New Insights into Neuroscience Research
Using nCounter® Technology for Comprehensive Gene Expression Profiling and Biomarker Identification

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NanoString Technologies, Inc., Seattle, WA 98109
Authors: Katie Buchanan and Joseph M. Beechem
Introduction

Diagnosis and treatment of neurodegenerative disease has long been impeded by the difficulty in accessing the affected areas of the brain. Unlike a suspicious mole on the skin or inflamed tissue around a wound, the brain presents a greater challenge to examine. Most neurodegenerative disease diagnoses have relied on post mortem confirmation of disease pathology or medical imaging during disease progression. Early diagnosis and treatment have remained elusive but have accelerated using advanced molecular biology methods including gene expression and profiling, mRNA analysis, and even investigations of single cells derived from neural cell lines.

One challenge facing all these advanced techniques is the scarcity of sample. To accurately analyze brain tissue, blood samples, or microvolumes of single cell supernatant requires highly sensitive and robust technology beyond traditional methods. Using NanoString’s nCounter technology, scientists have been able to profile specifically curated gene expression panels with minimal hands-on time to generate accurate, insightful, and repeatable results. Already researchers are making significant strides in understanding neurodegenerative disease and mechanisms of neuroinflammation. The following five publications represent groundbreaking research in neuropathology, including Alzheimer's disease, Parkinson's disease, and Amyotrophic Lateral sclerosis. In each study, NanoString technology was the cornerstone for research and results beyond today’s standards of qPCR and microarray analysis. The studies are leading to a more comprehensive understanding of neurodegenerative disease and treatment.

Comprehensive Analysis from FFPE

Researchers unlock gene expression data from many different sample types

There is an increasing body of evidence that noncoding, expanded repeats in RNA can contribute to the pathogenesis of neurodegenerative and neuromuscular disorders. In the case of familial amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) patients, a newly discovered hexanucleotide GGGGCC repeat expansion in the noncoding region of the C9ORF72 gene has been confirmed in over 40% of patients. While the function of the C9ORF72 protein remains unknown, it represents a potential candidate for screening and therapeutic intervention. In this study by Jeffrey D. Rothstein et al., the group characterized C9ORF72 to determine if expansion of the repeat contributes to the disease progression1.

The team directly assayed samples of patient-derived human brain tissue, ALS fibroblasts, iPSCs and IPSNs to determine the levels of C9ORF72 RNA. NanoString RNA detection allows highly sensitive, direct screening of tissue without any nucleotide amplification and confirmed C9ORF72 iPSCs (and ALS patient tissue) exhibit the GGGGCC repeat expansion and have reduced C9ORF72 RNA levels. Complicating matters is that there are three mRNA products transcribed from the C9ORF72 gene: C9ORF72 Variants 1, 2, and 3 (V1, V2, V3, respectively). The roles of these three variants is also unknown but V1 and V3 contain ORFs upstream of the expanded GGGGCC repeat. To detect the levels of each C9ORF72 RNA variant and to compare each variant between samples, the group generated two 50-mer NanoString probes for each transcript that targets one of the C9ORF72 variants. In order for the specific RNA variant to be detected, each 50-mer probe must bind in tandem to the transcript. C9ORF72 ALS patient tissue and IPSNs showed approximately a 50% reduction in expression of C9ORF72 V1 & V2 (Fig. 1). Patient-derived fibroblasts showed low expression with no differences between control and diseased tissue.

The group then used microarrays to assess other genes that have different expression levels in C9ORF72 iPSCs, fibroblasts, and human motor cortex. Genes were selected that code for proteins expressed in the CNS and that are predicted to be secreted, thus allowing for easy detection and monitoring in patient CSF samples. This led to the successful identification of sixteen aberrantly expressed target genes in C9ORF72 ALS patient tissues, seven of which showed similar dysregulation patterns when compared with IPSNs. These seven genes, whose expression mirrors the C9ORF72 disease state, can be potential candidates for disease biomarkers to monitor therapeutic intervention and are easily collected from patient blood or CSF samples. The NanoString profiling will continue to facilitate analysis of gene expression in human patient samples without the need for amplification steps. This not only reduces error and variability, but maximizes the amount of data that can be gathered from these patient samples and biomarkers.
Further Reading: Using Nanostring’s nCounter Human Inflammation Gene Expression panel, researchers at the University of Alberta studied the expression of 184 inflammation-related genes from FFPE brain samples to compare host innate immune response and bacterial polyglycan (PGN) presence in MS and non-MS samples. They found that PGN presence was inversely correlated with the intensity of LFB (luxol fast blue) stained sections of MS lesions, and host gene expression analysis revealed the induction of immune genes NFKB1, RIPK1, and IL-12A, demonstrating the highest immune response correlation with PGN in MS brains. They concluded that demyelination, PGN, and inflammatory gene expression accounts for 86% of observed variance in MS, and inflammatory demyelination could contribute to underlying disease mechanisms2.

