

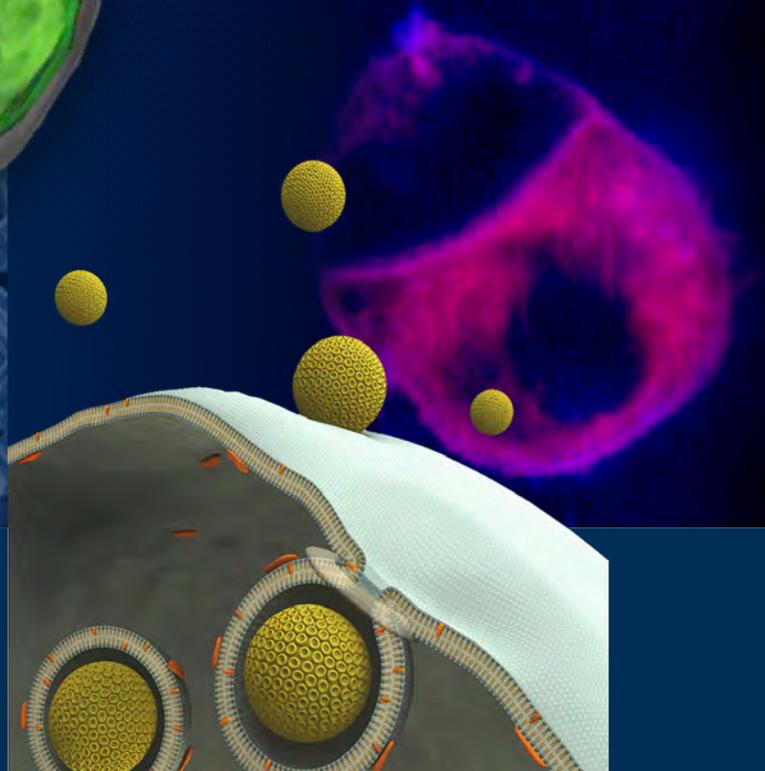
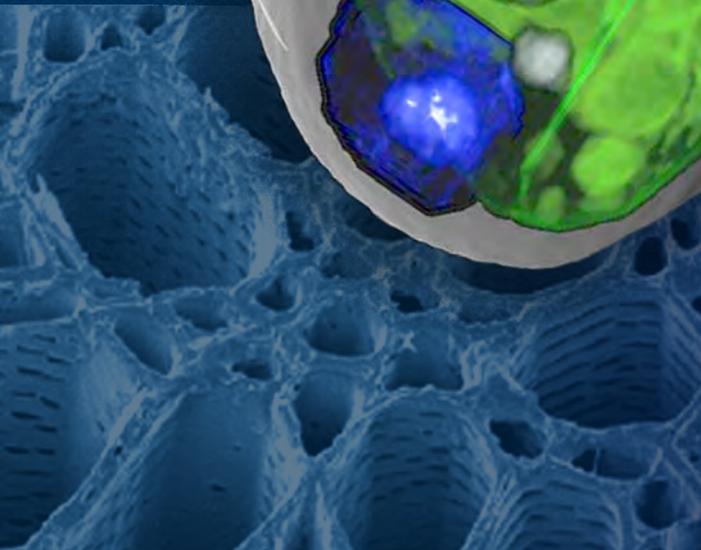
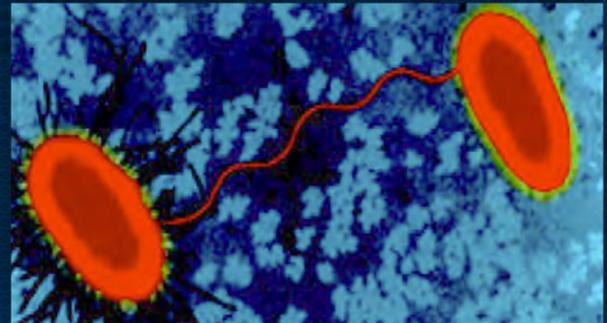
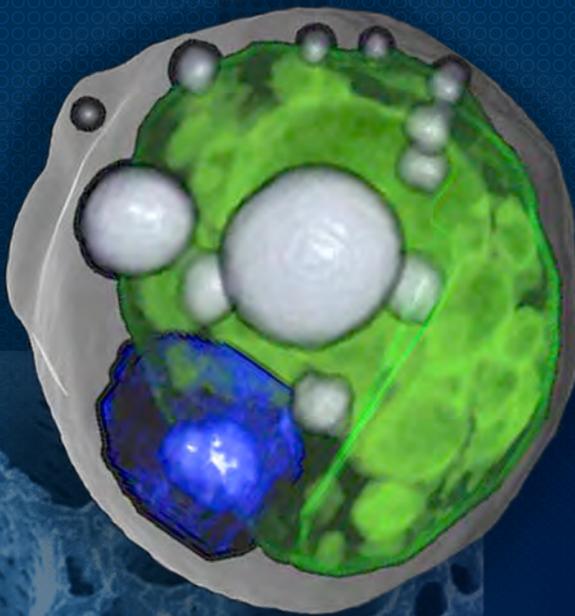
Exceptional service in the national interest



Sandia National Laboratories

LDRD IMPACTS ON SANDIA BIOSCIENCES

Biothreats to Biofuels



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From the Chief Technology Officer

The LDRD Program Innovates for Sandia's Biosciences Mission

The face of Sandia's Biosciences program is undergoing dramatic change and growth as the role of this foundational area in U.S. national security becomes increasingly important, for example in the areas of new biothreat organisms (arising both naturally and through genetic engineering), and the exciting prospect of multiple potential routes for improving biofuel production. Simultaneously, the scope of biomedical knowledge itself continues its recent pattern of explosive growth.

This state of affairs poses both an exciting opportunity and a major challenge to the Sandia Biosciences program in general and to its LDRD investments in particular. LDRD projects focus on R&D at the leading edge of scientific disciplines, which in the case of bioscience, is advancing at an astonishing pace. Sandia Bioscience LDRDs are thus faced with the dual task of addressing Laboratories mission needs while simultaneously innovating at and beyond that inexorably advancing leading edge.

LDRD projects enable Sandia scientists and engineers to both execute Sandia's broad national security mission and advance the frontiers of science and engineering, as they seek improved understanding of biological processes at the cellular and molecular levels and develop technologies to improve global biothreat reduction, public health, and biofuels production. Since its inception by Congress in 1992, Sandia's LDRD program has been a major contributor to scientific understanding and technological innovation across the breadth of science and technology disciplines found at the Laboratories. Although the history of bioscience R&D at Sandia is relatively short, the Laboratories' LDRD Bioscience portfolio has risen to meet these high standards.

This brochure describes some of the contributions of Sandia's LDRD program to U.S. bioscience initiatives. In all cases, the technical impact on the biosciences program derives from multidisciplinary fundamental science and engineering research—from cellular biology to analytical chemistry to microfluidic engineering, to high performance computing, and even to global outreach for threat-reduction partnerships. This is the hallmark of LDRD-funded projects: fundamental scientific and engineering research leads to enhanced understanding and ability to manipulate physical and biological systems, in turn, leading to the application of novel capabilities to crucial challenges in the bioscience arena. DOE/NNSA and the Nation benefit substantially from these LDRD-initiated innovations that contribute to improved public and environmental health, and national security.



Sandia National Laboratories

LDRD IMPACTS ON SANDIA BIOSCIENCES

BIOThREATS TO BIOFUELS

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Introduction: LDRD-Funded Bioscience at Sandia

Mission-Related Underpinnings

Bioscience Research activities at Sandia — including those projects funded by the LDRD Program — fall under the aegis of the Bioscience Research Foundation, and are largely linked to two Sandia Strategic Management Units (SMUs): Energy, Climate, and Infrastructure Security (ECIS) and International, Homeland, and Nuclear Security (IHNS). For a research arena that is really only a bit less than two decades old at the Laboratories, the breadth and depth of research has grown with remarkable rapidity. Such research has encompassed collaborations with other strong, established areas of laboratories' investment in the physical sciences, thereby gaining a foothold from which to launch significant bioscience investigations.

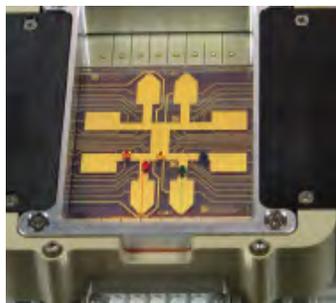
In alignment with SMU and Sandia national security mission space, LDRD-funded bioscience research — largely at the level of cell and molecular biology — can roughly be categorized under three overarching themes:

- Domestic and international rapid biothreat detection and countermeasures
- Nano-bio engineering, including biothreat mitigation, novel therapeutics, and areas such as vaccine studies and water quality
- Biofuels research, from both terrestrial and aquatic biomass sources

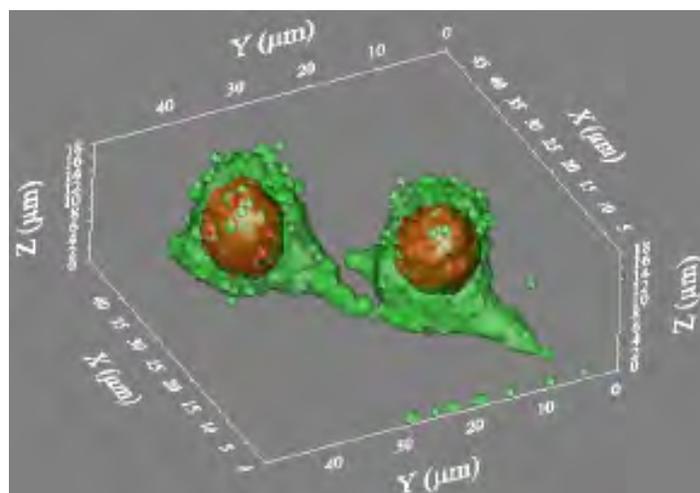
In discussing the impacts of the LDRD program on the missions of Sandia's Bioscience Research Foundation, and its ECIS and IHNS SMUs, several research technologies stand out as keys to multiple bioscience research endeavors. Among these, two are especially noteworthy: microfluidics technology enabling single-cell interrogation, and hyperspectral imaging with multivariate curve resolution analysis using Sandia-developed software packages. With so many LDRD-funded bioscience projects employing one or both of these technologies, they become an important introduction to bioscience research at Sandia.

MICROFLUIDICS

One aspect of Sandia's adeptness at engineering in the micro- and nano-realms, microfluidic systems are those employing microscale conduits (aquifers) — complete with microvalves and other microscale features — for the transfer, mixing, reacting, processing, and separating aqueous solutions in microliter or nanoliter volumes. While these systems enable the performance of chemistry and biochemistry under reagent-saving conditions, the systems have taken on greater significance for Sandia bioscientists in that they afford the ability to interrogate the physiology of individual cells. This ability was spawned to a great extent from two grand challenge LDRD projects, MicroChemLab and MISL (the Microscale Immune System Laboratory), a project that studied white blood cells of the innate immune system (such as macrophages) but, as importantly, demonstrated the utility of this single-cell interrogation methodology. Research into cellular physiology — such as cellular/metabolic/genetic responses to hormones or cytokines (chemical messengers) — most often entail the average response of a population or populations of cells, and therefore fail to take into account cell-to-cell variability. Such variability might well play an important role in vivo. Beyond this, the luxury of being able to interrogate a single cell — to converse with the fundamental unit of physiology — presents biologists with a remarkable microlaboratory with which to probe cellular and molecular biology. This is particularly true when cells can be studied then released still alive and unharmed (see, for example, p.31).



from a specimen; for example, proteins will fluoresce at approximately 353-nm when excited with 280-nm light. Note that this is related to but different from the use of fluorescent molecules such as green fluorescent protein (GFP), which through a variety of genetic, cytological, and immunological techniques, can be used to fluorescently label cell structures. Hyperspectral imaging, relying on autofluorescence, does not require such pre-labeling, but is also more complicated and depends on the algorithms used to process the collected light. Employing such computational algorithms developed in-house, this fluorescence light is analyzed in spectral bands, and cell structures correlated with their particular wavelength(s) of fluorescence. Structures are thus “fingerprinted” in microscope images, the technique capable of discovering all emitting structures present. Because changes in cellular physiology are often accompanied by changes in the spectral fingerprint of a given cell, hyperspectral imaging presents a non-invasive way of tracking such changes. Sandia scientists have made incredibly productive use of this technique, often in conjunction with microfluidics technology (see, for example, p.27). ■



HYPERSPECTRAL IMAGING AND ANALYSIS

Although the technique itself has been employed for some time, Sandia modifications to hyperspectral imaging, particularly in the area of image-analysis algorithms, have improved and further diversified its utility in both direct excitation and reflectivity applications. In direct biological applications, cells are irradiated with laser light and through a microscope, light, derived from intrinsic fluorescence (autofluorescence) of cell structures is collected

Part I: Rapid Biothreat Detection and Countermeasures

Introduction

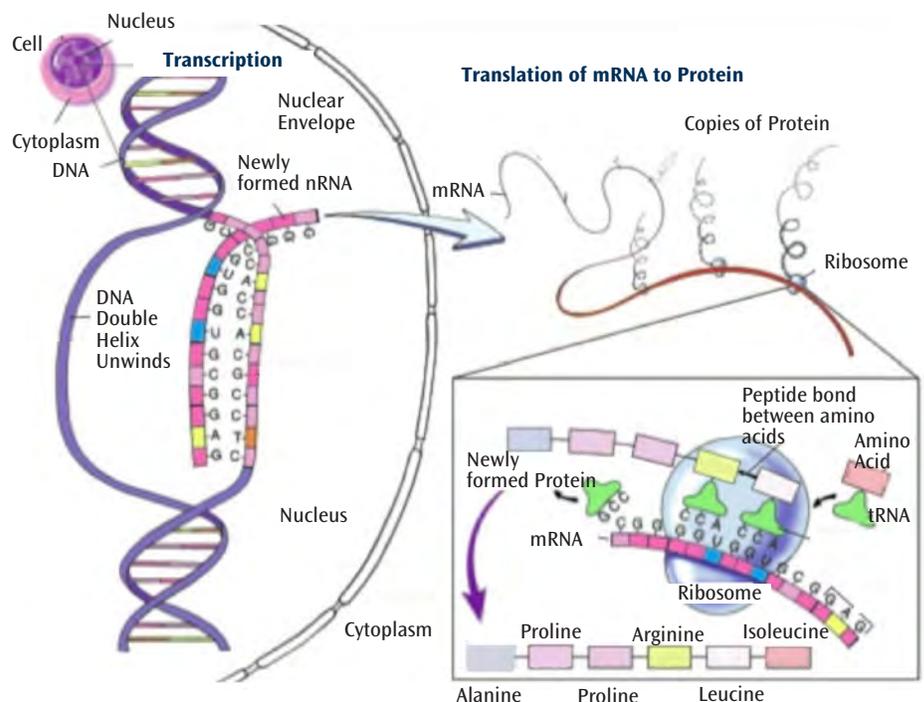
Sandia's biothreat reduction mission comprises several components. First and foremost is the development of methods for rapid detection of pathogenic microorganisms, their spores, and their toxins, with the ultimate aim of detection prior to significant human population exposure.

