WHITEPAPER

Multiplexed Infectious Disease Analysis

Using nCounter® for Simultaneous Pathogen and Host Gene Expression Profiling

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Multiplexed Infectious Disease Pathogen and Host Profiling Using nCounter

Introduction

Infectious disease researchers face critical challenges in their efforts to better understand how pathogens function and how hosts respond. Infection samples are scarce, complex, and usually dominated by host origin cells; pathogens typically constitute only a tiny fraction. Efficient techniques are needed to track transcriptional profiles *in vivo*, where gene expression may differ significantly from patterns observed *in vitro*, and to assess the host's immune response. Finally, current clinical methods of identifying pathogens and determining antibiotic resistance are too slow, causing many patients to receive ineffective treatment.

NanoString's nCounter platform provides a simple workflow to enable rapid, precise, and cost-effective infectious disease research and diagnosis. This direct hybridization-based digital counting technology offers several key advantages:

- Easy sample prep that involves no enzymes or amplification
- Compatible with complex input, including direct lysates from various tissue types
- Quantifies up to 800 targets simultaneously from multiple pathogens and host cells
- Highly reproducible data that does not require technical replicates
- 15 minutes of hands-on time; sample to data in less than 24 hours
- Straightforward automated "biology-guided" data analysis with free nSolver software package

Scientists around the world are taking advantage of nCounter to answer a wide range of infectious disease research questions. Teams have used NanoString technology to study the bacterial and viral pathogens involved in acute respiratory tract infections, the *in vivo* expression profile of the major human fungal pathogen *Candida albicans*, the host's inflammatory response to West Nile virus across multiple tissues, and changes in T cell function in tuberculosis. Others are pursuing diagnostic applications: for instance, one group has analyzed microRNA patterns to improve detection of human enterovirus 71, and another team is developing tests to quickly identify pathogens and assess antibiotic resistance in blood infections.

Identifying Multiple Bacterial and Viral Species in a Single Assay

Researchers probe for a range of pathogens in acute respiratory tract infections

Camila I. de Oliveira, an infectious disease researcher at the Oswaldo Cruz Foundation (Fiocruz) in Salvador, Brazil, and her colleagues wanted to characterize the pathogens involved in acute respiratory tract infections in local patients. These infections commonly affect children less than five years old and can be fatal if they progress to pneumonia. Although many viruses and bacteria had been connected to this illness, studies tended to focus on one type of pathogen at a time. "We don't see many studies that describe the many pathogens that can be present," de Oliveira says.

Working with physicians at the Federal University of Bahia in Salvador, de Oliveira's team obtained samples of nasopharyngeal aspirates from young patients. Initially, the researchers planned to test for pathogens using RT-PCR. But when the clinicians suggested running a large panel to detect many species, she realized that this approach was impractical. "When we got the list of pathogens, we said, 'This is not possible to do by RT-PCR,'" she recalls. "It's just going to be too much."

Instead, the team used NanoString's nCounter platform to simultaneously test for more than a dozen bacteria and viruses, including rhinovirus, parainfluenza, *Staphylococcus aureus*, and *Haemophilus influenzae*. With collaborators at the Rega Institute for Medical Research in Leuven, Belgium, the researchers found they could accurately identify the species in samples spiked with pathogen RNA; the nCounter method gave results similar to those obtained by real-time PCR experiments. The team then probed 61 patient samples and found that 55% contained only bacteria, 44% had both bacteria and viruses, and 1.4% had only viruses. The results were published in 2015 in the *Journal of Clinical Virology*[1].