Cell Type Profiling
Detection of protein in neurons and astrocytes down to a single cell
Amyloid precursor protein (APP) is an integral membrane protein found in high levels in the synapses of neurons. APP can be cleaved into soluble amyloid precursor protein-α (sAPPα); alternatively, APP can be cleaved to generate the insoluble amyloid β-protein (Aβ). Alzheimer’s disease is neurodegenerative disease characterized by accumulation of Aβ, creating extracellular amyloid plaques, presenting as lesions on the brain. To understand why either sAPPα or Aβ are generated, most research has utilized heterologous cell lines that were not derived from neuronal cells. As a result, the contributions of different cell types to the overall disease progression has not been well characterized.

There is an increasing body of evidence that specific cell types may secrete products that contribute to the pathology of AD. Tracy L. Young-Pearse et al. had already established protocols to differentiate human induced pluripotent stem cells (iPSC) to neuronal and glial cells found in the forebrain3. In order to examine the roles of individual cells in more depth, they adapted a technique known as microengraving to their studies of AD. In microengraving, cells are cultured in a dense array of nanowells, allowing analysis of secreted products from individual cells. In particular, the group examined the differential processing of APP from single living neuronal and glial cells derived from iPSCs.

Each nanowell array contains 84,672 wells on a glass microscope slide; each well is only 50 x 50 x 50 μm. Each nanowell constitutes an individual experiment and because the cells are live, they can be revisited over time. Optimizing data collection from this undertaking requires tools that are sensitive, robust, and can handle large amounts of data. The team used NanoString’s Single Cell Multiplexing capabilities to analyze gene expression of single neurons and glia. The custom-designed 150 gene NanoString CodeSet amplified gene products from each microwell, targeting cell fate markers, AD-related genes, and housekeeping genes. When they analyzed the data with the nCounter Digital Analyzer and software, they found they were able to detect Aβ and sAPPα from these individual cells at multiple time points (Fig 2).

FIGURE 1: C9ORF72 ALS-Specific Gene Expression1 Researchers performed targeted gene expression analysis of aberrantly expressed genes on NanoString’s nCounter platform, and revealed dysregulation of seven genes that were also found to be similarly dysregulated in C9ORF72 iPSNs.

FIGURE 2: Examination of gene expression profiles after detection of sAPPα and Aβ from single iPSC-derived neural cells3 HiPSC-derived neural cells were lysed, cDNA was synthesized and hybridized to a custom NanoString codeset for analysis. A heat map of expression data of select genes are shown for each cell, as well as for pools of 500 cells each.
Whereas previously only pooled groups of cells could be studied, now individual cells can be profiled to understand how they contribute to disease progression. More importantly, demonstrating that iPSCs are compatible with microengraving can reduce the reliance on immortalized cell lines that are less relevant or possibly even misleading. Taken together, this paper presents groundbreaking new methodology and a turning point in the study of AD and other neuronal diseases.

Further Reading: Researchers at UC Irvine published in Neuron in 2017 a process for producing induced microglia-like cells (iMGLs) from induced-pluripotent stem cells (iPSCs). In order to demonstrate that genomic integrity was maintained after differentiation, the team used NanoString's nCounter Human Karyotype CNV panel and found a high correlation (r2 > .92) between the iMGL and iPSC genomes. This study demonstrates that iMGLs can be used as a renewable source of patient-derived cells for determining gene function in neurodegenerative diseases.

Gene Signature Generation

nCounter platform allows rapid identification of microglia gene signatures

Microglia are a key component of the immune response in the central nervous system. Studying the role of these cells in the immune response is hampered because they lack clearly identifiable features that would distinguish them from infiltrating macrophages. Howard L. Weiner et al. used extensive gene and microRNA (miRNA) array screens and were able to identify a unique signature dependent on TGF-β1 in both mouse and human microglial cells. To this end, the team worked with NanoString to create a custom mouse codeset containing 354 microglial enriched genes, 40 inflammation-related genes, and six housekeeping genes. This chip, referred to as MG400, formed the basis of their studies.

The MG400 chip analysis enabled the group to analyze immune cell populations and create both a heatmap and clusters of the key microglial molecules as grouped by cell location and function (Fig. 3). Six of the genes identified showed little to no expression in immune cells or macrophages from other organs—they were specific to microglial cells. Additionally, the genes displayed conserved expression patterns in human microglia.

