if not performed correctly or tailored to specific biological samples. Experiments were performed using both commercially available kits and LCM specialized protocols to determine the most effective and reliable method for RNA amplification. The first strand

synthesis step of RNA amplification is crucial since it creates the basic template for amplification. Most methods are based on a 3' bias selection for this step of amplification. This selection technique is beneficial for reducing other RNA species such as rRNA and tRNA from the sample prior to mass amplification. This selection reduces potential bias toward more abundant RNAs since mRNA and non-coding RNA are significantly outnumbered by other RNA species in the samples. For samples collected from LCM, this 3' bias could reduce fidelity in amplification. Using frozen human tissue for these studies means that optimal RNA quality is never achieved because of decay that occurs during the brain collection process. A 3' bias selection could inadvertently exclude mRNAs that are susceptible to 3' degradation. Unlike other kits available on the market, NuGEN amplification kits create a first strand using 3' and random primers giving better transcriptome coverage and reducing potential bias. This feature makes these kits ideal for LCM samples based on its tolerance for less than optimal RNA integrity and a small RNA input requirement (picogram amounts). These kits provide robust amplification typically resulting in microgram amounts of RNA from very little template input. After the completion of NuGEN's innovative short protocol that requires very little hands-on time, enough RNA is produced to use in any downstream analysis.

*RNA-Seq Methods*

Frozen tissue blocks containing BA24 from eight ASD donors and nine typically developed control donors were obtained from BrainNet (formerly Autism Tissue Program, Harvard Brain Tissue Resource Center, Belmont, MA) and NeuroBioBank (formerly NICHD Brain and Tissue Bank for Developmental Disorders, Baltimore, MD). Additional subject samples were prepared but excluded from analysis at various stages of preparation due to poor sample quality factors such as low RIN or insufficient sequencing reads. Superficial white matter was laser captured from BA24 brain sections (10 μm thickness) mounted on PEN membrane glass slides (Life Technologies, Grand Island, NY). Superficial white matter was defined as the white matter area directly adjacent to gray matter and within 3 mm of the white/gray matter border area. Multiple large circular areas were captured for each sample. Pyramidal neurons and white matter astrocytes were stained and captured from BA24 cortical layer 3. Neurons were visualized by staining frozen 10 μm thick sections with the Histogene staining kit (Life Technologies; Grand Island, NY) according to manufacturer's instructions. Astrocytes were identified using a modified glial fibrillary acidic protein (GFAP) rapid immunohistochemistry protocol as previously described[1,2].

RNA was isolated from the captured samples using PicoPure RNA Isolation Kit (Life Technologies, Grand Island, NY) with the additional RNase-free DNase kit (Qiagen, Valencia, CA) step outlined in the manufacturer's protocol. The Ovation Single Cell RNA-Seq System (NuGEN, San Carlos, CA) was used to generate RNA-Seq libraries from isolated RNA. Extensive quality control was performed. Pooled libraries were sent to David H. Murdock Research Institute for sequencing. The HiSeq2500 instrument (Illumina, San Diego, CA) was used for 100 base paired reads with indexing sequencing using the instrument's high output sequencing run.

*Bioinformatics from David H Murdock Research Institute (DHMRI)*

Following sequencing, base calling was performed with CASAVA (v1.8.2) (Illumina, San Diego, CA). Filtering and trimming of reads consisted of removal of Illumina Adapter Library and trimming in the CLC Genomics Workbench 7.0.4 (Qiagen, Valencia, CA). Reads were then aligned to the human genome (latest version, hg18/GRCh38, assembled on December 2013, annotations updated in June 2014) using CLC Genomics Workbench 7.0.4 using the CLC's RNA-Seq package. The Baggerly Beta-binomial test [3] was performed for group comparisons using the control donors as the reference. A false discovery rate (FDR) correction was used to further correct p-values achieved using the above proportion-based tests. We used both paired and unpaired statistical comparisons of control and autism gene expressions for these preliminary data understanding that with the small sample size, neither approach is likely to produce data with high statistical confidence.