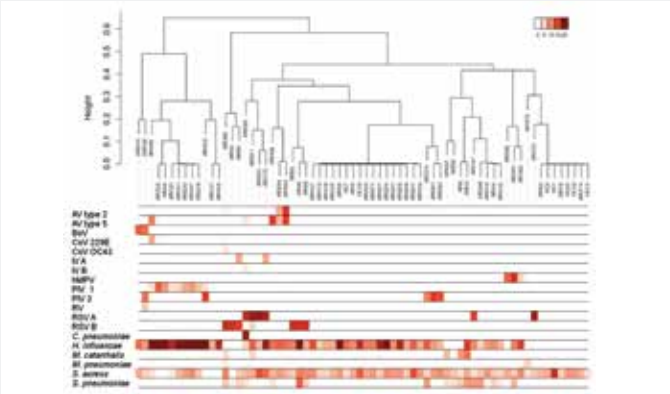

FIGURE 1: Simultaneous detection of multiple pathogens in acute respiratory tract infection (ARI) patients. nCounter was used to detect 13 types of viruses and six bacteria species in nasopharyngeal aspirates from 61 ARI patients and seven healthy controls (HC).

Darker colors correspond to higher probe counts.
Reprinted from *Journal of Clinical Virology* 69, Fukutani KF et al. "Pathogen transcriptional profile in nasopharyngeal aspirates of children with acute respiratory tract infection." 190-196, 2015, with permission from Elsevier.

The researchers are now expanding the analysis to about 600 samples and hope to correlate specific pathogens with clinical outcomes and patient demographic data, such as socio-economic factors. De Oliveira's team also has used nCounter on the same samples to assess patients' immune responses. The technology delivered quick results on many targets without generating an overwhelming amount of data. It was "very informative for us," she says. "We had a lot of parameters being detected in the same samples, all at once."

Further Reading: A team at the Stanford University School of Medicine in California assessed nCounter's ability to identify and quantify 10 fungal species. The results show that "an amplification-free technology can detect multiple fungal pathogens at the species level with acceptable specificity, sensitivity, and reproducibility within a 24-hour turnaround time," the researchers wrote in *Diagnostic Microbiology and Infectious Disease*[2].

Studying a Fungal Pathogen's Gene Expression In Vivo

Analysis reveals Candida albicans' response to drug treatment

The fungal pathogen *Candida albicans* often causes bloodstream infections in hospitalized patients, particularly those who have AIDS or are undergoing chemotherapy. Many studies of the fungus' gene expression have been performed *in vitro*. But Wenjie Xu, a microbiologist who studied C. *albicans* at Carnegie Mellon University in Pittsburgh, Pennsylvania, decided to investigate how the pathogen behaved in the host environment. "To understand how the pathogen survives inside the host and how it responds to a drug, we have to do it *in vivo*," says Xu, now a technical services scientist at NanoString.

Studying *in vivo* samples is challenging because more than 99% of the RNA from infected tissue is from host cells. The host RNA contributes to high background on microarrays and dominates sequence reads from RNA-Seq. In contrast, NanoString's nCounter system allowed Xu's team to easily work with mixed RNA, and the workflow was simple and fast enough for the scientists to process a large number of samples from a time course study.

Using nCounter in Infectious Disease Research: A Step-by-Step Video Guide

Xu W *et al.* (2016) *J. Vis. Exp.* 107:e53460.

In the *Journal of Visualized Experiments*, NanoString scientist Wenjie Xu and his colleagues provide a detailed guide for using nCounter to study gene expression in infected tissues[4]. The article includes a step-by-step video demonstrating the procedure.

