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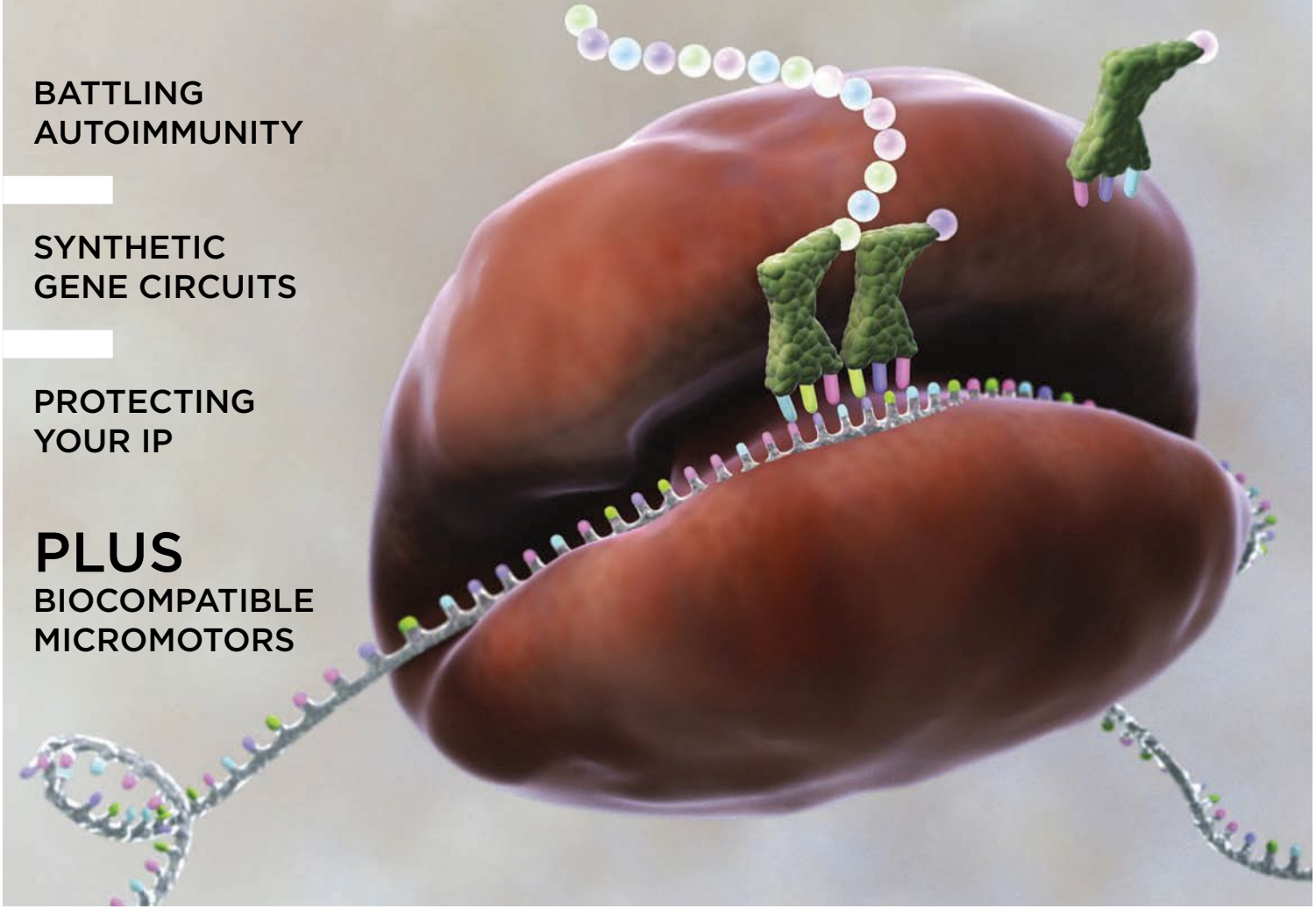

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FOR REFERENCE

Bolognesi, C. et al. Digital Sorting of Pure Cell Populations Enables Unambiguous Genetic Analysis of Heterogeneous Formalin-Fixed Paraffin-Embedded Tumors by Next Generation Sequencing. Scientific Reports 6, 20944 (2016) doi:10.1038/srep20944