*Sequencing Quality Based on Phred Score*

The first step in the analysis of sequencing is base-calling. This process consists of taking the multiple single nucleotide reads and composing the sequence of the fragment clusters. Using the Phred scoring methods, a cut-off score of around 30 is considered an acceptable quality for base-calling. A Phred score of 30 translates to a 1 in 1000 probability of an incorrect base-call or a 99.9% accuracy in sequence detection [4,5]. The PHRED score for all samples (white matter, neuron, and astrocyte preparations) exceeded this cut-off by reaching an average score of 35 to 40. A score of 40 translates to a probability of 1 in 10,000 incorrect base-call or a 99.99% accuracy in detection.

*Mapping and Alignment*

Mapping Percent and Total Reads. White matter containing mixed populations of cells, pyramidal neurons, and GFAP-positive astrocytes were compared to determine if there was a difference in mapping percentages based on sample type. There was no significant effect of sample type on the type of read produced by the samples (Figure 1). White matter and neuron samples were further analyzed to determine if there was a difference in the mapping between control and ASD subject samples. No difference was found between control and ASD samples for white matter (Figure 2A, $p = 0.64$) or neuron (Figure 2B, $p = 0.63$) preparations.

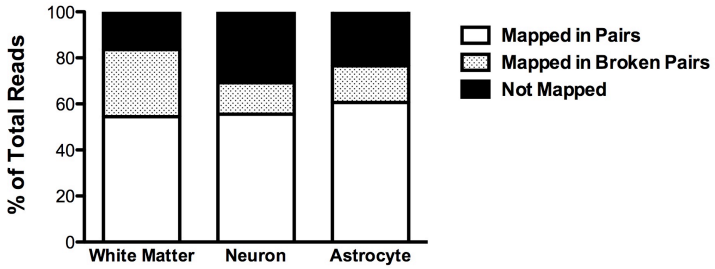


**Figure 1.** Division of read types of the white matter, neuron, and astrocyte RNA-Seq samples. The percent of reads mapped in pairs (white bars), mapped in broken pairs (shaded bars), and reads not mapped (black bars) were plotted as a percent of total reads for all samples.

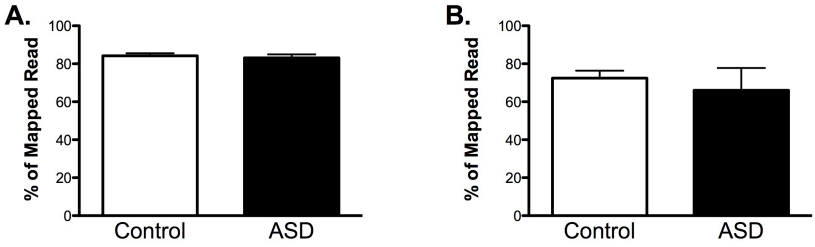


**Figure 2.** Comparison of read mapping between control and ASD samples for white matter (A) and neurons (B). The percentage of total mapped reads (mapped in pairs and broken pairs) of total reads was plotted. No significant difference was found between control and ASD samples for white matter or neuron samples.

Paired Read Mapping. For reads that were mapped as pairs, an analysis was done to investigate where those reads aligned (Figure 3). Approximately 75% of all mapped paired reads aligned to intron regions for all sample preparations. This was also the case when examining the alignment pattern between control and ASD samples for white matter (Figure 4A) and neuron (Figure 4B) sample preparations. To ensure that observed intronic read mapping was in agreement with previously reported brain sample RNA-Seq data, the percentage of intron reads was reported (Table 2) for known high intronic genes [6]. Each gene examined had a 52 to 100% intron mapping percentage.

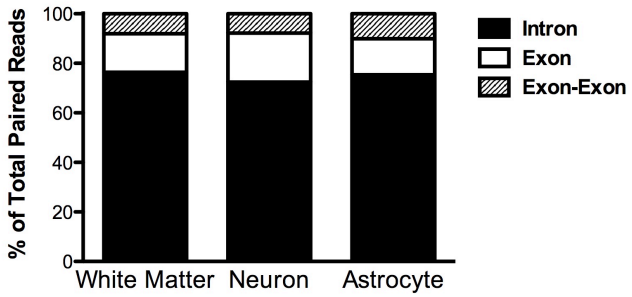


**Figure 3.** Comparison of read type for paired reads in white matter, neuron, and astrocyte samples. The percent of exon (white bar), exon-exon (shaded bar), and intron (black bar) reads of total paired reads were plotted.