Critical to such detection is the problem of novel genetic organisms (previously unknown) that may have arisen "naturally" through the now well-recognized ability of microorganisms to exchange DNA (genes) in a variety of ways. An equally daunting issue is that of known organisms that have been highly genetically engineered — potentially by adversaries — to the point where they essentially comprise unknowns. An affiliated challenge is improving the processes that extract cellular nucleic acids (DNA and RNA) and prepare them for analysis, usually via ultrahigh-throughput sequencing (UHTS).

In addition to maintaining fidelity of these processes, it is important to accelerate them to whatever extent possible, given that detection and positive identification must outpace a pathogen's incubation period that precedes the appearance of symptoms, and during which the potential for cross-infection and spread is great. Meanwhile, the initiation of more effective therapeutic (and possibly prophylactic) measures is an active area of LDRD-funded research, in the person of projects that seek to better understand and potentiate immune system responses, particularly those that operate in the reemergence of diseases with pandemic potential, such as drug-resistant tuberculosis.

Genomes and Gene Expression

This crude diagram indicates the fundamental processes involved in what biologists term, "gene expression." A classical gene is the information in a discrete segment of double-stranded DNA in a human cell's nucleus, or a bacteria's nucleoid (or a discrete segment of DNA or RNA in a virus). That information specifies the order of amino acids polymerized to synthesize one specific cellular protein (out of thousands within each cell). Some stretches of DNA may be transcribed into RNA, but do not encode protein-structure information, and cannot therefore be thought of as classical genes. This DNA has other functions in a cell.



“An amazing team of engineers, biologists, and bioinformaticists really galvanizes what we can do.”

RapTOR and the Digital Microfluidic Hub

This Grand Challenge LDRD under the leadership of PI Todd Lane approached the issue of pathogen identity in complex situations, and where pathogens might be novel; that is, where their genetic identity might not conform to pathogens previously identified and databased by their genomic sequence — for example in GenBank, a sequence database of the National Center for Biotechnology Information (NCBI) of the NIH. Such deviation from databased genetic identity could occur in several ways. Most simply, a pathogenic bacterium or virus may have significantly mutated (as often happens by DNA transfer of pathogenicity islands, (see p.12) to an extent that its identity might be in question. But even more germane to biowarfare stratagems, malicious genetic engineering may have occurred at the hands of adversaries, to surreptitiously render an avirulent or moderate pathogen into a so-called “super-bug,” one capable both of causing great individual pathology and of creating a pandemic because of both its pathogenicity and the ease by which it might be spread interpersonally.

The RapTOR (Rapid Threat Organism Recognition) project began from the assumption that the only way to deal with such a previously unknown pathogen would be to quickly isolate or amplify its DNA or RNA and prepare it for UHTS. The first challenge was biological, the second, micro-engineering, and both challenges were addressed. The biology had to do with the necessity to isolate the genomic DNA or RNA of an unknown (heavily mutated, genetically engineered) pathogenic bacteria or virus. Problematically, this DNA/RNA would occur against a background of immense quantities of the DNA/RNA of the infected human host. Further compounding the problem is the fact that most clinical specimens are “contaminated” with nucleic acids derived from nonpathogenic microorganisms that live in symbiotic relationships with humans, such as the hundreds of bacterial species that populate the human intestine, the “human microbiome.” Far more difficult than searching for a needle in a haystack, this situation would be

In the central dogma of molecular genetics, a classical human gene’s information is transcribed (“copied”) into a single strand of pre-messenger RNA (pre-mRNA), which is then further processed (details unimportant to this overview) and transported out of the cell’s nucleus into its cytoplasm, there to be translated into the primary structure (amino acid sequence) of some specific cellular protein (the gene is said to encode that protein). Although slightly different mechanistically, the overall principles are the same in bacteria.

Note that sequencing the information in a gene or genes (or their mRNAs) yields information about the protein they encode, while sequencing all the DNA in a cell (its genome), both protein-coding and -noncoding provides a molecular-level unique fingerprint about that cell, be it human, animal, plant, or bacterial. Thus we speak of the human genome, the genome of an anthrax bacterium (*Bacillus anthracis*) or the genome of Smallpox virus. Many other viruses, such as HIV (AIDS), Ebola, and Rift Valley Fever Virus (RVFV) have RNA rather than DNA genomes; nonetheless, sequencing those genomes does provide the same molecular-level fingerprint. The key point is that a complete sequencing of a cell’s or bacterium’s or a virus’ genome can give an unambiguous identity: human cell versus dog cell or monkey cell, or even normal human cell vs. human cell carrying a mutation predisposing to a particular cancer; *E. coli* bacterial cell vs. *B. anthracis* bacterial cell; and even more important, non-pathogenic (avirulent) *E. coli* vs. potentially deadly pathogenic (virulent) *E. coli*.

With the advent of ultrahigh-throughput DNA and RNA sequencing (UHTS), entire genomes can be accurately and rapidly sequenced in a few days. Sandia LDRD research has developed approaches and technologies to take advantage of this capability.

In addition to extracting and purifying DNA or RNA and sequencing them in order to learn the genetic information contained in these nucleic acid molecules, there are several ways in which biologists can intervene in or manipulate these fundamental cellular/viral processes in order to both provide identity information and to utilize this information in generating countermeasures and therapies. Some LDRD projects pursue these avenues as well.

more akin to searching for a particular fragment of hay in that haystack.

The challenge was therefore to try to selectively eliminate or subtract as much as possible of that overwhelming background of human and microbiome DNA, no trivial task. This task, known as normalization suppression was only one aspect of the RapTOR project, which ultimately expanded into several associated areas for pathogen detection, the activities spanning the range from biochemical manipulation of nucleic acids through clever engineering of automation, and to creation of data analysis tools and an informatics architecture that would facilitate identification of discovered pathogens rapidly enough for decision-makers to effectively respond to an attack or outbreak of a bioterror organism.

The initial challenge to enrich pathogen nucleic acids in a sample also containing a vast background of human and microbiome DNA employed, first, normalization. This makes use of the feature of double-stranded nucleic acids (for example the DNA in human and most bacterial chromosomes) to “find” its partner (or “complementary”) DNA (or RNA) strand under suitable chemical conditions. Such finding relies on the fundamental “rules” of base-pairing, whereby all the As (adenines) on one DNA strand pair with Ts (thymines) on its complementary strand, and vice-versa (in double-stranded RNA, the pairing is actually A-U (uracil); and where all the Gs (guanines) on one DNA strand pair with Cs (cytosines) on its complementary strand; and vice-versa. Hence in a long stretch of double-stranded DNA that is chemically or thermally separated into its two single strands, the high likelihood is that these two “complementary” strands will “find each other” via complementary base-pairing (“re-anneal” to double-stranded DNA [dsDNA]) under the right conditions). In the RapTOR project’s normalization technique, double-stranded nucleic acids are dissociated at high temperature and then allowed to re-anneal at lower temperature; the most abundant double-stranded nucleic acids (typically host- and microbiome-derived) will quickly recognize and base-pair with their complementary partners. Their removal using hydroxyapatite chromatography (HAC) thus reduces their abundance relative to the original sample amongst the remaining single-stranded (ss)DNA. By adjusting conditions and reagents in HAC, researchers can isolate and separate rapidly re-annealing dsDNA from ssDNA and more slowly re-annealing dsDNA. The micro-HAC column — whose

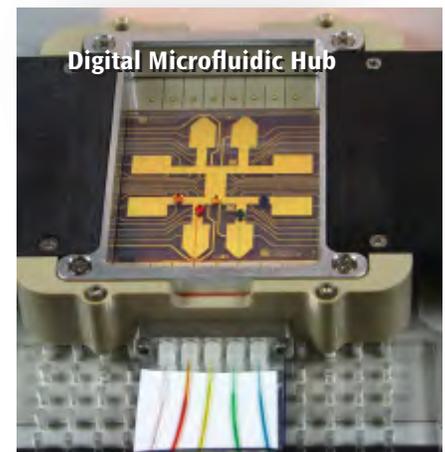
performance less-expensively rivaled other methods of subtractive hybridization — used convenient re-packable HC cartridges and was designed with a mind toward integration into an automated system, the system ultimately constructed around the digital microfluidic hub.

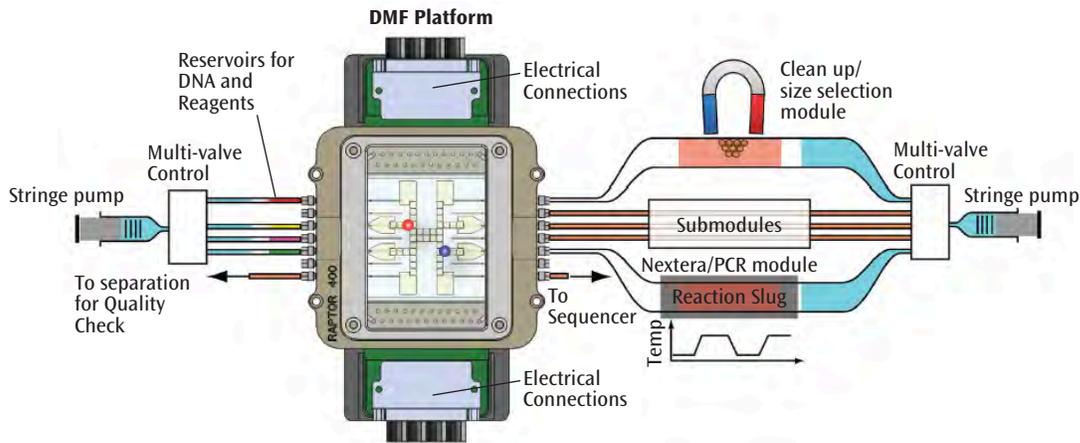
Having removed great quantities of the undesired human and microbiome DNA, the technique then proceeds to the capture stage of the process that depends on the extremely strong affinity of the protein streptavidin for biotin (or vitamin B7). A standard technique in molecular biology, this procedure is used to label and remove much of the remaining human or microbiome nucleic acids. With the pathogen’s DNA now greatly enriched, this now allows the sequence of steps necessary to prepare the DNA for UHTS, a process that can normally occupy a technician for a full day.

Digital Microfluidic Hub

But not in the RapTOR project! A key innovation is the project’s development of digital automation with the concomitant ability to carry out these procedures in a microfluidic environment at microliter volumes, including the ability to locally concentrate and mix specimens at membranes within microchannels. The RapTOR project included the engineering of a modular microfluidic system referred to as its Automated Molecular Biology Platform, the heart of which is the R&D100 Award-winning Digital Microfluidic Hub (DMH) under the leadership of Sandian Ken Patel. “It usually takes a full day of really intensive work to get DNA from a clinical blood sample into a format that’s compatible with sequencers,” says Patel in lauding the system’s ability to achieve that outcome in a fraction of that time.

Microdroplets are transferred from one location to another upon Teflon-coated glass above a grid of electrodes, (via a process known as “electrowetting”). Microliter microdroplets





carrying nanograms of DNA cargos or reagents can be merged and split, and the hub surface can be temperature controlled. Hence, the hub itself can execute a number of sample processing steps as well as intelligently shuttling materials to and from other sample-processing modules peripherally arrayed around the hub. Several key engineering advances include development of novel interconnects that support microfluidic communication between the hub and modules. Another important innovation was the use of transparent indium-tin-oxide electrodes for the DMH, providing optical access to the microdroplets on the hub, enabling real-time monitoring of microdroplet trafficking. This is of particular importance when, for example, fluorophore labeling is employed. The platform offers tremendous flexibility in the timing and ordering of processing reactions, as well as in incorporation of new functions, enabling rapid assembly and reconfiguration of a wide variety of sample processing trains. It is an outstanding exemplar of Sandia's microfluidics expertise.

The hub couples the flexibility of droplet-based digital microfluidics with the modularity of continuous-flow microchannel devices. Between its Teflon-coated glass plates, microliter-sized droplets of samples and reagents are trafficked and routed by a system of electrodes whose changing electric fields provide the motive force for trafficking. In turn, computational algorithms control the series of signals sent to the hub to effectively move, merge, and separate microdroplets and manipulate nanograms of DNA with precise temporal and spatial control. The central hub can connect both electrically and via microfluidic capillary tubes to a diversity of other specific-task modules. The output of this processing is a pathogen-DNA-enriched sample ready to be introduced into an automated DNA sequencer. The hands-free platform minimizes evaporation and contamination.

The target interval for clinical sample-to-pathogen identification turnaround is 24 hours, including UHTS. Providing detailed information about a known or genetically modified pathogen's genome in this timeframe would enable far faster and more effective public and military responses to infectious disease outbreaks. Ongoing work to improve the automated molecular biology platform is focused on multiplexing sample processing trains and accelerating the slowest reaction step, with the goal of completing end-to-end sample processing within 4 hrs. (current turnaround time is ~12 hrs.), then rapidly sending that prepared sample to UHTS. The standard bench-scale approach requires multiple workdays from a skilled technician. Part of this project and its ability to provide that rapid identification traces back to its parallel development of a new informatics pipeline for identification and characterization of known and novel pathogens based on sequence information.

In addition to the R&D100 award, the Society for Laboratory Automation and Screening selected the system for a \$10K innovation award. An eight-plex HAC platform to fully automate suppression methods has been developed and a cooperative research agreement with the DNA-sequencing company, Eureka Genomics, has been initiated. Additionally, a collaborative project with the Defense Threat Reduction Agency (DTRA) and the Centers for Disease Control (CDC) will examine the use of RapTOR technologies to detect food-borne pathogens. Samples of food will be spiked with biopathogens and then split & provided to both RapTOR's automated molecular biology platform & to another more-standard DNA-sequencing protocol, to see if Raptor can demonstrate its superiority in speed with superior data, with the potential outcome technology transfer. "An amazing team of engineers, biologists, and bioinformaticists really galvanizes what we can do," Patel comments.

Sandia LDRD bioscience efforts are also making numerous contributions to the ATP₃ project, an Arizona State University-led \$15 M DOE initiative. This consortium of research institutions and companies will pool resources in a way that allows new algae technologies, strains, and techniques to be tested and evaluated for their potential to succeed at large-scale biofuels production. It comprises a network of institutions and private companies with a range of necessary expertise from those (like NREL and Sandia) involved in R&D on the genetic/biochemical manipulation of different algal species and strains to those like Cellana with extensive experience at scaled-up cultivation of algae. RapTOR's automated molecular biology platform will search algal ponds for algal pond-crash-provoking pathogens with no a priori knowledge of what they might be, in order to discover pathogens that may have been missed or ignored in prior studies of detrimental factors in pond health/pond crashes. "We customized everything about the system to ensure flexibility. It impacts multiple areas . . . biofuels to biodefense," Todd Lane points out.

SPIN DX™

But what about rapidly keeping track of *known* algal pond pathogens in the ATP₃ project? AnLDRD-derived technology known as SpinDx™ has that covered as well. This application was not the target toward which SPINDx was initially developed and directed, so this adaptation to ATP₃ is testament to its versatility.