The team then used a NanoString mouse miRNA panel of 600 miRNAs to assess microglial cells with respect to other immune cells. This identified eight miRNAs highly expressed in microglial cells, three of which were unique to microglial over immune cells, resulting in the identification of a miRNA signature specific to microglia. Subsequent investigation showed that the signature is specific to developing microglial cells in vivo.

Surprisingly, this signature is not present in monocytes recruited during neuroinflammation nor is it expressed in established microglial cell lines in vitro. The team determined that the signature is inducible by culturing adult microglia cells in the presence of TGF-β1, and confirmed this by showing a loss of microglia in mice deficient for TGF-β1 in the CNS.

While an undertaking of this magnitude is possible using standard qPCR techniques, it was no doubt greatly enhanced and expedited by utilizing the NanoString nCounter platform. This extensive transcriptomic comparison of sorted mouse cells to identify the microglia gene signature required the efficient analysis of over 400 gene targets in at least 30 distinct cell populations, as well as 600 miRNA targets in 24 immune cell types and multiple CNS cell subsets. Now that this signature has been identified, the role of microglia in protection and damage to the CNS can be characterized and eventually modulated to potentially combat neurodegenerative disease.

FIGURE 3: Identification of a miRNA microglia signature. NanoString 600 miRNA nCounter chip was used to create a heatmap and hierarchical clustering of differentially expressed miRNAs in microglia, organ specific macrophages and immune cell populations to identify an novel miRNA gene signature for microglia.
Further Reading: A team of researchers led by Sellgren at Massachusetts General Hospital set out to generate human microglia-like cells from human somatic cells and demonstrate psychiatric disease-relevant application. The team isolated total RNA for a custom panel of probes developed with NanoString, and a miRNA panel of 800 miRNAs to compare the patient-derived human microglia-like cells to human fetal microglia cells. They were able to demonstrate that patient-derived human microglia-like cells performed similarly to actual microglia cells and they provide an opportunity for high-throughput drug screening that is not possible with post-mortem tissue.

Microglia Activation

Researchers demonstrate a therapeutic approach for neurodegenerative diseases by regulating microglia activation

Early studies on the brain and its cellular structure described it as a static environment, where neurons did not regenerate and non-neuronal cells played a minimal role. However, that view is changing rapidly, with studies revealing neuronal regeneration and the critical and varying roles played by supporting cells. One of those supporting cell types, microglia, are particularly important in maintaining a healthy environment as the brain changes during development or in response to damage.

Microglia are the phagocytes of the brain. They detect damaged or dying tissue, engulf the cellular debris, and clear the area so that normal growth or repair can occur. In addition to phagocytosis, microglia can enter either a pro- or anti-inflammatory state. In the anti-inflammatory state, microglia are thought to aid healing by minimizing inflammation. Conversely, microglia in the pro-inflammatory state are believed to contribute to cell damage, even while they continue to remove potentially damaging cellular debris. To further complicate the role of microglia, phagocytic cells produce reactive oxygen species (ROS) to break down ingested debris. However, the ROS produced can be damaging to nearby cells. It remains unclear whether these activities are independent or interrelated. A clear understanding of the molecular mechanisms regulating microglia activity could provide new therapeutic targets for a range of neurodegenerative diseases and cancers.

In a recent publication in the Journal of Neuroinflammation, Siddiqui et al, sought to address the question of how microglial states and activities interact and whether their state can be altered.

The authors took advantage of NanoString’s high throughput nCounter system to analyze mRNA expression levels of six independent microglia cultures for each of three conditions: control, pro-inflammatory and anti-inflammatory; examining each with or without myelin.

NanoString designed a custom panel with three housekeeping genes for normalization and 36 genes selected by the authors that have links to activation by microglia, phagocytosis, ROS production, and inflammation.

One key result from this study is that activated microglia can be stimulated to switch from pro- to anti-inflammatory. Initially, changes in mRNA expression levels demonstrated that resting glia could be consistently activated into pro- or anti-inflammatory states by treatment with cytokines (Fig. 4).

Next, the same treatments were applied to activated cells. Using the genetic markers that characterize either the pro- or anti-inflammatory states, the authors show that activated glia can change to the opposite state.

For example, expression of TNFα mRNA increases when glia are activated into the pro-inflammatory state (Fig. 4, blue box). When the activated pro-inflammatory cells are treated with a protocol to induce an anti-inflammatory state, TNFα levels decrease. Activated glial cells can be reversed to the other direction as well, from anti- to pro-inflammatory (Fig. 4, Red box).
The extent of changes in expression levels varies by gene, but the changes are consistent. This suggests that regulating the activation state of microglia cells could be a therapeutic approach to supplement current therapies for cancer and neurodegenerative diseases.