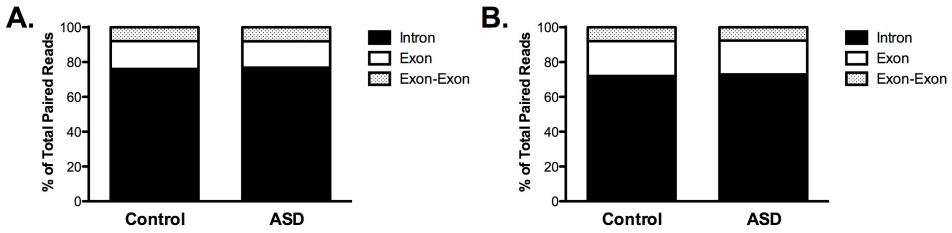


**Figure 4.** Comparison of read type for paired reads in control and ASD white matter (A) and neuron (B) samples. The percent of exon (white bar), exon-exon (shaded bar), and intron (black bar) reads of total paired reads were plotted for white matter and neuron control and ASD samples.

*Bioinformatics from Maverix Biomics*

Due to what we perceived were inconsistencies in the data received from DHMRI, we contracted Maverix Biomics to perform additional bioinformatics services. This company provided a specialized open source pipeline specifically tailored to our samples. We were fortunate to be paired with a project manager who had been involved with the creation of our RNA-Seq library prep kit and understood the challenges associated with our sample type. Since this bioinformatics analysis was meant as a check for the first round of analysis, we only submitted the pyramidal neuron samples to Maverix Biomics. Fastq sequencing files obtained from DHMRI were submitted to Maverix. The sequencing data was subjected to quality analysis before and after trimming. Much like the DHMRI results, our samples showed above average quality after trimming and filtering. TopHat was used to map our samples to human genome assembly hg19. Our mapping percentages mostly fell between 54.34% and 94.4%, excluding an outlier at 8.55%. This outlier was detected in both the DHMRI and Maverix analysis. Read amounts indicated a 1x plus coverage of the genome. Next, the reads were divided among read types. Each of the neuron samples seems to have similar patterns of read types with a larger percentage mapping to introns. All samples also included exon reads, intergenic reads (to varying degrees), 3' UTR exons, and 5' UTR exons. Lastly, differential expression genes between control and ASD samples were determined using CuffDiff and EdgeR analysis

One of the advantages of the Maverix platform was the ability to visualize sample read alignment using their integrated genomic browser. It was obvious after seeing the alignment why the data from DHMRI had been so variable. Despite high sequencing quality, gene coverage was inconsistent and generally low. This resulted in reported gene expression changes that were solely based on a lack of coverage. In addition to low coverage, PCR overamplication was also detected in all samples. PCR amplicons represented 96% to 99% of reads in each sample. This could help explain the deficiency of coverage for the genome.

*PCR Confirmation of RNA-Seq Data*

Even with the obvious coverage issues, PCR confirmation was attempted for several genes from both the DHMRI and Maverix analysis using laser captured pyramidal neurons. Seven pairs were used

for confirmation included the four pairs used for RNA-Seq analysis. For PCR confirmation of RNA-Seq data, RNA was isolated from the captured samples using PicoPure RNA Isolation Kit (Life Technologies, Grand Island, NY) with the additional RNase-free DNase kit (Qiagen, Valencia, CA) step outlined in the manufacturer's protocol. RNA samples were reverse transcribed into cDNA using the Superscript III kit (Life Technologies; Grand Island, NY) that contained oligodT and random hexamer primers. Gene specific primers were purchased from a vendor (Qiagen; Valencia, CA). To quantify transcripts, endpoint PCR was used for RNA isolated from laser captured cells as previously described[2,7]. Endpoint PCR data was computed as relative values generated from the ratios of amounts of target gene expression to a reference gene. Afterwards, endpoint PCR data were analyzed by the paired Student's t-test.