Underlined by recent nuclear facility accidents, such as the 2011 disaster at Japan's Fukushima Daiichi power plant, the need for rapid, on-the-spot diagnosis for workers acutely exposed to ionizing radiation is an ongoing issue for both power facilities and also for plants in the nuclear weapons complex. This Sandia LDRD project took a giant step toward addressing that need of rapid identification of exposed workers for rapid triage.

The basis of this identification lies with quickly testing the body for characteristic signs of ionizing radiation exposure, a

protocol known as radiation dosimetry. Animal testing has revealed a panel of biomarkers in blood that appears in response to radiation damage and can be used to estimate exposure dose across broad dose and time windows. These blood markers include both the up-regulation (increase in blood concentration) of a group of proteins deriving from several different physiological pathways and the depletion of white blood cells due to bone marrow damage. SpinDx was initially designed to evaluate this panel of biomarkers to assess radiation-exposure dose in a rapid, multi-parameter fashion.

Spin Dx™ injected engineering novelty into existing immunological techniques to measure such alterations in biochemistry, based upon immunological techniques such as the ELISA and sandwich immunoassays (see sidebar, p.11). While these techniques were quite specific, capable of identifying a diversity of changes in blood chemistry, they were also somewhat cumbersome, particularly in the sense of their being slowed down by the necessity for several wash steps during the procedure. This also increased the volumes of blood sample and reagents necessary to run the assays and, therefore, the probability of errors — an unacceptable

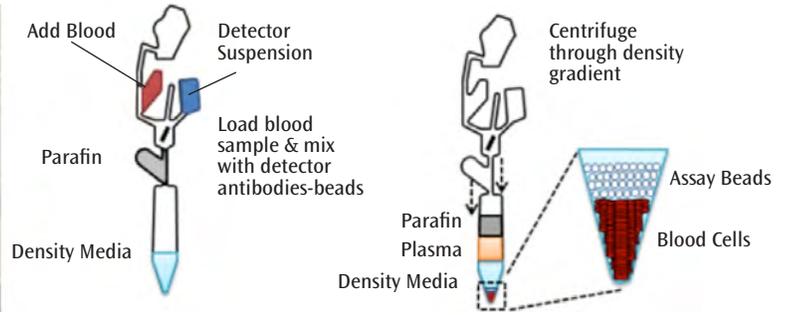
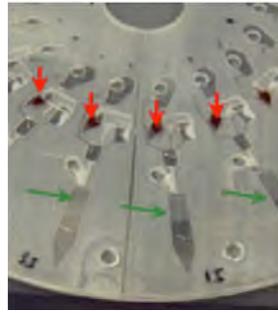
outcome when one is attempting to quickly triage individuals exposed to toxic chemical, radiation, or biological environments. Together, these drawbacks demanded a better method of screening individuals in danger, one that could diagnose very rapidly, had a diminished error rate, and could be initiated by testing a single drop (about 1 microliter [μL]) of blood.

Beyond the use of the immunological ELISA and sandwich assay, the basic idea relies upon the physics of centrifugation through a density medium, which separates the various entities in blood based upon their density. With one of the sandwich antibodies attached to inert beads (see figure in sidebar) and the density of the medium properly chosen, the technique can separate blood plasma, blood cells, and beads holding the immunological sandwich. A key feature of this process is that during centrifugation through the density medium, the sandwich and the blood cells are essentially washed clean of any contaminating molecules that might interfere with detection of the substance sandwiched by the antibodies. This obviates the need for the wash steps and the higher volumes of blood analyzed

SpinDx™



Flat disk with multiple microfluidic channels (green arrows) each loaded with a tiny droplet of blood (red arrows).



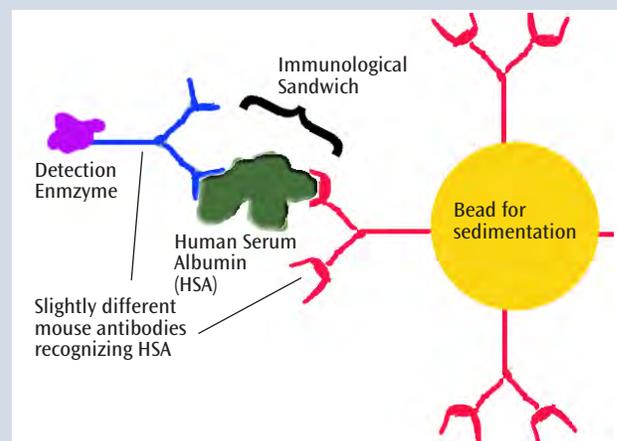
in traditional ELISAs — thus yielding a significantly accelerated testing regimen using a tiny droplet of blood.

The actual test device is a small disk onto which have been etched microfluidic channels (essentially, micro-test tubes) in which the immunological reactions and the centrifugation occur. Whole blood is introduced into the channel, mixed with the antibodies to form a bead-attached sandwich by spinning the disk at low rpm. Then, released into the density medium, the centrifuge spins the disk at higher rpms to effect sedimentation of the bead-attached sandwiches to a place in the density gradient that depends on the properties of the particular bead that is used. Typically, blood cells either sediment to the bottom of the channel or are trapped near the top of the density gradient—depending upon the exact density medium employed — blood plasma remains layered above the density gradient, and the bead sandwich somewhere within the density gradient. Extensive research has delineated the array of changes that are consistently observed very soon after acute exposure to radiation whose high energy causes breaks in DNA molecules of white blood cells in both peripheral blood and bone marrow, which, in turn, elicits the appearance of DNA repair mechanisms, such as specific repair enzymes. Other physiological changes also rapidly occur, including the appearance in blood of inflammatory proteins and certain cytokines (hormones of the immune system), as well as of a radical rise in blood concentration of cfDNA (cell-free DNA), that is, DNA released by damaged cells committing suicide (apoptosis) and also, an overall depletion of white blood cells. The kinetics of this cfDNA release appears to be specific to acute radiation, rising in blood up to 9 hours post-exposure, then falling to baseline levels.

Because human blood assays will often require detection of these multiple altered parameters to detect physiological impact of radiation, a chemical toxin or other situational exposure, this technique can separate several bead

ELISAs and Bead-based Sandwich Immunoassays

These techniques rely on the exquisitely specific chemistry of antibodies binding to the molecules (generally known as antigens) that stimulate their production by the immune system. For example, if one immunizes a mouse with (the antigen) human serum albumin (HSA) a human blood protein), the mouse will produce antibodies called “anti-HSA,” that can be used to specifically recognize the HSA protein. This antibody can be conjugated to an inexpensive enzyme, which will facilitate a chemical reaction such as a color change, thereby serving as a marker of this very specific binding reaction identifying the presence of HSA. This is called an ELISA test (enzyme-linked immunosorbent assay). In an adaptation of this test, some mouse anti-HSA antibodies can be attached to beads, while others are attached to the detection enzyme (in actuality, these antibodies are slightly different, but that need not concern us), and these would bind to different portions of the HSA molecule, trapping it in what is known as an “immunological sandwich.” This sandwich ELISA assay forms the basis of some of the detection schemes in Spin Dx™ operation.



sandwiches simultaneously, as well as detecting changes in the blood count and/or biochemistry of white blood cells, often an important marker of exposure. This is accomplished by using beads with different sedimentation properties, so that each sandwich — detecting a specific blood protein or altered blood cell, for example — will sediment, after centrifugation, to a different place in the sedimentation gradient. Theoretically, several hundred distinct bead layers could be discriminated by this method.

“The system is fast, quantitative, and extremely sensitive: one to two orders of magnitude more sensitive than regular ELISAs,” noted Greg Sommer, the technology’s developer and PI of the LDRD project. A mechanical engineer who developed microfluidic technologies for protein detection at the University of Michigan, Sommer’s postdoc at Sandia with Anup Singh, placed him in a great position to display his ingenuity in developing the microfluidic systems and the density gradient centrifugation used in SpinDx. After demonstrating under LDRD funding that they could simultaneously detect different human blood proteins with a sensitivity an order of magnitude better than a standard ELISA, the project team turned to the critical issue of point-of-care radiation biodosimetry, employing the multichannel approach, under post-LDRD funding from the Armed Forces Radiobiology Research Institute, Uniformed Services University, Bethesda, MD.

By performing a quantitative multichannel assay in a droplet of blood from a finger prick, this technique—with a sample to-answer time of less than 20 minutes—can assess early radiation exposure in large populations of potentially exposed individuals, allowing point-of-care triage that, through early identification of exposed individuals, is very likely to save lives and mitigate radiation damage by beginning treatment immediately. The portability of the device, makes it ideally suited for such point-of-care diagnostics.

And now its advantages are being applied to algal biofuels as well. In addition to fueling a startup company, illustrating the long reach of LDRD impacts, SpinDx has been tapped for DOE’s ATP₃ project. It will search for and quantitate the population of known algal pond pathogens, even while RapTOR’s automated molecular biology platform scans for novel/unexpected ones.

Bacterial Pathogenicity Islands

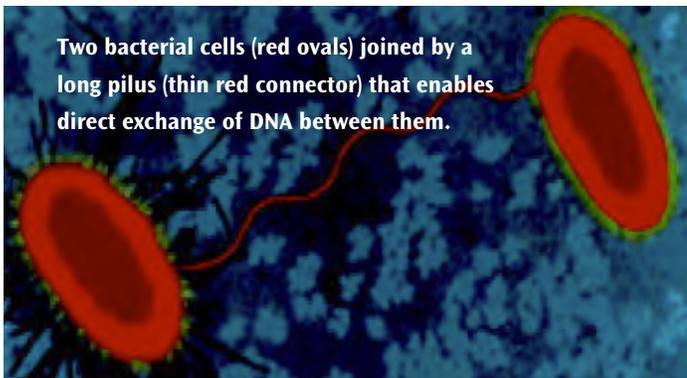
Battling an ever-changing adversary

Why such a focus on the detection of unknown pathogens? One reason is the remarkable fluidity and ease with which bacteria and viruses move DNA among themselves. Such DNA transfer is highlighted in a current LDRD project. PI Kelly Williams — who has been studying horizontal gene transfer for a decade at Indiana University and the Virginia Bioinformatics Institute of Virginia Tech — is investigating one mechanism of what is known as horizontal gene transfer, that is, the exchange and genetic variation/scrambling of DNA between bacterial cells of the same generation. This distinguishes such genetic variation from the long-recognized role of sexual or asexual reproduction as vehicles for vertical genetic variation, which transpires as one cell or generation passes its DNA to its progeny, with several mechanisms of DNA variation serving as the substrate for natural selection, the traditional mechanism for species evolution. For example, in vertical evolution, when antibiotics kill most bacteria in a population within a human body — leaving only the antibiotic-resistant ones alive — these will pass on the genetic antibiotic resistance to their progeny, which will then take over the population, evolving it to predominately antibiotic-resistant bacterial cells. Horizontal gene transfer is, in contrast, a mechanism for genetic variation through DNA exchange that can be independent of such reproduction.

Research has discovered that a favored method of horizontal gene transfer is through exchange of mobile DNA segments known as DNA islands. These stretches of DNA, which can exit as discrete genetic elements within a bacterial cell, tend to contain a number of genes and also commonly contain DNA sequences that allow their recognition as homologous to (“matching”) certain positions on the main bacterial chromosome that encode transfer RNA (tRNA) molecules.* Because of this match, enzymes called integrases can snip the bacterial chromosome at those sites, and incorporate the DNA island into the bacterial chromosome, thus genetically and physiologically transforming the bacterial cell. The island may originally enter that cell in one of several ways: through direct gene transfer from another bacterial cell (via a cell-to-cell bridge/tunnel known as a pilus); through infection by a bacterial virus (a phage) that contains and delivers that island

* tRNA molecules are essential participants in the genetic process of translation by which a messenger RNA containing a copy of the genetic code in a gene is translated into a protein.

to a bacterial cell upon viral infection, and even by way of a bacterial cell ingesting free DNA in its environment (such as that which might be released by the death of another cell). This mobile genetic element version of DNA transfer and genetic variation is, of course, evolutionary in scope, and operates along the same principles. For example, the human intestine serves as a particular niche in which survival and reproduction among the bacteria present may be promoted by possession of a given island. Williams points out that islands often contain a “payload gene,” one that can be physiologically beneficial to the host, “a way of paying rent to the host,” as he frames it.



Of special interest to Williams and Sandia’s IHNS SMU are pathogenicity islands, that is, stretches of DNA that carry genes encoding toxins or other mechanisms making the bacterium more virulent, more able to cause human and animal pathologies. For example, the difference between the *E. coli* bacteria that live in mutualistic symbiosis in our digestive systems and the strain of *E. coli* found in contaminated hamburger and implicated in outbreaks of hemorrhagic colitis, was discovered to be the pathogenic strain’s possession of a pathogenicity island. This is but one example of the general outlook for the potential emergence of new bacterial strains that provoke human diseases. Naturally, this process of horizontal DNA transfer of pathogenicity islands provokes cause for concern regarding bioterrorism, whereby terrorists

“We’re harnessing the incredible amount of data made available by advanced genetic sequencing.”

could utilize these islands to create super-pathogenic bacteria whose introduction into a human population could readily cause lethal pandemics.

One area of research that appears to be lacking, and which can serve as a base from which to recognize maliciously engineered pathogens is the deep understanding of how such islands are phylogenetically related and what are the patterns by which they are naturally moving around among related or unrelated bacteria. As Williams frames it, “what bacteria are talking to whom and what are the pathways by which transfer is occurring.” Hence this research is bioinformatic in scope. Drawing from the immense amount of data derived from DNA sequencing that has produced complete sequences of about 2700 bacterial genomes (complete with plasmids [extra-chromosomal DNA circles] and phages), Williams is organizing his data by scoring bacteria, the score representing parameters such as the “naturalness” of a given organism. “We’re harnessing the incredible amount of data made available by advanced genetic sequencing,” he comments, emphasizing that organization of this data may also be relevant to biofuels, given that some islands contain genes for catabolic pathways.[†]

However with pathological organisms the primary concern, the project is employing as much off-the shelf technology as possible, for example, Mugsy a free-download, whole-genome alignment tool that compares genomes on the basis of shared DNA sequences and has been used to order and visualize multiple genomes of bacterial genus *Brucella*. “Once you have a pathogenicity gene, you can get machine learning to find another,” Williams points out. The databases resulting from this effort both support Sandia’s growing effort in the area of multipurpose bioinformatics and make it more likely that engineered pathogenicity islands might possibly be detected. Because bioengineers have become more sophisticated and often leave scant evidence of what was formerly readily detectable engineering, the database assembled in this project will offer a more comprehensive methodology and related ability to deduce the “un-naturalness” of a pathogenic bacterium through the assessment of its relationship to more-dependably natural routes of island transfer. It is clearly a highly important approach, one whose impact will undoubtedly manifest in years to come.