Further Reading: In a 2017 study in Translational Psychiatry, Muhie’s team showed that PTSD activates and sustains inflammatory pathways by analyzing differentially expressed genes on Nanostring’s nCounter Analysis System. Sustained neuroinflammation appears to drive the developmental and behavioral manifestations of PTSD. Blood samples show promise as a suitable and accessible stand in for brain specimens for clinical translation8.

Early Predictors of Parkinson’s Disease
Analysis of SNCA isoforms as potential early stage biomarkers

The α-synuclein (SNCA) gene has traditionally been thought of as a “neuron-specific” gene because high levels of the α–synuclein protein accumulate in the brain in Parkinson’s disease. When it was discovered that high levels of α–synuclein are found in circulating blood cells, including red blood cells, it raised interest in this gene as an easily accessible indicator of the disease. Researchers began investigating how transcripts of α–synuclein in the blood could be used to track disease progression.

Under the direction of Clemens R. Scherzer, a team of researchers evaluated the levels of SNCA mRNA in three independent biomarker cohorts9. Using qPCR, microarray analysis, and NanoString technology, the group found that SNCA transcripts were consistently reduced in patients with Parkinson’s disease in each cohort. This finding seemed counterintuitive—the α–synuclein protein accumulates in the brain in Parkinson’s disease and yet there was an overall decrease in SNCA transcripts. It is notable that there are several isoforms of the SNCA transcript and some of these isoforms have been thought to have a role in Parkinson’s disease. Taken together, this led the group to extend their studies into patients who were earlier in the disease progression. The Parkinson’s Progressive Markers Initiative (PPMI) identifies individuals who are clinically at the early stage of Parkinson’s but not yet at the full disease state. Detailed multiplexed digital analysis of 340 patient samples using the NanoString nCounter Analysis System demonstrated that disease-relevant SNCA transcript isoforms were already reduced in the blood samples at this early stage in the PPMI cohort.

Specifically, a long 3’ UTR-SNCA mRNA transcript and E4E6-SNCA mRNA (a transcript skipping exon 5) were reduced by 27% and 19%, respectively, whereas other Parkinson’s related transcripts (such as DJ-1), were unchanged (Fig. 5). It is possible that these specific isoforms could be used as sensitive biomarkers of early stage disease.

Using NanoString technology, the researchers were able to examine the SNCA isoforms directly from the blood sample without the need for additional steps such as reverse transcription or PCR amplification. Additionally, NanoString data were precise and reliable; template controls generated no signal and reference RNA counts were highly correlated with R2 > 0.999 within and between different plates. Moreover, when a subset of samples was randomly resampled to verify the retest reliability of the technique, the average correlation value R2 was 0.98. Taken together, NanoString nCounter technology demonstrates a clear advantage over existing methods to identify early disease markers in Parkinson’s disease.

Further Reading: A team of scientists led by the Cure Huntington’s Disease Initiative (CHDI) used Nanostring’s nCounter to demonstrate the benefits of PDE10 inhibition for Huntington’s disease mouse models. They found that PDE10 inhibition showed improvements and partial reversal of deregulated transcripts and the prevention of neurophysiological deficits related to Huntington’s disease10.
**Advanced Neuroscience Research Starts Here**

The pace of neuroscience research is accelerating and personalized medicine allows new therapeutic approaches that are highly targeted to the individual disease state. Whereas previous generations of patients faced a dim prognosis when diagnosed with a neurodegenerative disease, current technology allows patients to be diagnosed faster and treated more effectively. The publications discussed here represent new potential avenues for early detection, improved diagnosis, and better clarity of disease pathogenesis.

As more biomarkers are identified and specific cell contributions are better understood it creates opportunities to transform knowledge into actions. NanoString’s nCounter platform is playing a significant role in this progress by enabling discovery on a cellular and molecular level. NanoString technology is faster than PCR and more convenient and targeted than NGS. Researchers can work with NanoString scientists to custom configure their codesets and primers to expedite data collection and optimize analytical software to maximize their time interpreting results.

These advancements have the promise of earlier detections and therapeutic intervention for those impacted by neurodegenerative diseases. As new research explores neurodegenerative pathways, processes, and cellular profiles, there is greater potential to develop personalized therapies for patients that are more successful and less toxic than previous treatments.
REFERENCES

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NanoString Technologies, Inc.
530 Fairview Avenue North
Seattle, Washington 98109
T (888) 358-6266
F (206) 378-6288
nanostring.com
info@nanostring.com

Sales Contacts
United States: us.sales@nanostring.com
EMEA: europe.sales@nanostring.com
Asia Pacific & Japan: apac.sales@nanostring.com
Other Regions: info@nanostring.com

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