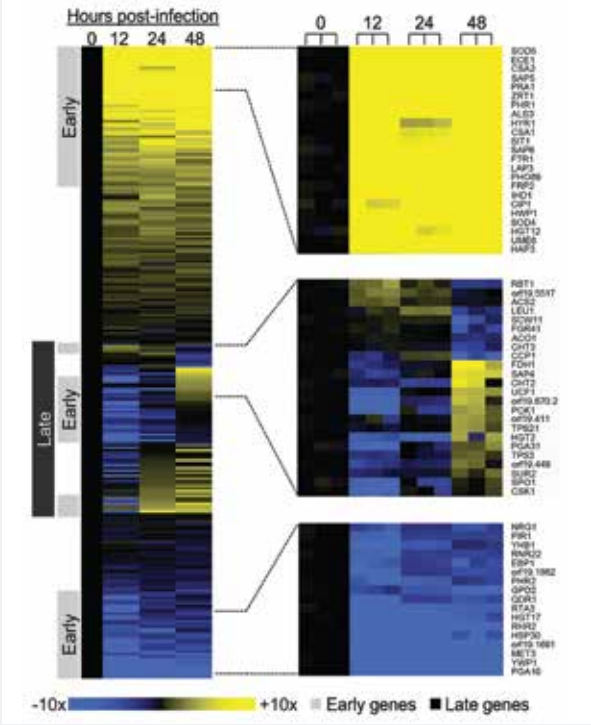

FIGURE 2: Stages of *Candida albicans* gene expression *in vivo* from 0 to 48 hours after infection. Researchers used NanoString technology to track 248 environmentally responsive genes in kidney samples from infected mice. Fully saturated color represents a 10-fold change in expression.

The researchers reviewed the existing literature to select 248 environmentally responsive genes that were suspected to play critical roles during infection. Next, the team collected kidney samples from mice at 0, 12, 24, and 48 hours after infection and assayed them for gene expression. The same samples also were tested for 231 C. *albicans* transcription factors and 46 mouse genes involved in responding to fungal infections. "We have perfectly matched host immune response and pathogen expression profiling from the exact same RNA prep," Xu says. "We have two sides of the same story."

Xu and his colleagues found that the pathogen and host's gene expression changed over the course of infection. For example, fungal genes upregulated in the early stage were related to nutritional limitation and hyphal growth, while genes upregulated later were involved in oxidative stress. When the team treated the mice with caspofungin, a drug commonly given to patients, the fungus' expression profile *in vivo* differed substantially from responses reported *in vitro*. Many of the induced genes overlapped with those that the pathogen had downregulated during early infection, presumably to increase its chances of survival.

"That's intriguing because it's almost like caspofungin knows the weak points of the pathogen," says Xu, who published the results in 2015 in *PLOS Biology*[3]. By learning more about the drug's mechanism of action, researchers can identify pathways to target with new treatments.

Further reading: A team at the Harvard School of Public Health in Boston analyzed *in vivo* gene expression of the parasite *Plasmodium falciparum* in tissues of children in Malawi who had died from malaria. The researchers wrote in *Genome Medicine* that the nCounter platform "is of great utility to the malaria community, as it is amenable to many different types of samples"[5]. In another study, researchers at the Dartmouth-Hitchcock Medical Center in Lebanon, New Hampshire and their colleagues used NanoString technology to track *Pseudomonas aeruginosa* transcripts in sputum samples, noting that this method "avoids the potentially confounding effects of *in vitro* culture conditions."[6]

Using MicroRNAs to Diagnose Severe Viral Infections

Human enterovirus 71 infection is linked to elevation of specific miRNAs

Patients may respond to pathogens by increasing or decreasing levels of certain microRNAs. Since these patterns are indirectly linked to infection, they could be used as a diagnostic tool to identify the pathogen in clinical settings. Robert Wang, a molecular virologist at Chang Gung University in Taoyuan, Taiwan, wanted to explore whether miRNAs could help physicians determine if a patient with hand, foot, and mouth disease is infected with human enterovirus 71 (EV71), which can enter the central nervous system and lead to neurological problems and death.

Wang chose to use NanoString's nCounter platform to characterize the miRNA patterns in EV71 infection because the system provides quantitative data. Next-generation sequencing would require amplification, which "can cause a lot of false positives," he says.

His team studied serum samples from four patients with mild infections, four with severe infections, and four healthy controls. After isolating RNA, the researchers used the nCounter Human miRNA Expression Assay Kit to detect 800 miRNAs. They found that levels of 44 miRNAs were at least twice as high in infected patients as in controls, and levels of 133 miRNAs were reduced by at least half. The experiments helped them identify a miRNA, called miR876-5p, that was upregulated nearly 10-fold in severe cases, Wang and his colleagues reported in 2016 in *Scientific Reports*[7]. Treating mice with an miR876-5p inhibitor before infection lowered viral replication, suggesting that this miRNA plays an important role in infection.