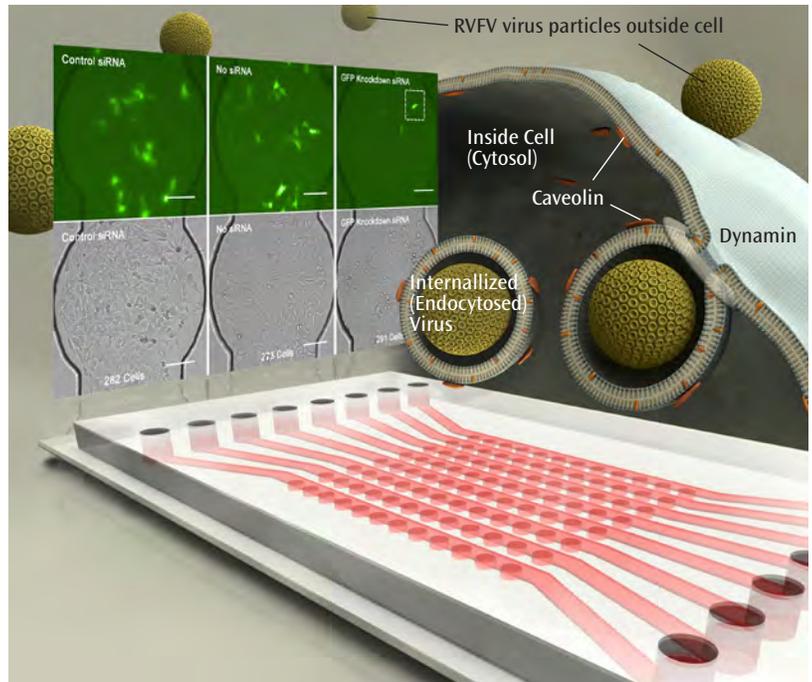
[†]Catabolic pathway genes encode a succession of enzyme proteins that catalyze the breakdown of larger molecules to smaller, often with liberation of energy.

Using siRNAs to Discern Routes of Biothreat Viral Infection

Whither the large amount of noncoding DNA mentioned previously — why so much DNA and so few classical genes in mammalian (including human) genomes? Originally thought to be junk, some evolutionary leftover, the past ten years have brought an amazing new level of genetic complexity to cells with the realization that at least some of this non-classical-gene DNA encodes (is transcribed by RNA polymerases) into a plethora of small RNA molecules that impose an additional level of the regulation of gene expression within cells. For example, micro-RNAs (miRNA) — only about 20 ribonucleotides in length — can negatively regulate the translation of mRNA into protein, by blocking ribosomes and by hastening mRNA turnover (meaning shorter lifetime, less frequency of translation, fewer proteins of that type); in some instances, they may positively influence translation, as well. Related but somewhat different, small interfering RNAs (siRNA), also about 20 ribonucleotide pairs in length can act in several ways to entirely silence (turn off) gene expression.

In addition to opening a new subfield of molecular biology research, siRNAs have provided biologists with the opportunity to specifically turn-off genes to assess the effect of such gene silencing on a variety of cellular functions. Sandia's Oscar Negrete has been utilizing siRNA technology in an LDRD project that is elucidating the biology of biothreat viruses in order to ultimately develop methods to block their infectivity and thwart their ability to damage human cells. Working with viruses such as Rift Valley Fever virus (RVFV), and collaborating with the University of Texas Medical Branch (UTMB), the team is characterizing the mechanism by which viruses enter cells, the obligatory step in initiating pathogenesis. For RVFV, potential pathogenic outcomes include encephalitis (brain inflammation) and lethal liver damage. Although this mosquito-borne pathogen is of interest because of its livestock mortality and its spread from Africa to Asia and potentially, Europe, its great potential as a bioweapon is as, if not more, critical, given the complete absence of therapies against it. For RVFV and related viral pathogens, therefore, understanding and thwarting entry into cells is an important line of discovery.

By engineering a microfluidic chip that increases efficiency and accuracy in siRNA gene-silencing studies and also



Upper right: a cell in the process of internalizing a virus through the process of caveolin-mediated endocytosis. Bottom: Microarray experimental setup for siRNA studies. Top left: evidence for siRNA-mediated inhibition of gene expression.

conserves precious cells and siRNA reagents while preventing cross-contamination in microarrays (by which different gene-specific siRNAs are tested for their ability to impact viral infectivity), the team has demonstrated that the entry of RVFV into cells occurs by a specific mechanism known as caveolin-mediated endocytosis. Essentially, the siRNAs are tested for their ability to silence the expression of genes that participate in alternative methods of viral internalization, systematically denying cells the proteins encoded by such genes, until the right combination of silenced genes defines a “switched-off” cellular function.

By elucidating this mechanism by contrast to several other potential mechanisms of viral internalization, this discovery has now opened the way for additional research aimed at discovery of prophylactic methods to block this cellular pathway to RVFV. Additionally, the chip technology developed in the project can now also be utilized in further siRNA-driven investigations into mechanisms of pathogenesis for other important biopathogens.

Addressing the Global Anthrax Threat

Perhaps no biothreat organism has received as much publicity as *Bacillus anthracis*, the causative agent in anthrax. And while the toxic proteins synthesized and secreted by this bacterium are well-known and well-studied — protective antigen, lethal factor and edema factor — the organism’s worldwide ubiquity makes detection of highly pathogenic strains of primary importance to both biothreat mitigation and public health. One current LDRD project has assembled a team of microfluidic/microsystem engineers, molecular biologists, and a project lead, Melissa Finley, with a deep understanding of the issues entailed with trying to accurately test for *B. anthracis* in resource-limited environments such as those in third-world countries.

The issue as expressed by Finley is attempting to “engage the veterinary segment in such environments,” whose interest is more local — protecting their livestock — but whose participation can be of assistance to a broader effort with which to stem the proliferation of this potentially lethal bioweapon whose spores, mailed to two US senators in 2001, caused one of our most publicized bioweapons incidents. Problematically for Finley, who has experience conducting one-week training classes in such settings, “it’s really tough out there. How can you do surveillance when the people you partner with are largely untrained?”

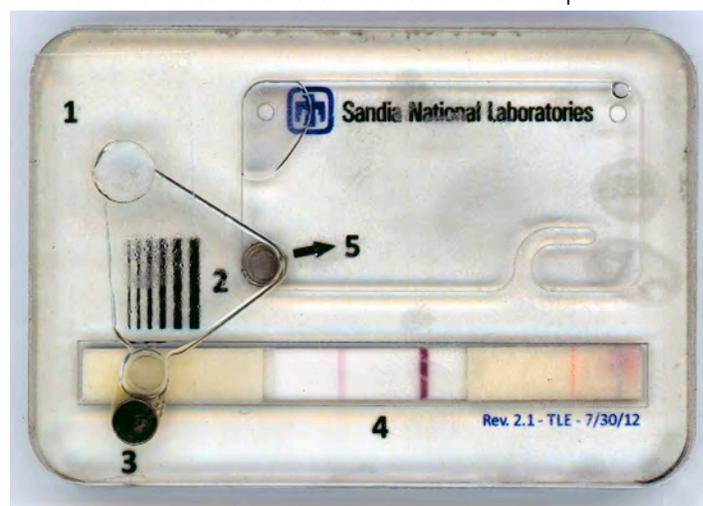
The answer, other than training, is to make detection simple,



A collection of long, rod-shaped, *B. anthracis* bacteria

requiring minimal sterile laboratory culturing technique and providing a strong, unambiguous colorimetric test for the bacteria. Such is the nature of this multifaceted LDRD project to ultimately minimize *B. anthracis* handling and mitigate the proliferation risk. The major thrust is to develop an inexpensive credit-card-sized chip requiring no power or other instrumentation, operable by individuals with minimal training, and self-sterilizing after a diagnostic assay for anthrax (or other organism) has been completed. The one opening in the chip, for inoculation, closes after that step rendering the device into a closed system. Designed and engineered by Jason Harper, the channel-free fluidics device mixes a sample inoculum with a tiny amount (0.3 mL) of culture medium, and after 24-hours of growth in the sealed microchamber, exposes that culture to a test chamber that changes color in the presence of anthrax and its toxic proteins. A magnetically controlled valve closes the growth chamber and opens the test chamber.

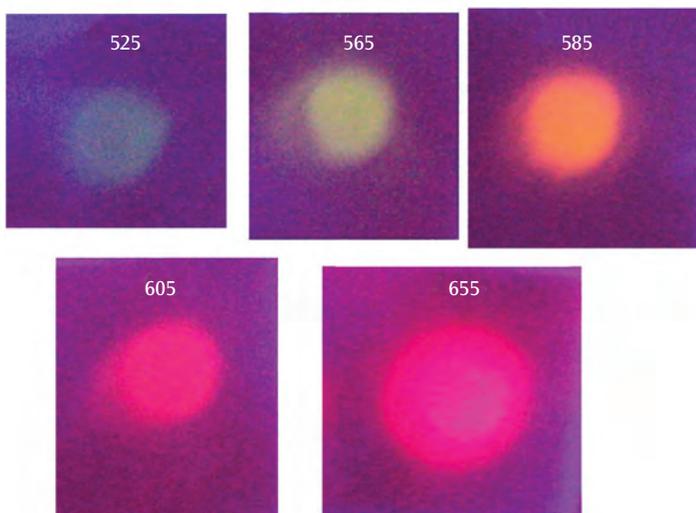
The fluid moves between chambers at different depths in



The anthrax detection chip, current iteration with commercial test strip.

the device by simple shaking or tapping. The final step after reading the test result is to transfer the sample to a chamber pre-loaded with bleach to destroy any contained organisms. There is no exit port.

With Finley’s experience and Harper’s clever engineering comes molecular-level research by George Bachand and Amanda Portillo-Carroll to design a better, more easily read test that does not rely on the more-expensive commercial test strips



Variety of emission wavelengths available from core-shell nanoparticles

that the project is currently employing. Their approach is to utilize conjugated-antibodies whose variable “business end” (the F_{abs} or antigen-binding sites) specifically bind to and identify *B. anthracis*’ toxic proteins or spores. One approach is to conjugate the antibodies at their F_c or constant end with gold nanoparticles, which when brought close together exhibit a red-to-blue colorimetric shift. A second, possibly more highly visible method is to substitute cadmium-selenium core-shell quantum dots for the gold, which, upon excitation, trade electron-hole pairs resulting in light emission as the electrons drop in energy. The wavelength of the emission can be tailored by varying the size of the quantum dot core. The dots have a “huge excitation profile and a huge emission”

“I can really appreciate why bio-surveillance is where it is.”

according to Bachand. This would merely entail providing an inexpensive LED along with the test device. A positive anthrax test would be revealed by a strong emission of light from the test chamber. The goal of this aspect of the project is to detect all four virulence factors, the three toxin proteins and a surface protein of *anthracis* spores by adjusting the nanoparticles to emit a different color for each of the different dot-conjugated antibodies. At that point, excitation with the LED would result in a maximum of 4 color bands, if spores were present along with all three toxin proteins. The methodology would be applicable beyond *Bacillus anthracis* to other biopathogens/ bioterror organisms, as well and would improve the target detection limit by several orders of magnitude.

“I can really appreciate why bio-surveillance is where it is,” comments Finley, in taking the long view of what the project is trying to accomplish against the backdrop of the lack of expertise in third-world countries. Clearly, the project’s impact in helping to thwart a global bioterror promises to be quite significant. ■

Part II: Bio-Nano-Engineering Initiatives

Nano-engineering solutions using Nature's models

Introduction

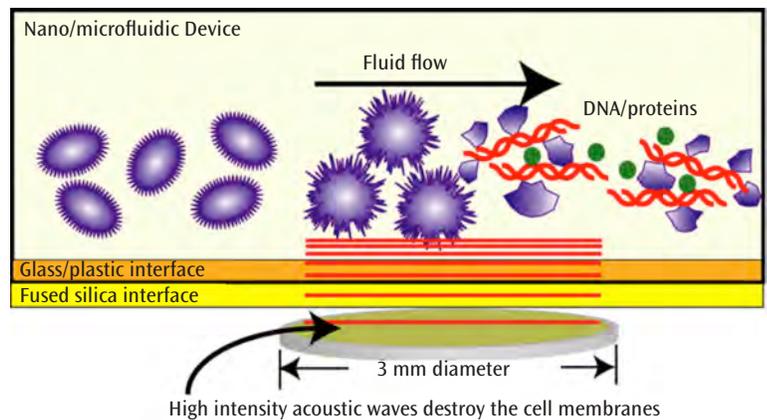
In addition to the obvious relevance of biothreat reduction activities, a number of other lines of LDRD-funded research have approached this contribution to reducing biologically related security threats through bio-engineering initiatives. For example, biomimetic approaches seek to find solutions inspired by evolution, by mimicking biological answers to questions like water purification. Some projects seek to use natural systems engineered for diagnostic or therapeutic purposes, in some cases engineering that combines breakthroughs in non-biological materials science with biologically derived or inspired micro- and nanostructures. Nanoparticle assemblies, mimicking aspects of biological systems, are being devised for novel therapeutic approaches to drug delivery and vaccine development. Biomolecular engineering projects now supported by the Sandia LDRD program display a great range of ingenuity and potential for reducing biothreats — both microorganismal and otherwise — and thereby improving public health.

Acoustic Lysis: Accelerating the Diagnosis of Drug-resistant Tuberculosis

CleanBurst — Sandia's miniature acoustic lysing system — transmits controlled amounts of acoustic waves remotely into nano/microfluidic devices to disrupt the cellular lipid bilayer and internal nuclear membranes of biological cells, including the outer plasma membrane, internal organelle membranes, and membranes associated with the cell walls of certain bacteria. Releasing the intracellular contents without the need for chemicals and simultaneously preserving molecular-biological structures, the analysis also does not require large laboratory equipment or time consuming processing. The acoustic technology directly interfaces with existing nano/microfluidic devices that are being used in a variety of applications — from laboratory and environmental assays to point-of-care devices for infectious disease analysis and treatment.

Developed by Darren Branch and collaborators, the miniature multichannel acoustic wave device disrupts (lyses) the cellular membranes of even the most difficult bacteria such as *Mycobacterium tuberculosis* (MTB) in seconds. MBT lives inside Macrophage cells of the immune system, cells, which, when activated are normally able to lyse and destroy most bacteria, but

which have difficulty eliminating MBT, a testament to MBT's lysis-resistance. The macrophages can only barely control MBT, which makes many people silent carriers. When the immune system is suppressed (as in AIDS patients), MBT grows and multiplies, resulting in active TB with pathological lung symptoms. Because of the worldwide AIDS epidemic and other factors, we are in the midst of a new worldwide TB epidemic. One in every three people worldwide is latently infected (about



Drawing showing CleanBurst's ability to lyse cells acoustically, with resulting release of their DNA and proteins

a third of the world has latent tuberculosis), until another event occurs to suppress immune functioning and allow pathology to manifest into active TB. About 12 million people worldwide had active TB in 2011 and about 630,000 had active multidrug-resistant TB.