For the DHMRI data, we were not successful in confirming any gene that had been found to be significantly different using their analysis. We determined this was due to variation within the sample pairs and created our own system of finding candidate genes. The basis of our search involved sorting genes based on a cutoff level of expression to ensure that the data could be reproduced as well as ensuring that all pairs exhibited similar trends in expression changes. Based on these criteria, a list was produced. One of the most interesting genes found using this method was *DLG4*, the gene for PSD95. Based on the most recent information from Maverix, *DLG4* had an increase in abundance in controls (83.304) compared to ASD (33.477) donors and resulted in a Log2 fold changed on -1.32. When PCR confirmation was attempted in seven pairs, *DLG4* was found to have no significant difference between control and ASD donors (Figure 5).

Based on Maverix's bioinformatic data, two genes were found to be significantly different between control and ASD donors using EdgeR paired analysis. *CALM1* was found to be reduced in abundance between control (577.653) versus ASD (351.831) donors. SYT1 was also followed the same trend with higher gene expression in control (525.397) compared to ASD (363.199) donors. However, neither of these gene were significantly changed using endpoint PCR confirmation methods in seven pairs (Figure 5).

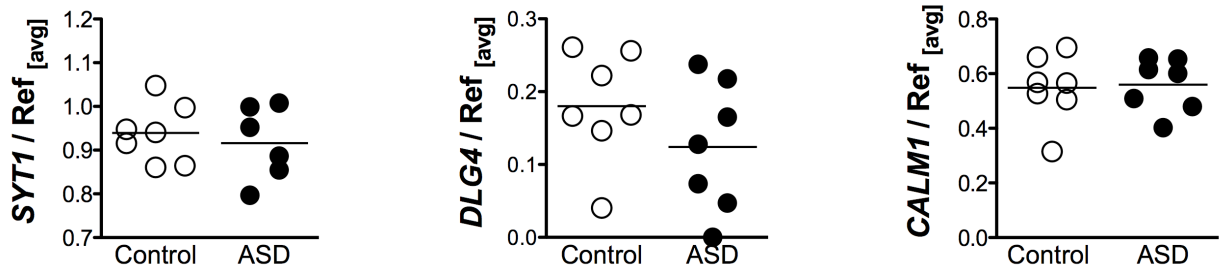


**Figure 5.** PCR confirmation of candidate genes in laser captured pyramidal neurons from BA24. Endpoint PCR was used to determine differences between control (open circles) and ASD (closed circles) donors (n=7). Data for each gene was normalized by the average of two reference genes (*GAPDH* and *RNA18S*). No significant difference was found for any of the genes.

It is also important to note the RNA-Seq bioinformatics data did not match previously differential expressed genes that were discovered by us using a targeted gene approach with standard PCR. In previous studies, we found *Ntrk2* to be significantly reduced in BA24 laser capture pyramidal neurons (Figure 6). A subset of the subjects used in that study were also used for the RNA-Seq analysis. Based on the latest Maverix-analyzed RNA-Seq data, no significant difference was found for this gene using sequencing analysis ($p = 0.98$). The lack of confirmation for this gene leads us to further doubt the validity of the data produced using the RNA preparation methods for RNA-Seq described.

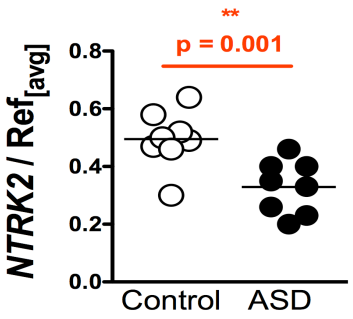


**Figure 6.** Significant reduction in *Ntrk2* in laser captured pyramidal neurons from BA24. Endpoint PCR was used to determine differences between control (open circles) and ASD (closed circles) donors (n=8). Data was normalized by the average of two reference genes (*GAPDH* and *RNA18S*). These data were not confirmed by RNA-Seq.