The work could eventually improve diagnosis and treatment. If researchers can narrow down the set of upregulated miRNAs to a smaller panel, clinicians could test for those miRNAs to determine if a patient is likely to develop severe disease. Patients also could potentially be treated with a miR876-5p inhibitor, Wang says. He is now extending the analysis to more patients, and he has performed similar studies using nCounter to identify miRNAs associated with Japanese encephalitis virus infection. "NanoString provides me with a whole picture of microRNA," he says.

Further reading: In a study published in the *Journal of Immunology*, a team at Ohio State University in Columbus investigated how *Mycobacterium tuberculosis* alters host miRNA expression[8]. Using nCounter, the researchers found 31 miRNAs that were significantly upregulated or downregulated during infection, including two that may weaken immune response.

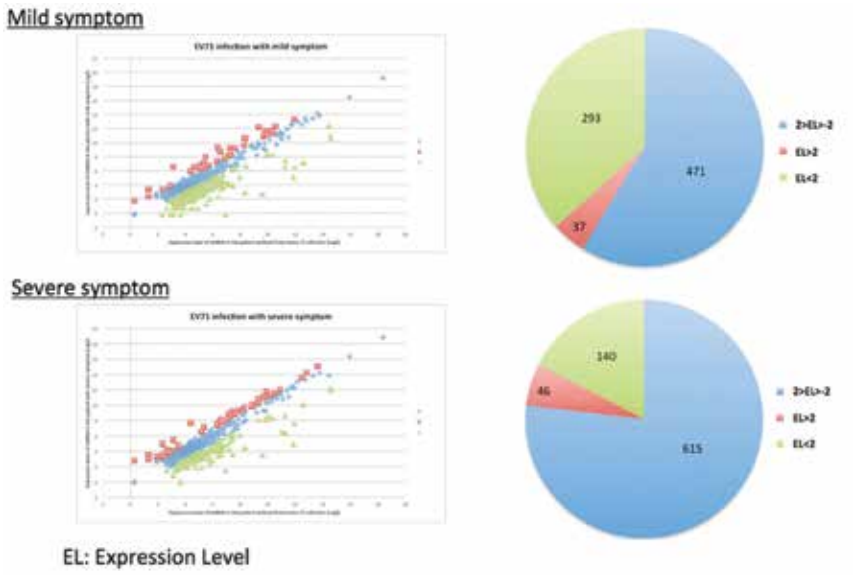

FIGURE 3: MicroRNA expression profiles in patients infected with human enterovirus 71 (EV71). The nCounter Human miRNA Expression Assay Kit was used to detect 800 miRNAs in EV71 infections. Levels in healthy controls (X axis) are plotted against levels in patients with mild or severe infections (Y axis). Red indicates a significant increase, and green indicates a significant decrease.

Investigating the Immune Response in Tuberculosis

nCounter experiment provides evidence for T cell exhaustion

In chronic *Mycobacterium tuberculosis* infections, bacterial recrudescence can sometimes occur. "Suddenly there's a breakthrough of infection," says Pushpa Jayaraman, an immunologist at the Novartis Institutes for BioMedical Research in Cambridge. The immune system "just kind of gives up."

While she was working at the University of Massachusetts Medical School in Worcester, Jayaraman and her colleagues investigated whether the host's T cells became functionally exhausted, a type of immune failure that had been observed with other pathogens such as HIV and hepatitis B and C viruses. "This is a very well-documented phenomenon," she says. "But it had not been shown for TB at all." In experiments with mice infected with *M. tuberculosis*, the team found that the T cells showed typical signs of exhaustion: for instance, their cytokine production declined, and they expressed inhibitory receptors such as TIM3 and PD1.

But the researchers wanted to characterize the cells' gene expression in more detail. They turned to NanoString's nCounter platform to measure RNA levels of about 200 genes involved in processes such as T cell activation and regulating transcription. The system allowed them to gather data with high sensitivity while avoiding potential problems such as preferential amplification. "It just gave us better data," Jayaraman says. The team also could expand their analysis beyond a few genes of interest, but not to such a large set that it became difficult to see trends.