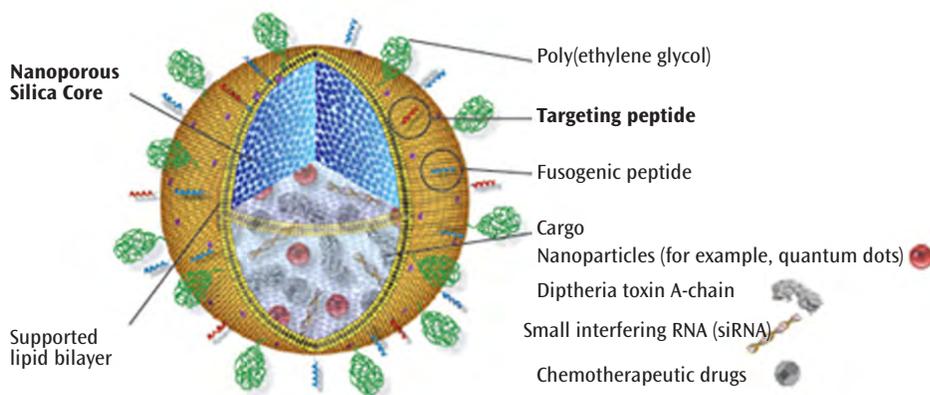
Even standard TB takes six months to cure with a four-antibiotic cocktail. Drug-resistant forms can take two years and require dangerously toxic drugs that cost \$5,000 or more per person; they usually emerge when public health officials fail to ensure that patients with regular TB take their drugs daily. Some of the more-potent drugs cause side effects like deafness and psychosis. Identifying individuals with drug resistance or the even more serious “extreme drug-resistance” can only be done accurately through DNA-sequence analysis of their infecting bacteria. CleanBurst enables this analysis by releasing DNA and proteins without the use of the normally harsh methods that can damage these macromolecules and cause diagnostic errors.

The technology allows such molecular diagnosis to be done quickly, portably, and without other cumbersome reagents both in traditional medical clinics or in those in underprivileged or third-world settings. CleanBurst easily interfaces with other micro-analytical devices, such as DNA sequencers; therefore, starting from, for example, a patient’s sputum sample, bacteria present in that sample can be analyzed right in a point-of-care clinical setting, with immediate treatment, thereafter. This means that a person infected by MTB can be immediately assessed for the seriousness of the infection, such as bacterial drug-resistance. This is true for other disease-causing bacteria, as well, such as distinguishing between the normal *E. coli* bacteria that exist harmlessly in every human being’s intestines and the “super-bugs,” such as those in tainted beef, which can crop up to cause serious human illness. These super-bugs often contain pathogenicity islands (see page 12), and this can only be detected by releasing and analyzing their DNA. With the recognition by biological researchers that the exchange of DNA is nearly ubiquitous among bacteria occurring by several different mechanisms, this diagnostic capability becomes that much more critical.

Protocells: Bio-organic and Inorganic Nanomaterials Engineering — Countering the “Internal Biothreat” of Cancer

Among its other discoveries that enable biomineralization and conversion of biological structures to function-retaining silicon equivalents, this LDRD project also presents an evolved route to the possible outcome of single-dose killing of cancer cells. Dubbed “protocells,” this ingenious nanoparticulate engineering of organic and inorganic materials goes one better on liposomes, an already FDA-approved method of drug delivery. While a liposome is simply a bag of aqueous solution encapsulated by a ligand-decorated lipid-bilayer membrane (the same type of membrane that encloses all higher (eukaryotic) cells and many of their internal organelles, a protocell is much more. The outer lipid bilayer bag encloses and is supported by an internal nanoporous silica nanoparticle that acts as a high-surface-area container-binder for a diversity of cytotoxic compounds—drugs, nucleic acids (such as small interfering RNAs [siRNA], see page 14), proteins, and peptides of varying water solubility, some of which are difficult to simply dissolve in the aqueous medium of liposomes.

Additionally, the membrane of the protocell is “decorated” with both specific and nonspecific binding agents (peptide and polypeptide ligands) that promote its adherence to receptors on a given cancer cell’s surface (in this instance, hepatocarcinoma), which subsequently promote its internalization into the cancer cell (via a process known



Schematic drawing of a protocell, showing a targeted lipid bilayer surrounding a nanoporous silica core that carries a cargo of chemical and biological agents toxic to cancer cells.

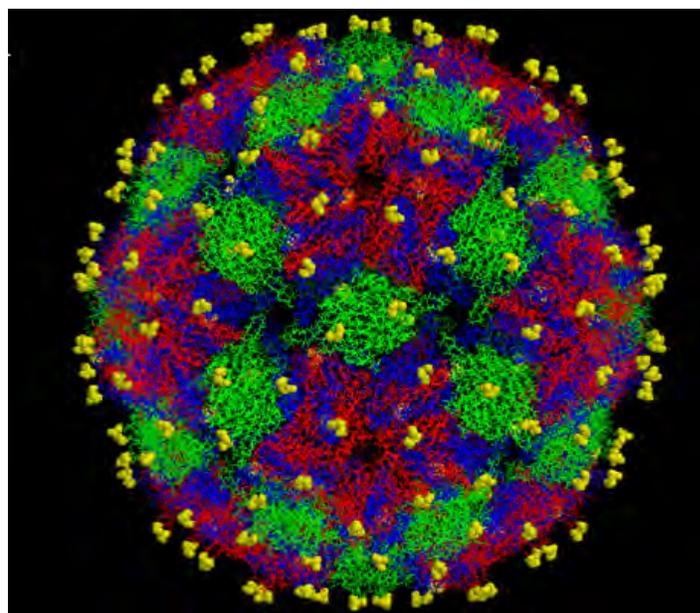
as receptor-mediated endocytosis). Once internalized and acted upon by the acidic environment of cellular lysosomes, the protocell releases a huge cargo of cytotoxins, a cargo that can be composed of such a disparate array of compounds — in terms of mechanism of action — that the probability of a kill is maximized, while the probability of the cancer cell's developing drug resistance is minimized. For example, classic cytotoxic drugs, such as alkylating agents, antimetabolites, plant alkaloids often act by interfering with either DNA and RNA synthesis or the actual mechanics of cell division (mitosis), or by chemically modifying other crucial biomolecules.

Meanwhile, siRNAs are capable of silencing gene expression in a gene-specific fashion, and other signaling proteins may be able to effect or set in motion the process of apoptosis or programmed cell death, by which cancer cells can be induced to commit suicide. Given all these possibilities, to combine a plethora of them in the protocell's internal nanoparticle core for release into a cancer cell provides incredible optimism about the possibilities for better therapeutic intervention. Equally important is the low side-effect profile, the minimization of killing of normal cells. Wrapping the lipid bilayer around the silica nanoparticle imparts highly desirable physical characteristics to the fluidity and stability of the lipid bilayer —favoring, with its array of binding agents, its highly specific binding to the cancer cell's surface, a prerequisite for internalization (endocytosis), and lowering its side-effect profile in binding to and killing normal cell types — in this instance, normal liver cells (hepatocytes).

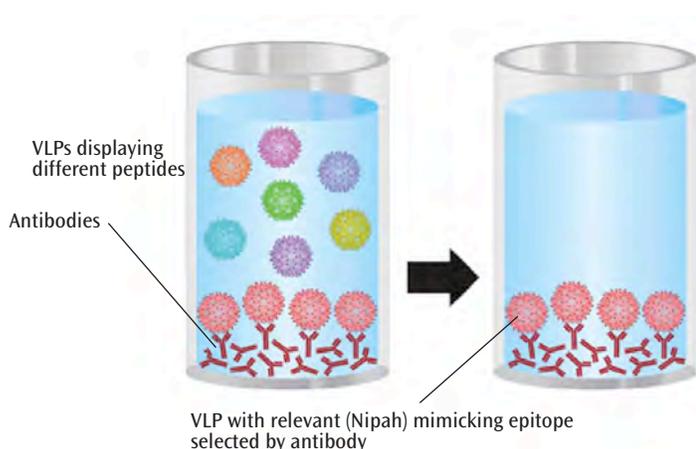
The surface of the protocell is engineered such that it is also quite soluble in blood and is tailored toward low immunogenicity, that is, it does not strongly stimulate the immune system to act against and remove it, both key characteristics of an effective agent in vivo. Although in vivo testing yet lies in the future, the in vitro results are stunning, showing that a single nanoparticulate protocell can deliver such a potent dose of cytotoxic agents so as to be effective at killing a hepatocarcinoma cell with a single dose, while sparing greater than 90% of normal liver hepatocytes. Given that cancer is a major biological threat to our society, such bioengineering activities form an important component of biothreat reduction.

Virus-Like Particles for Vaccines and Other Applications — Engineering the Immune Response for Threat Reduction

Advances in molecular biology have made it possible to use bacterial viruses (phages) such as M13 as platforms for displaying proteins and peptides for a variety of research and clinical applications. But the limitations of these platforms have provoked research in this project to develop superior vehicles, virus-like particles (VLPs), whose molecularly engineered outer capsid assembles — like that of a normal virus — into a multi-protein shell. But rather than viral proteins, this capsid (shell) can be made to display from one to many copies of researcher-selected proteins or peptides, with many copies of the same protein or many different ones. In the latter case, the VLP is used to find so-called “mimotopes” of particular pathogens. For example, by using a monoclonal antibody, known to bind to and recognize the outer shell of the biothreat Nipah virus, that antibody when exposed to a library of VLPs carrying different peptides will identify the peptide or peptides in that library that mimic the actual Nipah-viral molecular surface “shape” (epitope) that had stimulated the immune system to produce such antibodies in the first place. That identified peptide epitope in the VLP library, mimics the actual Nipah-viral peptide; that mimotope is now a candidate for a Nipah-virus-specific protective vaccine.



Virus-like particle capable of displaying up to 180 peptides



This procedure goes something like this (see the drawing above). Inject a mouse with Nipah virus; the mouse's immune system produces anti-Nipah antibodies that specifically recognize a surface peptide (portion of a protein) of the virus' capsid (outer shell). Researchers now have a useful antibody. Researchers create a library of different VLPs (different colored spheres in the drawing), where each VLP in the library has, built into its shell, a peptide of some type. They mix this library with the antibody. The antibody will hopefully stick to one or more of the VLPs (red spheres in the drawing), telling us that that VLP's peptide strongly resembles (at a molecular-shape level) the Nipah virus' capsid (shell) peptide; it is a mimic (mimotope) of that molecular shape of the virus. That VLP (red sphere) can now serve as a harmless injected vaccine to induce vaccine recipients to produce more of these antibodies (as well as immunological memory). These vaccine recipients are now protected against Nipah infection.

Nipah and its close relative Hendra virus cause respiratory and brain inflammation, and with mortality rates in people that can exceed 70%, these assassins are the deadliest paramyxoviruses known to infect humans. They are the only paramyxoviruses that are classified as biosafety level 4 (BSL4) pathogens owing to their extreme virulence and bioterrorism potential. Hence, creating effective vaccines against them is of the utmost importance.

This approach can be extended to any viral or bacterial pathogen, as well as to human cancers and other pathologies. In the opinion of President Harry S. Truman postdoctoral fellow, Carlee Ashley, the project's PI, "VLP

technology promises to be a remarkably powerful, universal technology that will enable rapid, cost-effective identification of vaccine candidates." By inducing immunological memory and hence protection against biothreats, vaccines are an important component of biothreat reduction in human populations.

"A powerful, universal technology that will enable rapid, cost-effective identification of vaccine candidates."

Engineering Cognition from the Bottom Up: Addressing Neuropathological Biothreats

The monumental advances in clinical neuroscience over the past several decades would not have been possible without the enhanced understanding of neuronal processes at the cellular and molecular levels. For example, the underlying basis of Parkinson's disease is now widely accepted as the loss of dopamine-secreting (dopaminergic) neurons in the brain's substantia nigra. Also, understanding the biochemistry involved in depression as somehow relating to neurotransmission in the lower brain's reticular activating (arousal) system — which projects axons widely over the cerebral cortex — has led to the development of the serotonin specific reuptake inhibitor (SSRI) group of drugs that successfully treat depression in some individuals. Although the serotonin theory is now recognized as incomplete, it does illustrate how modulation

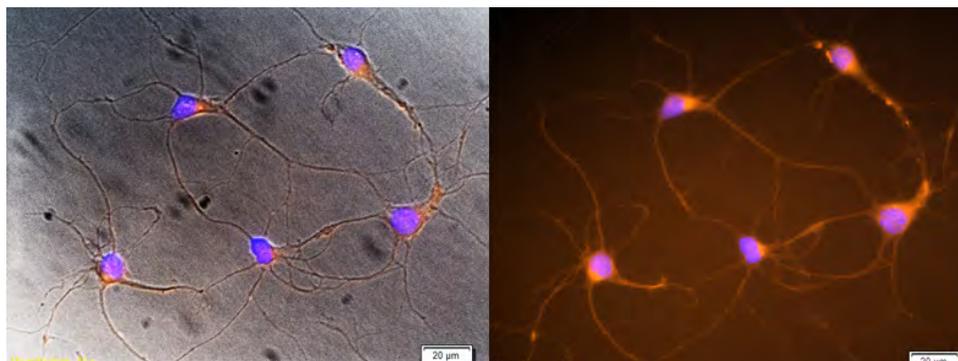
of biochemical processes at the level of the single neuron and its synapses (points of communication with other neurons) can offer an important target for therapeutic intervention in emotional and cognitive issues. Such issues relate to national security in numerous ways, both as they pertain to the optimal psychological functioning of civilian and military analysts and decision makers and to homeland security in ensuring protection of the public from the aberrant, antisocial behavior of belligerent, pernicious individuals.

Looking toward the future, biomedical scientists can envision research and therapeutic methods for selectively intervening in the metabolism of selected neuron types, particularly biochemistry related to neurotransmitter synthesis and secretion. Such methods could allow the correlation of cognitive functions with the activity (or

lack thereof) of selected brain neuronal systems. A recent short LDRD project has begun the investigation of a methodology to accomplish this type of intervention, and the project is notable for its use of genetic engineering methods combined with the engineering of novel inorganic nanomaterials. These so-called PIONs, porous iron oxide nanoparticles, have several advantageous features. First, their nanopores can accommodate and be loaded with other material cargoes, including nucleic acids. Second, they are biostable and biocompatible, and small enough when coated by a lipid bilayer, to enter neurons easily. They provide enhanced contrast in MRI imaging and can be induced to release their cargoes by radiofrequency stimuli.

The ultimate goal of this research was to demonstrate that the PIONs could be loaded with small interfering RNA

(siRNA) cargoes (see page 14 in Section I for an explanation of siRNA mechanism of action), which after uptake by a neuron, would then selectively turn off gene expression in critical genes



Bright-field (left) and dark-field (right) images of tissue-cultured neurons, where the cell bodies have taken up PIONs as evidenced by their violet light emission.

related to neurotransmitter synthesis and/or secretion. In its brief timeframe, the project illustrated successful uptake into the cell body of cultured neurons of PIONs carrying short stretches of DNA, about the size of siRNAs, thus setting the stage for the next phase of this research into novel methods for elucidation and ultimately control of brain mechanisms underlying cognition. One goal in test animals would be to test whether selective inhibition of gene expression in neurons could alter behavior by interfering in a specific neurotransmission pathway by which neurons communicate. Projecting futuristically, one might imagine such an intervention as serving a therapeutic role in altering behaviors where imbalances are present among different neurotransmitter systems. In either case, this project aptly illustrates the leading-edge nature of LDRD initiatives in Sandia's biosciences/cognitive sciences research in support of current and future national security objectives.