*Conclusions*

When developing this method, efforts were made to circumvent the potential pitfalls associated with analyzing LCM samples. We were able to control for the limitations of these samples by selecting protocols suitable for sample type and putting in controls for the biological variances of human studies. There is still more work to be done to produce a full-scale analysis of transcription regulation underlying ASD brain pathology. Based on our data and the lack of PCR confirmation results, we need to focus on further optimizing our RNA-Seq methodology. It is clear that with the PCR artifacts that exist in the samples that coverage will not be high enough to determine true changes in gene expression. A disadvantage to using LCM collected samples is the small amount of material that can be obtained. When coupling LCM with the use of postmortem brain tissue, restrictions such as cost, time, and limited availability of tissue does not allow for the collection of sufficient amounts of input materials needed for many downstream applications. Due to the limited amount of sample that can be obtained from LCM, sample amplification is an unavoidable preparation step in the transcriptional analysis. The amplification step in sample preparation is a sensitive process that can and will introduce experimental artifact if not performed correctly or tailored to specific biological samples. Experiments were performed using both commercially available kits and LCM specialized protocols to determine the most effective and reliable method for sample amplification. The first strand synthesis step of RNA amplification is crucial since it creates the basic template for amplification. Most methods are based on a 3' bias selection for this step of amplification. This selection technique is beneficial for reducing other RNA species such as rRNA and tRNA from the sample prior to mass amplification. This selection reduces potential bias toward more abundant RNAs since mRNA and non-coding RNA are significantly outnumbered by other RNA species in the samples. For samples collected from LCM, this 3' bias could reduce fidelity in amplification. Using frozen human tissue for these studies means that optimal RNA quality is never achieved because of decay that occurs during the brain collection and LCM process. A 3' bias selection could inadvertently exclude mRNAs that are susceptible to 3' degradation. Unlike other kits available on the market, the NuGEN amplification kits create a first strand using 3' and random primers giving better transcriptome coverage and reducing potential bias from degradation effects. This feature makes the kits ideal for LCM samples based on its tolerance for less than optimal RNA integrity and a small RNA input requirement of 100 picograms. However, these kits lack ribosomal depletion and mRNA selection steps which can reduce read depth of exons and bias samples to pre-mRNA or intron containing transcripts. Intron spanning regions are larger than exon regions and are more abundantly represented in the samples. It is still unclear if our samples produced enough exon based reads to truly reflect transcriptional changes at the mature mRNA level. We have recently obtained information for new methodology that will allow us to remedy our PCR overamplification issues. We plan to utilize these methods to continue optimizing our RNA-Seq experiments. This work is continuing in the lab using funds from our University. We have also submitted a grant to the NIH to support further study. We are confident that we will soon have technical issues worked out and will be able to conduct RNA-Seq analysis on laser captured single cell populations from the human brain.

**What opportunities for training and professional development has the project provided?**

Training
Work towards the completion of this project provided a training experience for the graduate student in the PI's lab. The work from the project was included in this student's dissertation to fulfill the requirement for her Ph.D. in Biomedical Sciences (see Products section for more details).

Professional Development
The travel expenses for this grant were used to send the key laboratory scientist, Dr. Michelle Chandley, and the PI's trainee, Jessica Crawford, to the 2014 International Meeting for Autism Research held in Atlanta, GA. This meeting was hosted by the International Society for Autism Research and other nonprofit agencies that both support and fund autism research. This conference allowed these individuals to meet experts, identify future collaborative projects, and gain exposure to other projects happening in the field.