The nCounter experiment showed that the T cells had distinct signatures depending on which inhibitory receptors they expressed. "This gives us a bird's-eye view of the differences," she says. For instance, cells expressing TIM3 but not PD1 showed higher transcription of pro-inflammatory cytokine and chemokine receptor genes than double-positive cells expressing both TIM3 and PD1 did. When the researchers compared the profiles to a set of genes associated with T cell exhaustion, they found that many of those genes showed similar expression patterns in the double-positive cells. The results, published in 2016 in *PLOS Pathogens*, suggest that treatments might need to target multiple inhibitory receptors to re-activate the T cells.

NanoString's technology enabled the researchers to examine gene expression patterns in the cells "in a more holistic manner," Jayaraman says. She also has used nCounter to study the functional profile of T cells[9] after vaccination and to analyze cytokine networks that may regulate T cell responses.

Further reading: A team led by scientists at Aarhus University in Denmark analyzed gene expression in bone marrow-derived cells with nCounter to characterize the innate immune response to murine gammaherpesvirus 68[10]. In studies of West Nile virus, researchers used NanoString panels to identify a genetic signature for susceptible patients[11] and to characterize host immune-related gene expression in the spleen[12] and brain[13] after infection.

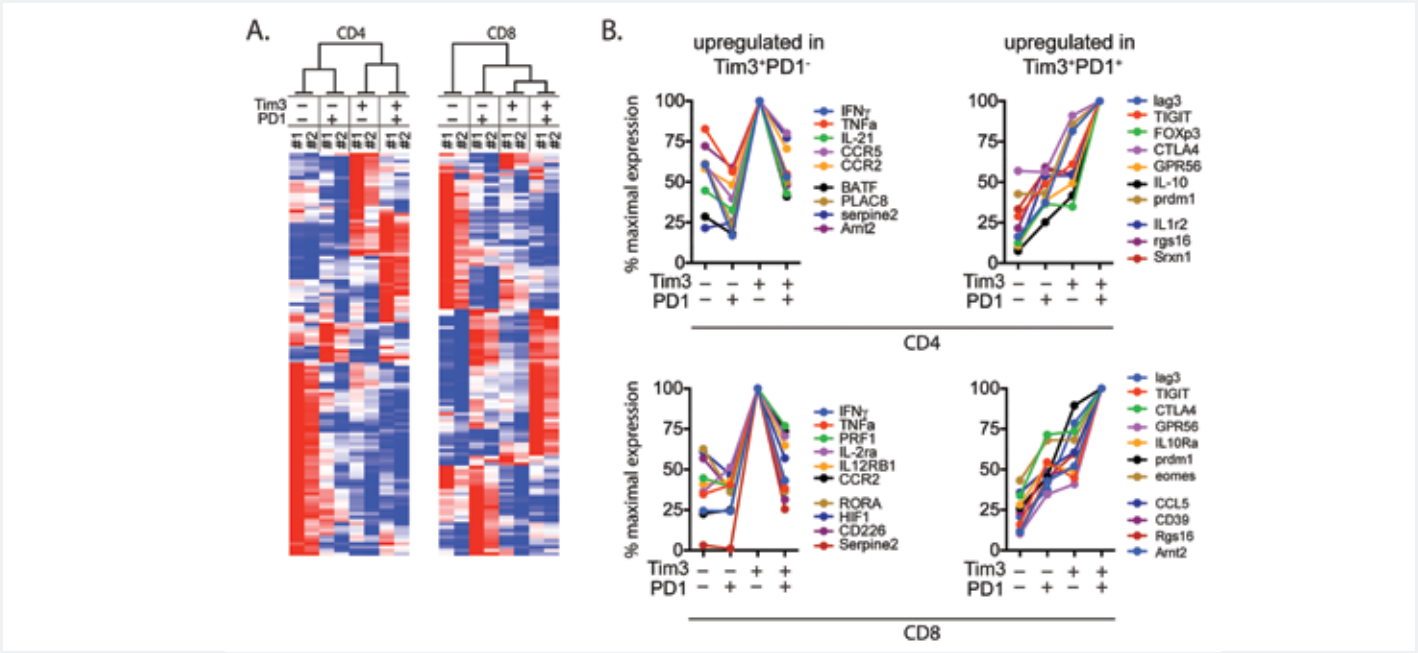

FIGURE 4: Distinct gene expression patterns in T cells after *Mycobacterium tuberculosis* infection. NanoString analysis revealed that T cells from infected mice have different transcriptional profiles depending on whether they express the inhibitory receptor TIM3, PD1, or both. The experiment provided evidence that T cells become functionally exhausted during infection.