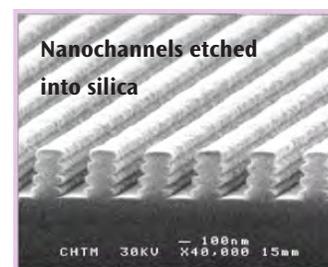
Mimicking the Wisdom of Cells and Their Channel Proteins

A major thrust of LDRD-funded biosciences research impacting national security has been a set of investigations into the structure-function relationships of channel proteins. Cells live in a surrounding aqueous fluid, which except for the brain, is fairly similar throughout the human body. This so-called extracellular fluid (ECF) is serviced by blood. Inside cells is another aqueous solution referred to as cytosol. The internal (cytosol) aqueous solution has a solute (dissolved substances) composition quite different from the ECF, and the lipid bilayer membrane of cells generally keeps the two solutions separate, given its limited permeability to water and dissolved substances.

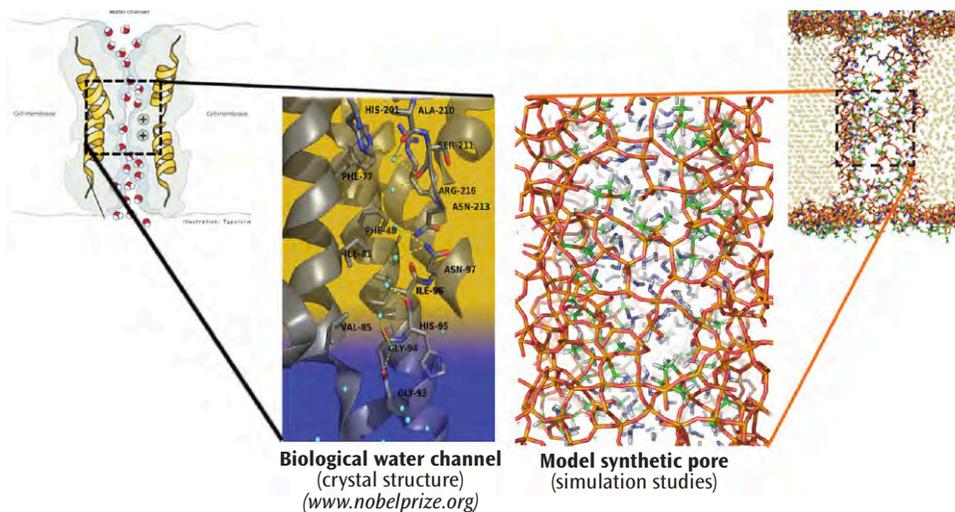
Under normal circumstances, however, a variety of cellular processes such as the nerve impulse and the contraction of muscle cells require a controlled exchange of substances across that membrane. Ions (salts) such as sodium (Na^+), potassium (K^+) and calcium (Ca^{2+}), and also water itself are exchanged between inside (cytosol) and outside (ECF) using proteins that form channels in the cell's membrane. Such ion and water channels are structured to provide nanopores that permit the transit of a given ion or water molecule across a cell's membrane, while rejecting the transit of other substances. Hence a potassium channel permits only potassium to transit, and a water channel (aquaporin) permits water to transit while rejecting the ions dissolved in that water. The physiological importance of such channels (and their energy-consuming relatives known as "pumps") is underscored by, for example, the genetic disease, cystic fibrosis, in which a mutated gene carries information for a defective chloride (Cl^-) channel pump, which, in its failure to adequately function, leads to a mucus that is thick and gluey, clogging the lungs and usually leading to premature death. Because ion channels are such key players in biological processes, the pharmaceutical industry has developed drugs that target specific ion channels. For example, to lower blood pressure, a class of drugs known as calcium channel blockers reduces the strength of heart-muscle contraction triggered by the flux of calcium ions from

extracellular fluid through its channels and into the cytosol of heart-muscle cells.

The bold yet methodical chemistry in these LDRD projects is testament to the combination of scientific ingenuity and hard work necessary to achieve these challenging goals. Beyond simply unraveling how



channel proteins work, the projects also succeeded in building inorganic mimics of an aquaporin, an accomplishment with far-reaching practical applications. PI Susan Rempe and her collaborators initiated nanoengineering in silica, creating physical nanochannels in Sandia's MESA microfabrication facility. Subsequently, with the help of computational modeling, they began chemically modifying the walls of these nanopores. Knowing that water molecules line up in a continuous chain through the narrow, approximately 2.5-nm pore of the biological aquaporin, they initially lined the pore with polar (or "hydrophilic") chemical groups to which water, because of its polar chemistry is attracted. And the water molecule did "like" the channel, but unfortunately, so did certain of the dissolved ions. In other words, the balance between water passage and ion rejection by the channel was not desirable. "We then greased-up the channel," Rempe recalls, lining it with bulky hydrophobic groups, chemical groups that, like oil, don't mix well with water or its dissolved ions. Now there was excellent ion



Schematic of biological aquaporin (left) and the chemical modifications made to a silica nanopore (right) to attempt to mimic the characteristics of the aquaporin.

rejection, but “the water molecules were unhappy”; they wouldn’t enter the channel much. The jousting for correct chemistry was clearly far more sophisticated, and further study of the aquaporin pointed to a staircase ladder of a mix of hydrophilic and hydrophobic chemical groups that also tapered the pore size down below 1 nm.

This resulted in an R&D100 Award-winning technology with multiple applications. First, was its ability to passage water while rejecting the dissolved ions (salts), exactly what reverse osmosis (RO) membranes are designed to do to yield potable water from either brackish, seawater, or contaminated water. On a lab scale, the project’s water channels outperformed commercial RO membranes by an order of magnitude, exhibiting a robust flow of purified water with flow varying linearly with applied pressure, and with that pressure requirement being 10 times less that for commercial RO membranes. “The ion rejection of the pores can also be tuned to reject say 85% of the salts to yield mineral water, rather than 99% rejection for pure drinking water,” Rempe points out. Given the opinion that water may be the “oil of the Twenty-first Century,” that is, a resource whose shortages may prove critical to national security, this nano-engineering accomplishment with a biological tutor is quite significant.

The technology’s superior performance has led to a memorandum of understanding (MOU) with Corning Glass, Inc., for a somewhat different application, namely osmotic power generation. With the project’s water channels separating fresh and salt water, fresh water will subsequently osmotically flow into the salt water, raising the column of salt water against gravity, and essentially transforming chemical gradient energy into gravitational potential energy, which can then be used to power some other process (such as turning a generator). Another application in the offing is an already funded cleanup of well water by removal of arsenic, perchlorates, and nitrates under California’s Proposition 50.

The LDRD-funded ion channel research did not stop at water channels. The team studied, modeled and experimentally investigated the involvement of potassium (K^+) channels in the immune response, beginning with the chemical basis for allowing the passage of K^+ through the channel while, somewhat counterintuitively, excluding the slightly smaller Na^+ ion. Although there are many varieties of K^+ channel among the different cells of the human body, this research focused on those regulating the innate immune response — the more evolutionarily primitive portion of the immune

“The oil of the 21st Century.”

system that responds quickly and relatively nonspecifically to invasion by bacteria. For example, all bacteria of the gram-negative variety possess a molecule, lipopolysaccharide (LPS), that triggers this innate response by activating scavenging white blood cells known as macrophages. This immune response includes a component of inflammation (by which blood vessels are “opened up” to the passage of these bacteria-scavenging white blood cells from blood into tissues where bacteria are located). Using a combination of fluorescently labeled probes and electrophysiology, the research identified potassium channels in macrophages that are involved in mediating their activation by bacterial LPS. In addition to providing a new drug target for pharmaceutical intervention in this immune-response component, the research also clarified the relationship between channel structure and activity (its gating of the flow of K^+ through the cell membrane).

Membranes for Biofuels

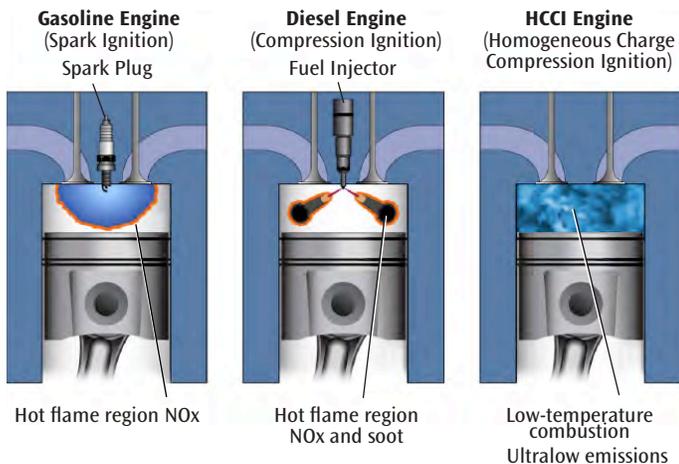
As a final illustration of the diverse impacts of this work — and a transition into Section III of this publication — the team is hopeful about a proposal to ARPA-E (Advanced Research Projects Agency—Energy) in collaboration with UNM and the University of Texas, El Paso for a clever cross-feeding system requiring several types of membrane development. The proposed project uses cross-feeding between cultures of microalgae and cultures of bacteria that can, with the appropriate membrane designs, generate energy via reverse electrodialysis, this energy later used to drive water purification membranes, while in the meanwhile, growing a culture of microalgae from which cells and their photosynthesized lipid are semi-continuously harvested as biofuels sources. This complex, ingenious system has the potential to reduce water and nutrient use for algal culturing and also reduce the requirement for extensive energetic input for algae harvesting and dewatering. If successful, the strategy outlined in the proposal would point the way to a greater efficiency in growing algae and extracting their photosynthetically produced lipids for conversion to biofuels. Water security *and* biofuels impacts — this work illustrates the broad reach of LDRD research endeavors. ■

Part III: Biofuels Research

Fungi to Thermophiles to Algae

Introduction

The essence of biofuels research is to optimize the efficiency of harvesting the chemical potential energy from carbohydrates and lipids originally photosynthesized by plants and algae and stored in their biomolecules (biomass). Sandia biofuels research initiatives span the investigation of biomass sources from terrestrial (e.g., switchgrass and agricultural waste) to freshwater and marine algae, both prokaryotic (cyanobacteria) and eukaryotic. The terrestrial-source research encompasses a variety of innovative genetic engineering approaches to both complex carbohydrate (lignocellulose) digestion (decomposition) and increased yield from simple sugar fermentation. Meanwhile, in addition to genetic engineering work, algal research employs novel diagnostics to understand and manipulate the entire range of problematic issues in algae cultivation and lipid biodiesel-precursor extraction and optimization, from single cell to industrial-scale. Sandia's advanced microfluidics systems, supplemented by laser-based devices for single-cell trapping enable sophisticated interrogation technologies to categorize differences in cells and their effects on biomass productivity. Included in this range of LDRD-funded initiatives are computational modeling activities that both complement experimental work and help to set the stage for new experimental approaches. Collaborations with corporate partners and with the Joint Bioenergy Institute (JBEI) and its member organizations figure prominently in much of this research.



Comparison of three internal combustion engine types

Codevelopment of Biofuels and Internal Combustion Engines

Although numerous LDRD projects are investigating novel approaches for terrestrial biomass optimization and processing (degradation to sugars and then ethanol or other organic fuel prospects), none is so uniquely Sandian as this initiative for codevelopment of biofuels and the engines that will combust them. This especially novel approach is possible only in a highly multidisciplinary research environment such as Sandia's, and it is based on the premise that current engines are hardly optimized for biofuels combustion, and therefore that the two lines of research should inform each other. This thread of LDRD-supported research is one of only a few taking this approach, worldwide. "Sandia is unusual in that we have strengths in both areas," says Craig Taatjes, the project PI.

The project encompasses genomics, chemistry, and engine combustion studies, both modeling and experimental. Multiple potential sources of terrestrial lignocellulosic biomass are available for conversion to biofuels. More importantly,

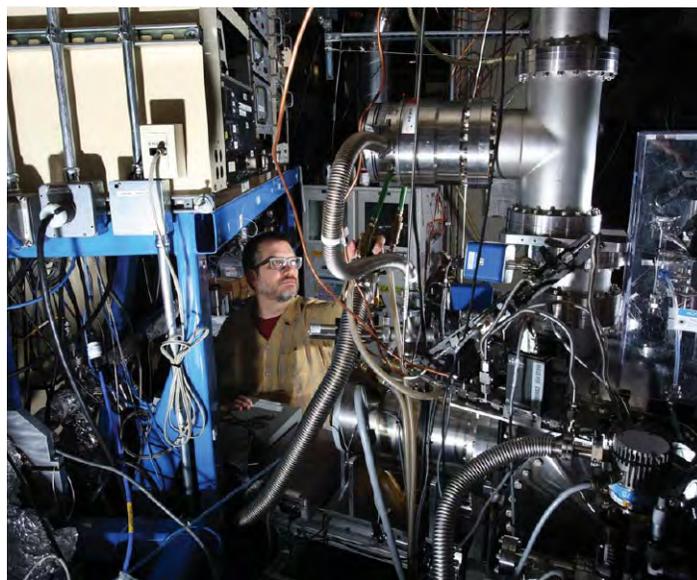
within the biofuels community, abundant mechanisms are being researched for enzymatic degradation (biochemical breakdown) of the biomass' polysaccharide cellulose and the polyphenol lignin to the simpler sugars, alcohols, and ketones that comprise a class of volatile organic compounds (VOCs) suited for combustion in engines. Many routes of genetic engineering of the bacteria and fungi that can degrade cellulose and lignin to VOCs are in process, worldwide. Most are attempting to optimize the biochemistry to minimize the chemical reaction steps and the cost of such processes,* while maximizing yield.

Wisely, this project has chosen to initially work with endophytic fungi, organisms that actually live symbiotically within the bodies of plants, and which therefore intrinsically possess genetically encoded enzyme systems to degrade cellulose to a variety of VOCs. The genomic aspect of this project will characterize the segments of DNA encoding enzymes and other proteins that appear to be important to the fungus' ability to perform such biochemistry, and subsequently, to express these fungal genes in convenient bacterial systems or yeast to ultimately assemble ready-to-use genetic modules.

In parallel with the genomic elements necessary to produce these VOCs, the VOCs themselves are being characterized in terms of their likely combustion chemistry. This project aspect collaborates with Massachusetts Institute of Technology (MIT), where a unique tool, the automated reaction mechanism generator (RMG) can predict the rates of chemical combustion based on the molecular structure of the VOC being characterized. There is also communication and cross-fertilization with a German project team at RWTH Aachen University.

The third parallel component of this project selects promising VOCs for actual engine combustion studies for low-temperature autoignition in Homogeneous Charge Compression Ignition (HCCI) engines. These engines function like diesels but with far lower emissions, what Taatjes describes as a "platonic ideal" because of the HCCP engine's very high sensitivity to fuel chemistry for engine combustion at low temperatures. General Motors has been experimenting with both HCCI engines and hybrid engines that include

spark ignition under certain conditions. By characterizing engine performance with the variety of VOCs produced by the fungi and comparing them with the RMG predictions, a great deal can be learned for both present and future endeavors. "We're really trying to develop a capability," Taatjes says. The prospects for doing so has won the work participation in CERC, the U.S.-China Clean Energy Research Center, a major nod toward the importance of the project's approach. It has also garnered the 2012 David A. Shirley Award for Outstanding Scientific Achievement.