**How were the results disseminated to communities of interest?**
Nothing to Report.

**What do you plan to do during the next reporting period to accomplish the goals?**
Nothing to Report.

## IMPACT

**What was the impact on the development of the principal discipline(s) of the project?**

Researchers including our laboratory have shown that analyzing gene expression differences between two groups of subjects (disease versus control) is significantly impacted by the resolution of the dissection of brain tissue. This seems obvious when considering the possibility of homogenizing and entire brain versus homogenizing a discrete brain region to search for gene expression differences between groups. This improvement in the detection of differential gene expression by increased resolution is also highly relevant when one dramatically enhances the resolution of inspection to the cellular level. Ginsberg et al[9] has shown significant disparities between gene expression differences found using homogenate brain samples (of a discrete brain region) versus laser captured single cell populations from the same discrete brain region. In that study, gene expressions found to be abnormal in hippocampal CA1 neurons in Alzheimer's disease were not found significantly altered when measured in a homogenized hippocampal sample. That study demonstrated the need to focus gene expression profiling on single cell populations in order to achieve an accurate understanding of factors and pathways involved in cellular pathology. As we have mentioned previously, all molecular pathology studies to date using ASD donor brain tissue have analyzed brain samples that contain multiple cell types. In this DOD-funded project, laser capture microdissection was used to capture specific cell populations in order to perform gene expression profiling using RNA-Seq. To date, we are aware of only one study in the literature that has succeeded in performing this type of study. The reproducibility of their approach remains to be determined. At the end of this DOD-funded project, we conclude that we have made significant advances in achieving the combined use of laser capture microdissection with RNA-Seq to differentially profile the transcriptome of single cell populations. However, we cannot claim success at this point. Although we were able to move the methodology forward, there remain adjustments and improvements that are needed to bring our results to a reproducible level. In this regard, we believe that we are very near having the technical issues sorted out with this approach and are continuing this work with funds from our University. Additionally and importantly, we were able to generate sufficient preliminary data to submit an R01 grant application to the NIH to use this method to study cellular pathology in ASD. This grant application will be resubmitted in March of 2016, when we hope to have additional preliminary data from current ongoing

research using the LCM/RNA-Seq approach. Ultimately, results of this project will not only bring light to unanswered questions of ASD pathology, but will also establish a method that has not previously been used to investigate the contributory roles of specific brain cell pathology in neurological diseases.

**What was the impact on other disciplines?**

Once this combined use of laser capture of single cell populations with RNA-Seq is optimized, we anticipate that it will be used widely to investigate the cellular pathology of many brain illnesses.

**What was the impact on technology transfer?**

Nothing to report.

**What was the impact on society beyond science and technology?**

Until the technology is further improved, nothing at this point in time.

## CHANGES/PROBLEMS

**Changes in approach and reasons for change**

Since the submission of this project's 2013 annual report, we have made improvements on the technology we chose to answer the overall hypothesis of this project. The project originally outlined the analysis of cell populations using microarray technology. We decided, with guidance from reviewers of this grant application, to use RNA-Seq technology instead. The use of RNA-Seq technology exponentially increases the amount of transcriptome data produced from each sample. Another benefit of RNA-Seq is the ability to compare the data from this study with other RNA-Seq based studies. RNA-Seq is analyzed as an absolute quantification of gene expression while microarray data is relative expression analysis, and its data can only be compared within a specific experiment. This modification in methodology did not alter the overall goal of the project, but it improves the data produced from this study.

In the original Statement of Work (SOW), we proposed to capture single cell glial populations for analysis. After extensive optimization for RNA-Seq analysis, we decided that we would analysis laser capture superficial white matter samples and pyramidal neuron instead. The confirmation of RNA-Seq findings would then be performed in the matching laser captured materials. This decision was made on both a scientific and financial basis. All superficial white matter and pyramidal neuron samples were captured, RNA-Seq libraries were created from RNA isolated from these samples, and samples were submitted for sequencing and bioinformatics analysis at David H. Murdock Research Institute (DHMRI).

**Actual or anticipated problems or delays and actions or plans to resolve them**

After receiving this award, the Arcturus Veritas LCM instrument that was to be used for cell capture became nonfunctional. After some typical administrative delays for purchasing such an expensive piece of equipment, we replaced the Veritas with another instrument. That new instrument (Leica brand) was deemed by us to be unusable for downstream mRNA studies and returned to the company. We then purchased a new Arcturus XT instrument and were able to resume our work. The details of this delay have been described previously, and are elaborated below.