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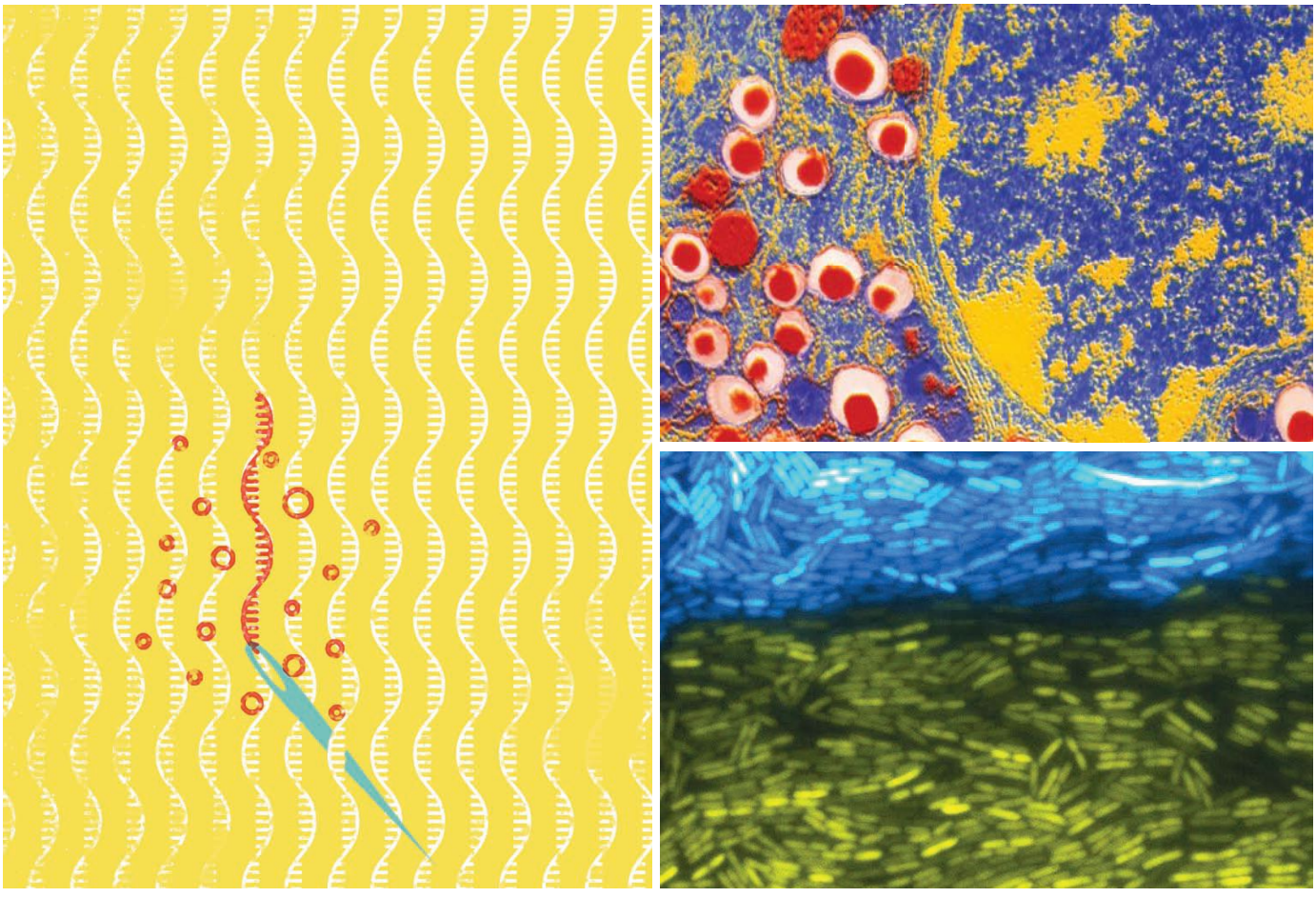

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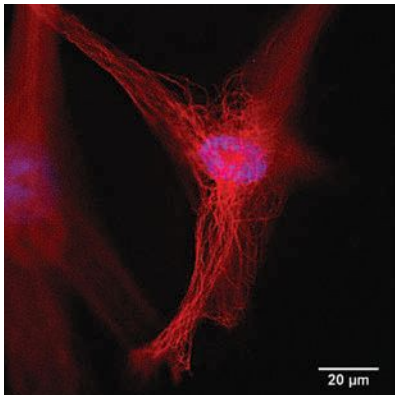

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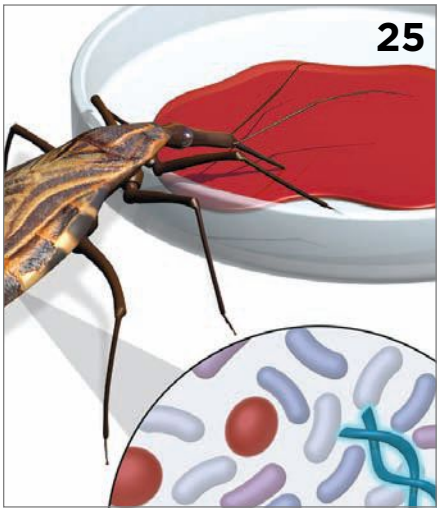