A Thorough Analysis of Inflammatory Response

Researchers track gene expression in multiple tissues after West Nile virus infection

West Nile virus (WNV) has spread to North and South America, and infection can lead to encephalitis and long-term neurological damage. José Peña, a virologist at Lawrence Livermore National Laboratory in California, and his colleagues, were seeking a method to track the inflammatory response to the pathogen in multiple tissues. In the past, due to the cost and labor involved in gene expression analysis, "most experiments focused on just spleen and brain," he says. "But if you get an infection, it's not just localized to one tissue."

The researchers decided to use NanoString's technology to profile the host inflammatory response in the lung, liver, kidney, spleen, and brain of infected mice. Collaborators at the University of Texas Medical Branch in Galveston collected samples from the animals' tissues each day for nine days. Peña's team then extracted RNA and analyzed expression of 179 genes involved in inflammation using the nCounter GX Mouse Inflammation Kit.

The team chose the nCounter platform because it provided a digital, quantitative analysis that did not require amplification. "It took one of the variables out: Is this an artifact or not?" Peña says. The method also was less labor-intensive than other techniques. The researchers processed about 24 to 48 samples per day and received the initial data within about two weeks of starting the experiment. The study "yielded a pretty large dataset that we could analyze internally without too much computational time," he says.

The team found many gene expression patterns that could serve as the basis for future studies. For example, expression levels of the chemokine gene *Cxcl10* and cytokine gene *Il12b* changed in most tissues, the transcription factor *Maff* was upregulated in the lung, and chemoattractant gene expression was reduced in the kidney. "The nCounter system and complementary methods employed here provide a powerful platform for detailed comparative analysis of the kinetics and magnitude of host responses to WNV infection," the authors wrote in 2014 in *PLOS Neglected Tropical Diseases.*[14]

Further reading: Using the nCounter GX Human Inflammation Kit, researchers at Stanford University in California studied the expression of 184 inflammation-related genes in patients who had been infected with hepatitis C virus and found that pegylated interferon treatment had long-lasting effects[15]. An international team from Brazil and the United States created a custom NanoString codeset to analyze 98 inflammatory genes in patients infected with the parasite *Plasmodium vivax*[16].