Craig Taatjes adjusts an experimental engine

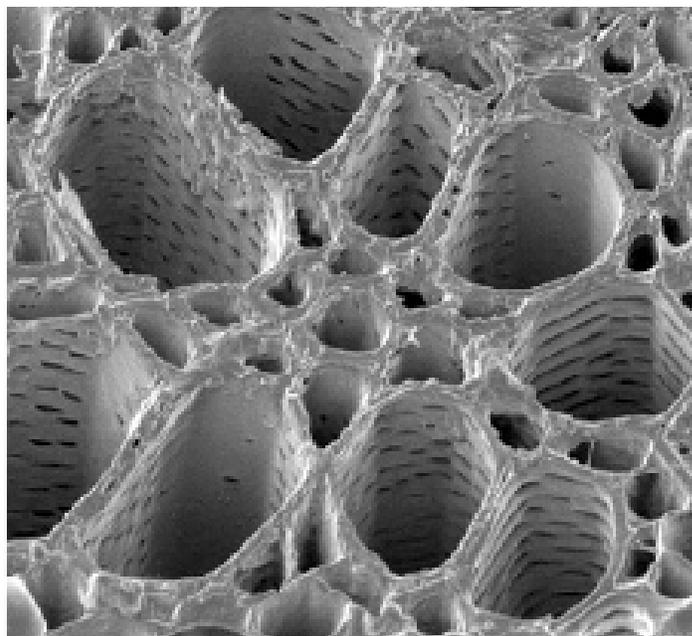
"Sandia is unusual in that we have strengths in both areas."

*Traditionally, these processes have encompassed two overall sets of biochemical transformations, the first to degrade the biopolymer polysaccharide cellulose to its component monomer sugars (glucose), and the second to ferment glucose to ethanol or other alcohols for combustion.

Production of Extremophile Bacterial Cellulases in Fungi

In another LDRD project aimed at improving the degradation of terrestrial biomass utilizing commonplace filamentous fungi such as *Aspergillus niger*, the common black bread/fruit mold, researchers are aiming to carry out these processes at higher temperatures. The advantages are first, the increase in kinetics (faster) because of the additional available thermal energy present to break bonds in splitting the larger polymers to their smaller monomers, and second, the inhibition of the growth of opportunistic microbial infections. However, higher temperatures denature† and inactivate proteins, including the fungal enzymes that catalyze these reactions. While filamentous fungi are desirable because they overproduce these enzymes, the enzymes are useless if denatured.

The solution: Certain microorganisms are thermophilic (“heat loving”), such as bacteria living in hot springs and hydrothermal vents on the ocean floor. Their enzymes are not denatured by excess heat. Hence if the bacterial genes encoding these enzymes — such as the genes for cellulose-degrading enzymes — could be rendered functional in filamentous fungi like *Aspergillus*, one would expect to elicit overproduction of such high-temperature-functioning (thermophilic) enzymes, which can then be used to degrade cellulose at the higher temperatures desirable to increase reaction kinetics (speed up the reactions). This project is aiming to engineer *Aspergillus* to express the thermophilic bacterial genes for a “cellulase cocktail,” including the enzymes endoglucanase, cellobiohydrolase, and β -glucosidase. Such a cocktail, operating at high temperatures would represent a significant advance in the attempts to increase the efficiency and lower the cost of using terrestrial biomass such as switchgrass and corn stover as inputs for biofuels production. Hence, this LDRD project can, in a sense, be seen as complementary to the fungal fuels/engine evolution research just described.



Micrograph of lignocellulosic plant cell walls, the substrate for most terrestrial biofuels.

† Denaturation means the loss of the specific three-dimensional shape that enzyme proteins require to perform their very specific catalytic functions.



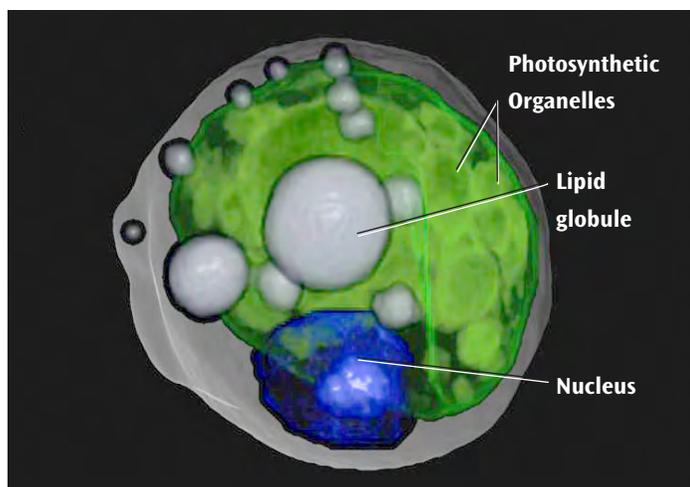
Algal Initiatives

Sandia is also vigorously pursuing biofuels initiatives based upon cultivating and engineering algae for biodiesel production, and the LDRD program is playing a significant role here, as well. Microalgae (which exist largely as single cells or microscopic colonies) may be cultured so as to promote individual cells to produce large quantities of triacylglycerol lipids, and commercial-scale processes exist to readily convert these lipids to biodiesel.

Benchtop to Raceway: Laboratory-Scale vs. Commercial-Scale Cultivation of Algae

Key among LDRD projects in this arena — in which hyperspectral imaging has played a huge role — is an LDRD project, led by PI Jeri Timlin, known as *From Benchtop to Raceway: Spectroscopic Signatures of Dynamic Biological Processes in Algal Communities*.

Small yet efficient, microalgae are attractive for many reasons, including their rapid, cost-effective, and resource-efficient photosynthetic production of biomolecules — mostly lipids under the right conditions. They can be cultivated on non-arable land with reclaimed water and can yield remarkable lipid production per cell — up to 20–50% of their total cellular dry weight, with examples of up to 70% reported under certain conditions. The water and nutrients used in growing microalgae can be easily recycled, thus conserving precious water resources as compared to other biofuels production routes. It has been estimated that lipid production in microalgae could be 30-times more efficient in terms of relative production of lipids per acre per year than any terrestrial plant oil feedstock.



Drawing of components of a single cell of eukaryotic algae

However, the fundamental issue dominating this project, and algal biofuel initiatives, in general, is the disparity between lab-scale and commercial-scale endeavors. For example, according to Timlin, a 60% lipid yield in the laboratory often crashes to a 10% yield in algal raceways, the quarter-acre (or larger) open ponds used to grow algae on a commercial scale.

To tackle this disparity, Timlin and her co-investigators have collected spectroscopic signatures of changes within algal cells under altered environmental conditions, which might range from changes in nutrients in the growth medium to infections of the algal culture by bacterial or fungal pathogens, so-called pond grazers. Results indicate variability that can be observed in, for example, the lipid content of the algal cells and the proportion of different photosynthetic pigments (chlorophylls a and b and carotenoids) within the algal cells, as conditions change. There are so many variables, that some of the tools — such as multivariate hyperspectral imaging — to observe such correlated changes were absolutely essential to collect meaningful data. By clever filtering of the autofluorescence hyperspectral signal, the carotenoid signal was utilized to quantify lipid content; hence, there was no necessity to add any fluorescent dyes to the culture, which would potentially have their own impact on algal growth and photosynthesis. This capability — conferred by hyperspectral microscopy — to allow collection and analysis of data from “clean cultures” has been crucial for making reproducible correlations of this type. Cells infected by viruses or fungi (such as by the *Chytridiomycota*) or suffering nutrient deprivation or imbalance can yield reproducible changes in these hyperspectral signatures.

Led by Thomas Reichardt, the team developed a clever technique to utilize spectroradiometry to measure growth and photosynthetic pigments in cultures of *Nannochloropsis salina*, a popular microalgal candidate species for biofuels production.

This remote technique collected data allowing calculation of the reflectance from cultures, which, in turn, contained information about the population density and about relative optical activity of the different photosynthetic pigments in the algae, information predictive of algal health.

The key transition point for the project was from benchtop to raceway, a raceway being the commercial-scale shallow oblong cultivation pond (reminiscent of a horseracing track) that may cover areas from a few square meters to as much as a quarter acre or more. The question now being investigated is a key issue for the commercial algae community, businesses that often survive by producing a mix of algal products such as pharmaceuticals, nutraceuticals, and cosmetics. Can the observations of correlated hyperspectral changes serving as markers of algal physiology in the laboratory be adapted and validated for raceways, so that it becomes economically feasible for commercial algae growers to direct the majority of their businesses toward biofuels production?

Sandia researchers now have access to Sapphire Energy Incorporated's raceways, with funding by a research grant from DOE's Office of Biomass Programs (OBP). In addition to her NIH New Investigator Bioscience Award to study protein-protein interactions in living cells at a fundamental level, Jeri Timlin is participating in this study, led by Reichardt, with Howland Jones the third key Sandian. Through a combination of hyperspectral confocal microscopy and the reflectance method described above, which can assess biomass growth at intervals as frequent as 5 seconds, the hope is that early detection of changes in the population and in individual cells will be able to detect both nutritional imbalances and nascent infections. This should enable rapid responses to thwart "pond crashes," the worst nightmare of the industry, in which a seemingly healthy population suddenly decrements to near-zero as a consequence of unanticipated massive cell die-offs.



Algal raceways for commercial-scale cultivation of algae

“A 60% yield in the laboratory often crashes to a 10% yield in algal raceways.”

Are All Algae Created Equal? Inquiries into Cyanobacterial Suitability

Many species of algae — both eukaryotic (plant-like, more-complex cells) and prokaryotic (bacteria-like, simpler cells) — produce lipids (fats), using energy from the sun in photosynthesis. In simplified terms, solar energy “activates” the chemically stable carbon dioxide (CO₂) and water (H₂O) in a complicated set of biochemical reactions (partly known as the Calvin Cycle), the result being the (photo)synthesis of bio-organic molecules—carbohydrates, proteins, lipids, and DNA/RNA. Some of these molecules are necessary for cellular housekeeping functions and cell growth and division to produce new algal cells. But the accumulation, inside the cell of lipids that are readily convertible to biodiesel is a feature that is the subject of intense study, particularly focused on the issue of whether one phylogenetic algal type or one particular algal species might be superior in this respect. This LDRD-funded, Truman Fellowship Project led by Anne Ruffing focused on genetically engineering the prokaryotic alga (also called cyanobacteria), *Synechococcus elongatus*, to increase its production of biofuel precursor lipids. Utilization of the simpler cyanobacteria is potentially advantageous given that their nutrient requirements are less demanding than that of eukaryotic algae, and that — like their non-photosynthetic bacterial cousins — they are easier to genetically manipulate. In addition to optimizing the amount of lipid produced by the cyanobacterial cells, a second important question addressed by the research was the impact of optimizing lipid biosynthesis on the remainder of the algal cell’s metabolism and physiology.

Using genetic engineering techniques perfected in bacteria such as the model prokaryotic species, *E. Coli*, the project team created numerous genetic variants designed to shift the cell’s metabolism toward increased free fatty acid (FFA) production. Because their chemical structures allow them to be converted in one fairly simple reaction step, free fatty acids are a quite convenient biodiesel precursor once they are excreted by the algae into their surrounding environment.

Certain secondary alterations observed in the cells’ metabolism made physiological “sense.” For example, the cells increased their expression of the genes encoding the information for FFA transporters, proteins that facilitate movement of FFAs from inside the cyanobacterial cell to its outside environment. Logically, this would be expected as a positive genetic/metabolic adaptation — if the cells are synthesizing extra FFAs, they must have an increased ability to export that excess.

Unfortunately, not all the news trended positive. For example, certain algae genetically engineered for higher levels of FFA synthesis/export showed reduced overall cell growth coupled with a decrease in photosynthetic capabilities. In turn, decreased photosynthesis was coupled to negative changes in the quantity and location of chlorophyll a in the cells. In addition to making algae and plant cells appear green, chlorophyll harnesses solar energy, converting it to chemical

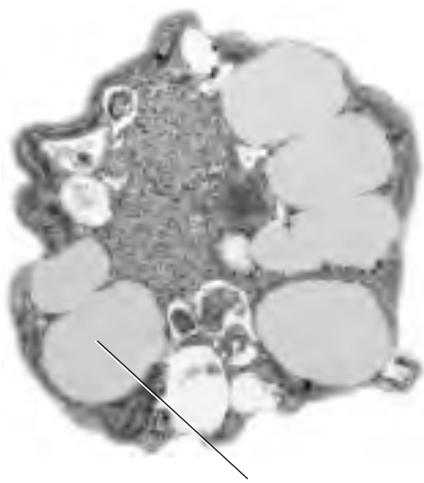
potential energy for synthesis of FFAs and cellular housekeeping molecules such as DNA and proteins. Logically, diminution of chlorophyll would be expected to be correlated with slower cell growth, since less solar energy would be used to drive the synthesis of housekeeping biomolecules.

Project measurements utilized Sandia’s capabilities in hyperspectral imaging, and the work entailed a collaboration with LANL and the Kansas Lipidomics Research Center.

First and foremost, the research demonstrated multiple genetic manipulations that can be used to engineer the simpler cyanobacteria for enhanced FFA

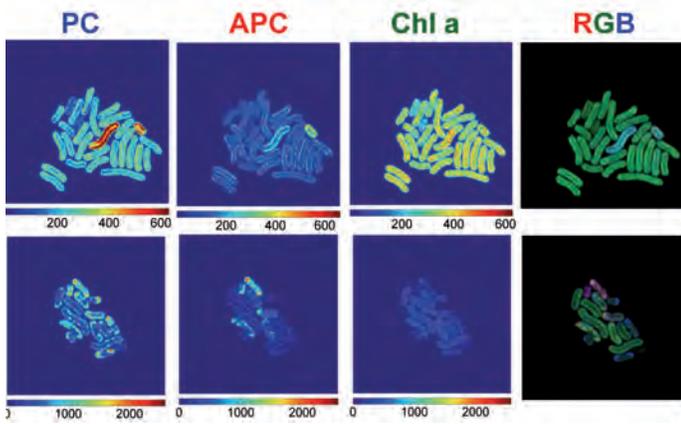
production and excretion (export to the external environment). In addition to identifying these genetic targets for engineering initiatives, concomitant identification of potential side-effects such as slower cell growth provides subsequent researchers with key knowledge about the most desirable physiological changes to yield optimal FFA production for biofuels. In other words, the ultimate outcome of this project and the suitability of cyanobacteria as biofuels producers remains an open question requiring further research.

Regardless of that outcome, the contribution of this work to the biofuels community was recognized by scientific peers: the



**Accumulation of Lipid
inside an Algal Cell**

research earned the cover story in the September 2012 issue of the journal *Biotechnology and Bioengineering*.