The Arcturus Veritas LCM instrument uses an infrared laser to adhere tissue to a polymer-coated cap. If the machine loses the ability to correctly place the cap or fire the laser, sample capture is not possible. Both of these instrument errors occurred at the beginning of this project. Since the instrument was still under a service contract, the company made several attempts to repair the instrument including a complete overhaul of the system. Because the instrument was no longer being sold, the company was unable service the machine to working order. The determination that the Arcturus Veritas machine could not be repaired was made several months following the awarding of our DOD grant.

In efforts to reduce downtime, we immediately started the process of purchasing a new LCM

instrument. Based on available purchasing funds, we wished to move away from LCM cap-based technology because of the added expense of consumables for this method of LCM. Our university selected the Leica LMD6500 instrument for purchase, primarily based on their claims that this instrument could be used for downstream gene expression research. Leica assured us that the instrument was superior and capable of providing quality RNA following cell capture. Following purchasing and delivery delays, the LMD6500 instrument was installed on May 8, 2013. Once the instrument was installed, we immediately started performing validation experiments to determine the suitability of this instrument for RNA analysis. Between May and September, we worked closely with the company to get the machine in full working order. There were several pieces of equipment on the new instrument that had to be replaced, which resulted in multiple service visits to repair the machine. The new software system on the instrument also posed problems thus hindering the ability to capture samples. The machine was nonfunctional for roughly half of this five-month period. The machine is still experiencing both hardware and software problems.

During the time we were trying to get the new LCM instrument working, we were able to perform enough experiments to determine if the LMD6500 instrument was capable of producing captured samples with high RNA integrity. RNA was isolated from captured tissue samples and analyzed using the Bioanalyzer Pico 6000 RNA chips (Agilent Technology) to determine both RNA quality and quantity. Acceptable RNA quality is defined as a RNA Integrity Number (RIN) of 5.0 or greater. This is the minimum requirement for our downstream RNA amplification process, and it is imperative that this RIN threshold is met. Studies show that RNA degradation can alter results by producing false changes in gene expression analysis[8–10]. We were unable to reliably produce captured samples with acceptable RNA quality using the LMD6500 instrument. In order to rule out RNA degradation due to tissue and sample preparation, studies were performed using different slides types, collection methods, tissue types, staining protocols, laser firing approaches, and RNA isolation kits. Based on our results, these preparation steps produced no difference in the RIN obtained from samples captured using the Leica LMD6500 instrument. In fact, after detailed analysis, we concluded that this instrument destroys RNA quality and that the use of the instrument's UV laser and its prism-guided movement is the causal factor.

Arrangements were then made to complete the grant using the facilities and equipment at David H. Murdock Research Institute (DHMRI) in Kannapolis, NC. The DHMRI employs experts in the field of laser capture microdissection and transcriptional analysis. This research facility has a Zeiss Palm Microbeam laser capture instrument as well as all the resources need to complete the transcriptional analysis outlined in this grant. DHMRI made the Palm instrument available to us on a fee-for-service basis. We considered this a possible alternative because the UV laser on the Palm system is lower energy and the laser cutting movement is guided by a different engineering technology than the UV beam on the Leica system. Work began immediately to validate the Zeiss Palm LCM system for RNA analysis. Various samples were collected to test the quality of post-capture samples. After analyzing the samples using the Agilent Bioanalyzer, it was concluded that a RIN of 5.0 or greater could not be achieved using this laser capture technology.

Based on the data produced from the Leica LMD6500 and Zeiss Palm Microbeam instruments, we concluded that instruments that focus the UV cutting laser through the microscope objective would not produce reliably high quality RNA. The reasons why are not fully understood, but it is possible that microscopic irregularities within the objective lens of these instruments may disperse the UV radiation resulting in RNA damage. Based on this conclusion, our university ordered an Arcturus XT LCM instrument. This instrument is an updated version of the Veritas model that we have utilized in the past to generate all the LCM data published from our lab[1,11]. Again, this instrument uses an infrared laser and we have previously confirmed that this method of cell capture does not damage RNA quality as much as UV lasers. Hence, despite our attempts to move away from cap-based LCM technology because of the cost of consumables needed for this capture method, we have determined Arcturus instruments are the only instruments that can provide reliable RNA quality using the LCM methods outlined in our Statement of Work. Our university purchased the Arcturus XT instrument allowing our lab to have constant access to this piece of equipment. Due to our previous experience with the Arcturus Veritas instrument, we were able to start immediately capturing the materials needed for the project.