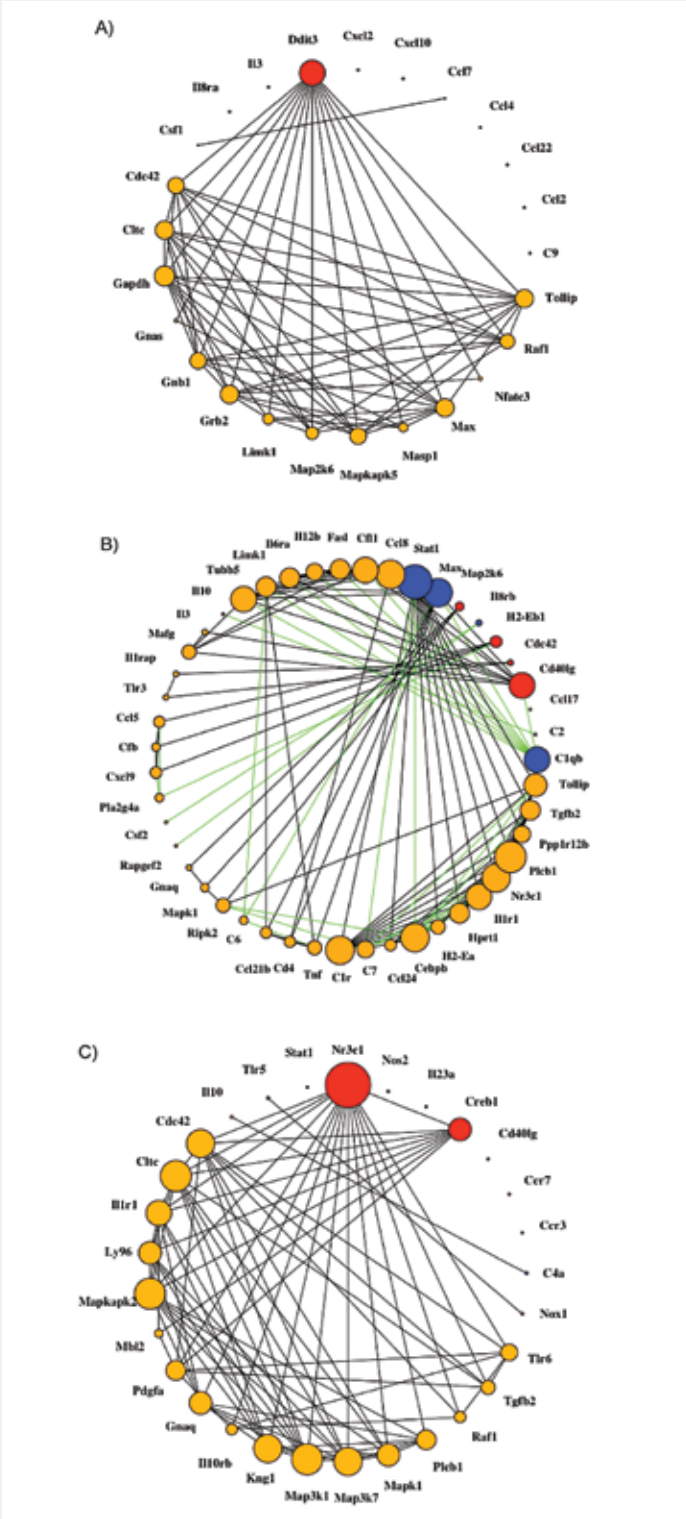

FIGURE 5: Expression of inflammation-related genes across multiple tissues in response to West Nile virus. Gene expression networks for the brain (A), spleen (B), and kidney (C) of infected mice were generated from data obtained with the nCounter GX Mouse Inflammation Kit. Blue genes are upregulated, and red genes are downregulated; black edges represent positive correlations, and green edges represent negative correlations.

Rapid Tests to Identify Pathogens and Assess Antibiotic Resistance

nCounter helps researchers analyze complex blood samples

Today, bacterial infections are typically diagnosed by culturing a sample from the patient, running biochemical assays, and testing the pathogens for growth in the presence of antibiotics to assess susceptibility.

However, the process takes about 48 to 72 hours, and clinicians often make treatment decisions before results are available. Researchers are now working with NanoString to develop a rapid diagnostic method to reduce the likelihood that patients are prescribed ineffective antibiotics. "There are mortality costs with getting a slow answer," says Deborah Hung, an infectious disease researcher at the Broad Institute in Cambridge, Massachusetts.

In a 2015 study published in *Lab on a Chip*, the team tested their approach on blood samples[17]. Blood is particularly difficult to analyze because it contains molecular factors that can interfere with amplification, and the pathogen abundance may be very low. "It is very challenging to detect pathogens from the blood," says Jongyoon Han, a bioengineer at the Massachusetts Institute of Technology in Cambridge, who collaborated with Hung. But rapid diagnostics are critical for bacterial blood infections because delaying the proper treatment can lead to septic shock. That's the situation "where you need to know the answer the fastest," Hung says.

Hung decided to use nCounter for the tests because the platform offers a multiplex, hybridization-based solution that works with cell lysates. "The beauty of NanoString is that you can do it on crude samples," she says. In addition, "NanoString is an easier rapid platform where you don't have to do as much optimization of every probe set."