Imaging of photosynthetic pigments in different genetic types (strains) of *Synechococcus* algal cells. Chlorophyll a is shown in the third panel from left.

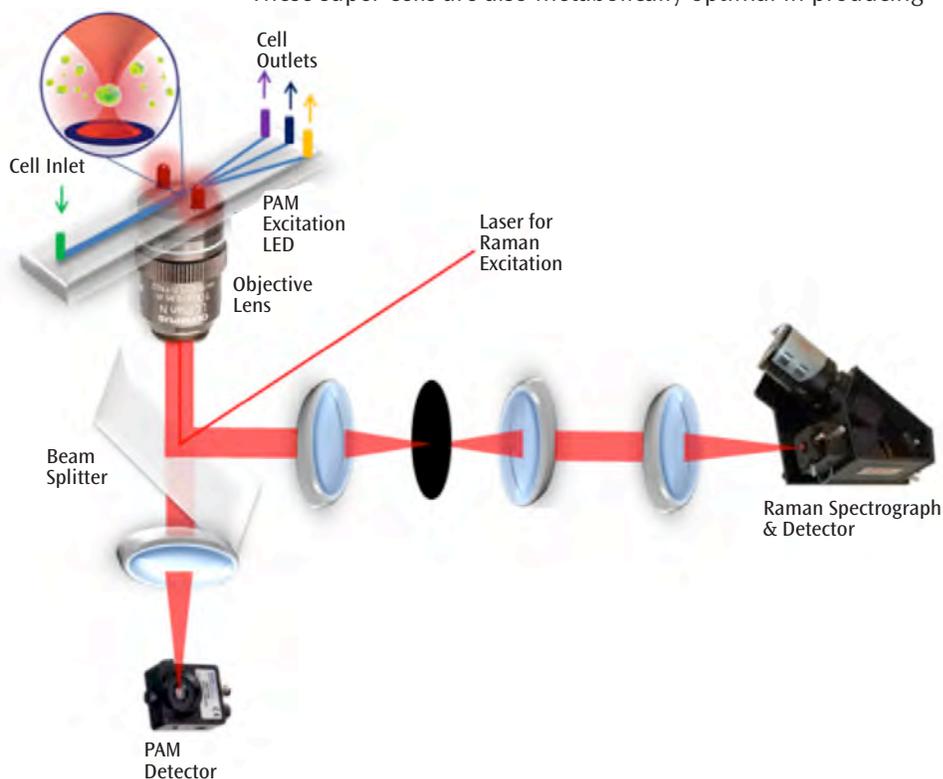
Catch and Release: Bioprospecting for Algal “Super Cells”

Referred to as the “Algae to Oilgae” Initiative, led by PI, Seema Singh, this LDRD-funded project has made some remarkable progress in rendering eukaryotic microalgae more viable as a source of biodiesel for domestic production of transportation fuels. In addition to research on the growth conditions and factors that appear to be optimal for yielding microalgae that contain large quantities (up to 70% dry cell weight) of lipid biodiesel precursors, this project has developed an ingenious technology for rapidly screening algal cell populations to identify such “super-cells” — both on species-, strain-, and individual-cell metabolic levels.

A combination of micro-Raman spectroscopy, laser “tweezers” for manipulation of single cells, a pulse-amplitude-modulated (PAM) fluorometry system, and a microfluidic flow system that rapidly delivers cells into the laser beam, this “bioprospector” carries out analysis of cellular contents in single algal cells in a timeframe of just a few seconds, enabling hundreds of cells to be interrogated every few minutes. Such interrogation produces what can be thought of as a “Raman fingerprint” for each cell, the peaks of the spectrogram at given wavelengths providing information about the types of lipids present in a particular algal cell. Raman spectroscopy, a nondestructive technique delivers photons that interact with the electron clouds of biomolecules within storage compartments (vesicles) of the algal cell — in this case, various lipids that the cell synthesizes via photosynthesis. Overall, the method is dubbed, “laser-trapping Raman spectroscopy (LTRS).

Such factors as the chain length (number of carbon atoms), degree of unsaturation (number of carbon-carbon double bonds) in the backbone of the fatty acids composing the triacylglycerides (TAG) stored within each cell, as well as the

melting point of these lipids, are crucially important to the quality of biodiesel derived from these lipids. These parameters therefore determine which algal species and which individual cells within that species are the optimal photosynthesizers. While the Raman profile informs researchers about the types of lipids (as well as carotenoid pigments, protein and carbohydrates), the LED-based fluorometry system interrogates the cells as to their photosynthetic productivity, detecting their content of chlorophylls. Taken together, this information allows the discovery of “super-cells” — cells containing the biomolecular apparatus to be the optimal photosynthesizers, producing the highest lipid content per photon of sunlight absorbed. These super-cells are also metabolically optimal in producing

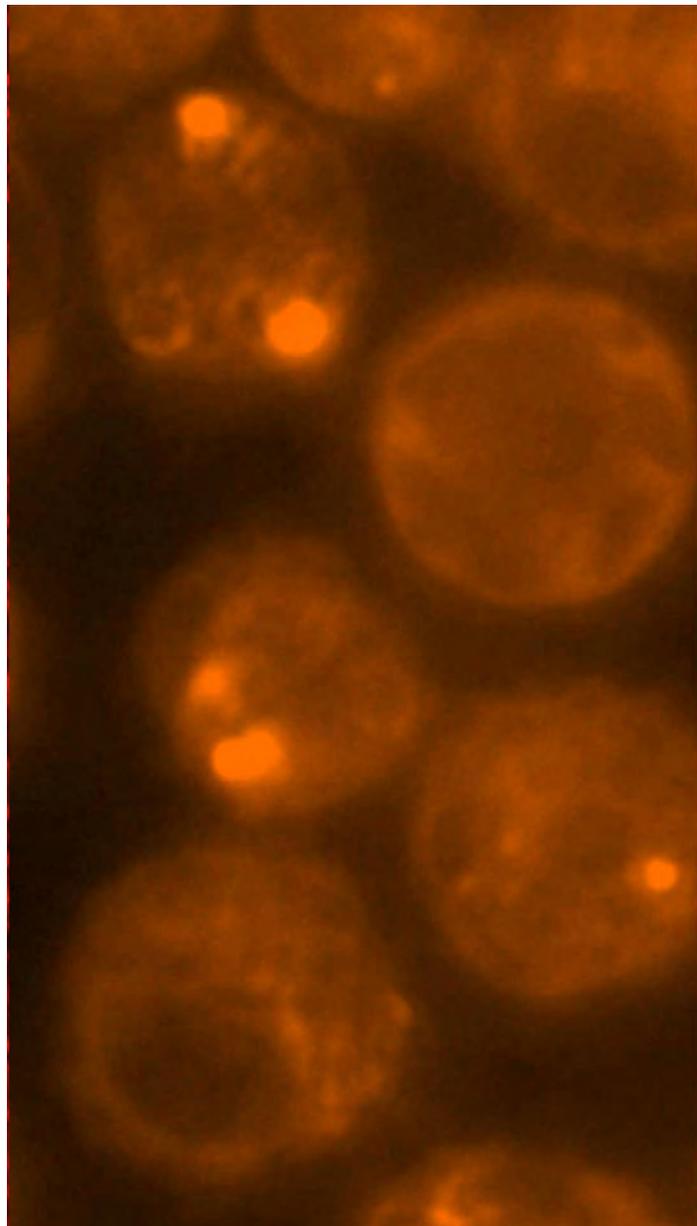


the most desirable lipid types under given defined growth conditions, while allowing researchers to define what growth conditions were optimal to yield such an outcome. In essence, “optimal” here defines cells that would most readily yield the highest output of high-quality biodiesel with the least possible effort in processing these lipids once extracted from the algal cells. It is important to note that the LTRS method itself is nondestructive and nontoxic. Unlike other methods of determining lipid content (lipid extraction and staining, for example), the cell is gently caught by laser tweezers,

interrogated, and then released. This preserves the best producers for later clonal cultivation, that is, growing a clone of such identical “super cells” with which to populate algal raceways for commercial-scale biofuels production. This method is far more efficient in terms of time, labor, and expense than other alternatives, most of which lead to alteration or destruction of the cells.

Beyond this nationally important application of the technology in biofuels R&D, the bioprospector can also be utilized as a research tool in other areas of biology. Many possibilities reveal themselves, in all cases, the goal to discover an optimal metabolically configured cell within a slightly diverse population. For example, in the immune response, there might be a form of so-called antigen presenting cells that, because of their specific type of major histocompatibility proteins are particularly adept at triggering a strong and substantial immune response. Raman screening might be amenable to detecting such “super immune-trigger-cells.”

Because of Sandia’s unique experience with Raman spectroscopy for microalgae, the Laboratories are partnering with BaySpec, a Bay Area company, to commercialize a pond-deployable algal analyzer configured with the operational elements just described. The project received a phase one National Science Foundation Small Business Innovation Research grant to assist in those commercialization activities.



Algal cells stained with Nile red to reveal intracellular lipid droplets.

Putting it All Together: A Complex Systems Model

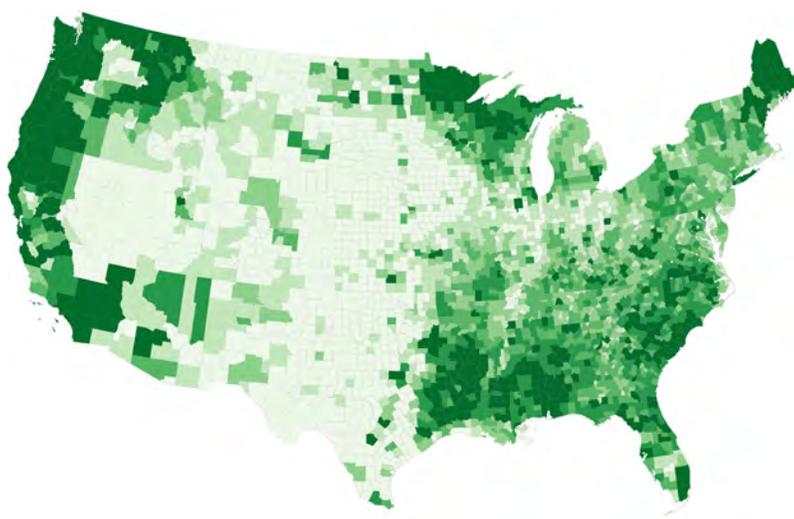
At first glance, it might appear that progress toward the increased use of biofuels will depend primarily upon improved methods of digesting lignocellulosic plant biomass and fermenting the resulting sugars to ethanol and other combustible hydrocarbons (VOCs), and alternatively, improving algal biomass yield and lipid extraction. However, the factors that come into play in this biofuels scenario are actually quite a bit more numerous and complexly interwoven, and Scott Paap has proposed—in an early career LDRD project—to computationally tackle this complexity from a complex systems standpoint. The ultimate objective is the development of models of biomass feedstock production and conversion to transportation fuels, beginning with the regional availability of feedstock biomass and following the supply chain through to biofuels production and beyond to fuel distribution and use by consumers. This holistic model should be simple and flexible, capable of incorporating multiple paths from agricultural and algal feedstock input to fuels, while remaining compatible with other models and analytical software.

Hence, a tractable (or “medium-fidelity”) model that takes biochemical and local and regional feedstock factors into account, and that also incorporates the key distribution/utilization steps in the entire process, should be a genuinely productive addition to the biofuels initiative.

During the course of the project, the team developed a modular, flexible modeling and analysis approach well suited for the evaluation of early-stage technologies and processes to convert biomass to liquid transportation fuels. The approach connects simplified models of individual process units in a unified framework, enabling the representation of multiple pathways from biomass to liquid fuels and accounting for the inherent uncertainty in process inputs and performance through the use of Monte Carlo analysis techniques. The modeling framework is sufficiently flexible to readily incorporate advances in technology, while still representing the important physical processes that govern the system’s behavior.

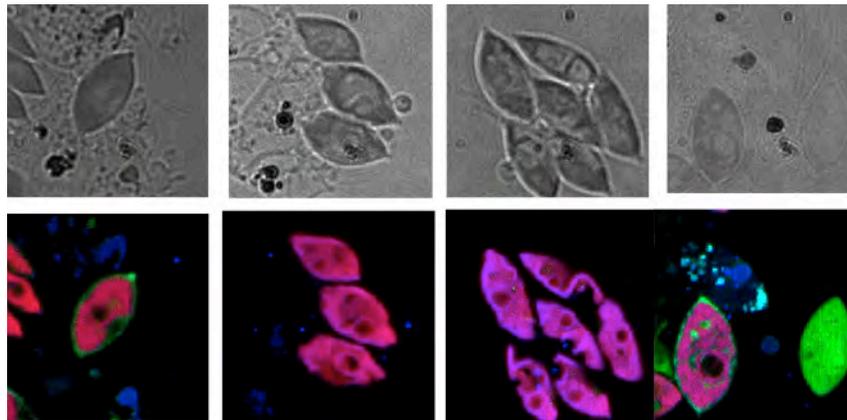
The project fits nicely into an ongoing collaboration between Sandia biofuels scientists — several of whom work at JBEI — and several Chinese academic researchers at the China

Automotive Energy Research Center (CAERC), in Beijing’s century-old Tsinghua University, an eminent biological research institution. ■



Amount of woody biomass by US county: a possible model input

Postscript: LDRD and the Complexity of BioNano World



An editorial in the January 18, 2013 issue of the journal *Science* captures a fundamental issue with biomedical research, framing both the intricacies of cells and the need to temper expectations regarding the rate of progress in this arena. The writer analogizes bioscience researchers to first-graders trying to translate a text with the sophistication and depth of Shakespeare. That sophisticated text is, of course, represented by the intricacies of the interwoven molecular biology and biochemistry of cells. Our first-grade-level vocabulary and experience represents the fact that our knowledge of these cellular processes — subsumed within hierarchical self-regulating nexuses — is both “rudimentary and piecemeal.”*

A multicellular organism, be it human or blade of grass is composed of different cell types, each with a unique expression of a subset of its genetic information and a complex program of *intracellular* regulatory relationships, and this further complicates matters. Not only does each cell intrinsically comprise an *intracellular* hierarchy of inter-regulatory relationships, but now different cell types — combining to form tissues and organs — create new levels of *intercellular* inter-regulatory relationships, such as those embodied in humans by hormonal and cytokine signals among different organs and their cell types. Add the fact that humans contain more symbiotic bacterial cells than actual human cells, and the inter-regulatory complexities take on an additional twist.

Considering these facts, it is not surprising that advances in biological sciences require enormous investments of time, energy, and resources. Moreover, by definition of the program, Sandia LDRD bioscience initiatives are expected not only to simply make advances, but also to advance the leading edge of the biological subdisciplines pertinent to Sandia’s mission. This is a quite ambitious goal — hence, it bears remembering that biothreats or biofuels, such advances are likely to be hard-won research endeavors that engage Sandia scientists and engineers across several disciplines from molecular biology through analytical chemistry, biophysics and bioinformatics, and microsystem/microimaging engineering. Such ambitious endeavors are the lifeblood of the LDRD program. ■

*H.Y. Zoghbi, “The Basics of Translation,” *Science* **229**, 250 (2013)

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