**Changes that had a significant impact on expenditures**

The grant that was awarded to us paid for supplies, sequencing and bioinformatics. It did not pay for any part of the salaries of the three main investigators, Drs. Ordway, Crawford, and Chandley. The East Tennessee State University paid these salaries. Now that the DOD funds have been expended, it is obvious that more funds are needed to achieve the goals of this proposal. Again, the East Tennessee State University is supplying these funds. Had everything worked exactly as planned, we believe the original award was enough to pay for the supplies, sequencing and bioinformatics. However, since this project is attempting to achieve that which only

**Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

Nothing to report.

## PRODUCTS

**Publications, conference papers, and presentations**

**Journal publications**

**Books or other non-periodical, one-time publications**

Crawford, Jessica D. "Cellular-based Brain Pathology in the Anterior Cingulate Cortex of Males with Autism Spectrum Disorder" December 2014. Doctoral Dissertation. Accepted. (Withheld from publication for 2 years)

**Other publications, conference papers, and presentations**

**Technologies or techniques**

**Other Products**

Whole transcriptome sequencing data was produced for 25+ samples. These samples represent different tissue/cell types as well as different subject demographics. This information will be release to an autism database following a full analysis by our lab.

An ASD-related R01 NIH grant application that includes RNA-Seq was submitted based on data generated in this pilot grant.

## PARTICIPANTS & OTHER COLLABORATING ORGANIZATION

**What individuals have worked on the project?**

| | |
|---|---|
| Name: | Gregory A. Ordway, Ph.D. (No Change) |

| | |
|---|---|
| Name: | Michelle J. Chandley, Ph.D. (No Change) |

| | |
|---|---|
| Name: | Jessica D. Crawford, Ph.D. (No Change) |

**Has there been a change in active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

**Ongoing Research Support**

**The following grants have ended for the PI:**

5R01 MH 46692 Ordway (PI) 09/01/91-03/31/13
NIMH "Noradrenergic System in Depression"
This research tests the hypothesis that dysfunction of noradrenergic neurons is closely associated with glial disruption in major depressive disorder. Laser capture microdissection and quantitative PCR methods are employed to study gene expression along specific pathways in noradrenergic locus coeruleus neurons, astrocytes and oligodendrocytes from assiduously matched control and major depressive disorder subjects.

Distinguished Investigator Award Ordway (PI) 2/9/09-2/8/11
American Foundation for Suicide Prevention. "Glutamatergic signaling in the locus coeruleus in depression and suicide"
The major goal to examine the quantitative expression of glutamate receptor genes in noradrenergic neurons in the human locus coeruleus from victims of suicide that had major depression at the time of death, and to determine whether glutamate receptor gene expression changes are also observed in the entorrhinal cortex.

AS#7330 Ordway (PI)
Autism Speaks "Glia Pathology in Autism" 3/1/11-2/28/13
The goal of this project is to measure levels of expression of several genes associated with glutamate transmission in pyramidal neurons and surrounding astrocytes in postmortem anterior cingulate cortex of young adult autism subjects and matched normal control subjects.

**The following grant has been awarded to the PI:**

SRG-0-100-13 Ordway (PI)
American Foundation for Suicide Prevention; "Oxidative DNA Damage in Brainstem Oligodendrocytes in Depressed Suicide Victims; 6-3-14 to 7-1-16
This project examines whether the relative density of noradrenergic innervation to a brain region affects the susceptibility of oligodendrocytes to telomere shortening and oxidative stress as observed in depressed suicide victims. To examine this, oligodendrocytes will be captured from the region of the brainstem locus coeruleus (high norepinephrine) and occipital cortex white matter (low norepinephrine) from depressed suicide victims and matched psychiatrically normal control subjects.

**What other organizations were involved as partners?**

Nothing to report.

## SPECIAL REPORTING REQUIREMENTS

Nothing to report.

## APPENDICES

### REFERENCES

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