In the study, the researchers first separated the pathogens from blood using a microfluidic device developed by Han's team. By running the sample through a spiral-shaped channel, the scientists could isolate the bacteria from red and white blood cells based on cell size. Hung's team then used nCounter to test for RNA from *Escherichia coli, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa* and identified the species at levels as low as 100 CFU/mL. The researchers also exposed *E. coli* to the antibiotic ciprofloxacin and accurately determined each strain's susceptibility to the treatment based on RNA signatures.

The test currently takes about eight hours, and the researchers are collaborating with NanoString to reduce the time to a few hours. "The whole field is trying to push toward rapid diagnostics," Hung says.

Further reading: In a 2012 study in *Proceedings of the National Academy of Sciences*, Hung's team showed that nCounter could identify many other pathogens in lysates, including *Mycobacterium tuberculosis,* influenza virus, herpes simplex virus-2, HIV-1, the fungus *Candida albicans*, and the parasite *Plasmodium falciparum*[18].

The Future of nCounter: Advanced Diagnostics and Immune Response Profiling

One of the strengths of utilizing nCounter technology for translational research, is that one can utilize the identical reagents and instrumentation also, for full diagnostic testing (after appropriate studies/approvals). NanoString's rapid, multiplex technology has been deployed as a diagnostic tool in the clinic. In 2013, NanoString's Prosigna™ Breast Cancer Prognostic Gene Signature Assay, which uses the nCounter platform, received 510(k) clearance from the U.S. Food and Drug Administration (FDA). Since this original FDA clearance, three additional multiplexed gene expression assays have received clearance on the nCounter platform to perform patient stratification in cancer treatment clinical trials. As described in this report, multiple studies have demonstrated that nCounter can accurately and simultaneously detect multiple bacterial, viral, and fungal species, as well as transcriptional profiles of antibiotic susceptibility. NanoString experiments also could improve diagnosis by identifying immune response signatures for specific pathogens.

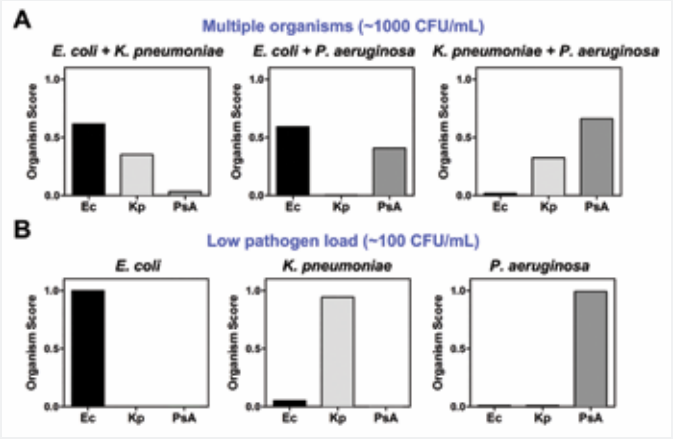

FIGURE 6: Detection of three bacteria species in blood samples. Blood was inoculated with *Escherichia coli, Klebsiella pneumoniae*, and/or *Pseudomonas aeruginosa* at 1000 CFU/mL (A) and 100 CFU/mL (B). Pathogens were isolated from blood cells using a microfluidic device, then analyzed with NanoString's rRNA detection assay.

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In the future, infectious disease treatment may follow a trajectory similar to that of cancer treatment. Cancer researchers previously focused on developing drugs to target the tumor; now, many successful treatments do not interact with the tumor-cells directly, but are designed to interact with the host, by inducing the immune system to more effectively eliminate cancer cells. Similarly, most infectious disease treatments currently target the pathogen, but new drugs could potentially be developed to modify the host immune response to better recognize and destroy infectious agents. Just as NanoString's nCounter PanCancer Immune Profiling Panel has enabled researchers to effectively study the immune response to tumors, other nCounter products can help track the immune response to bacteria, viruses, fungi, and parasites, providing insight into potential treatment pathways. The highplex capability of the nCounter system allows for the flexibility of multiplexing both pathogen and host-specific responses simultaneously, ushering-in a whole new capability in the study of infectious disease at the basic-research, translational-research, and (ultimately) diagnostic level.

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