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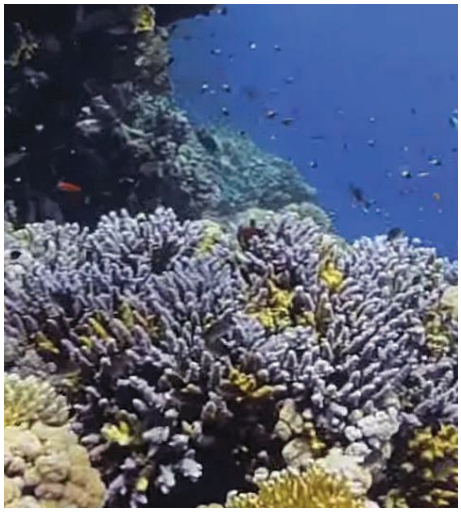

THIS MONTH AT THE-SCIENTIST.COM:

VIDEO
Bertozzi the Editor
Stanford University biochemist Carolyn Bertozzi discusses her role at the helm of *ACS Central Science,* a cross-disciplinary, open-access chemistry journal.

VIDEO
Myriad Genetics Post Mortem
David Schwartz of the Illinois Institute of Technology-Chicago discusses the impact of the US Supreme Court's unanimous decision negating patents on *BRCA* genes and mutation tests.

VIDEO
Coral Currents
Researchers at MIT and the Weizmann Institute of Science discover the utility of coral cilia in creating water currents that bring food and move molecules around the colonial organisms.

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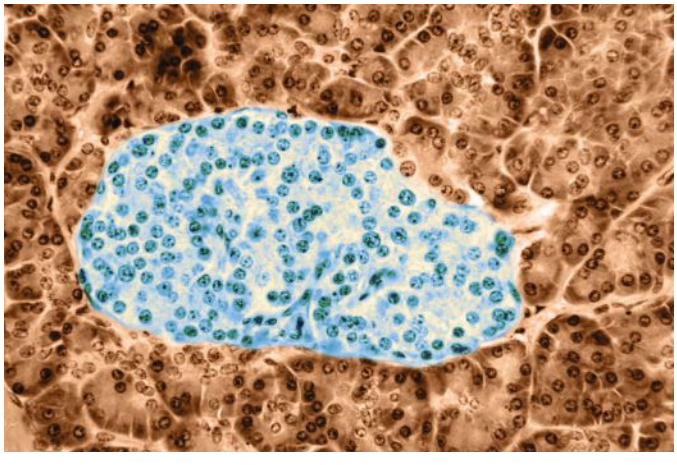
- What is the true driver of type 2 diabetes?
- How global marine changes affect the spread of disease among the ocean's organisms
- Interactions between the immune system and stem cells
- Composite endpoints in clinical trials
- Becoming better data scientists

AND MUCH MORE



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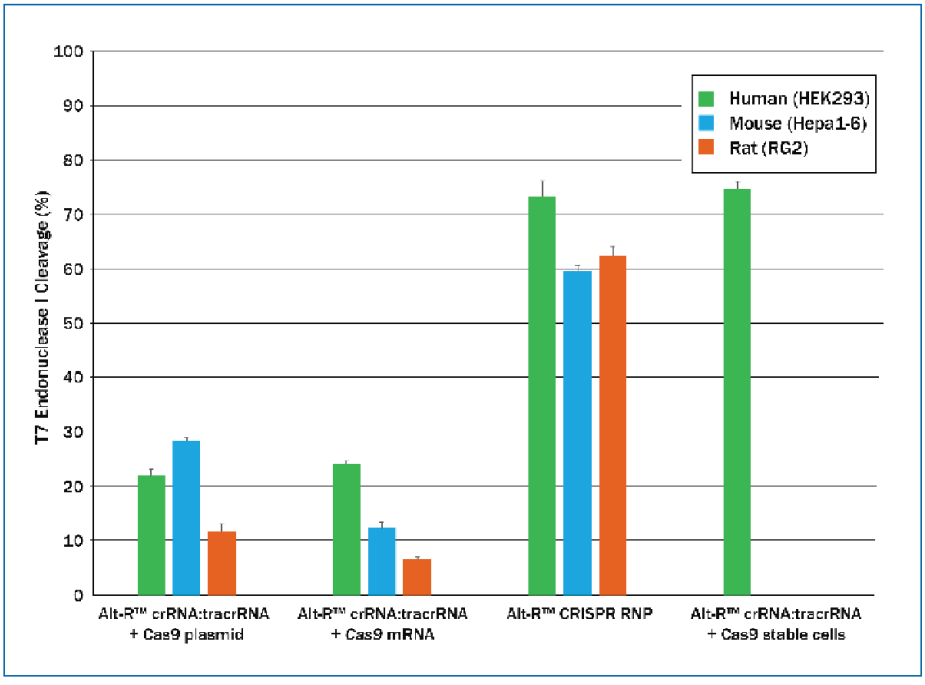

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Figure 1. Lipofection of Alt-R™ CRISPR-Cas9 System components as a ribonucleoprotein outperforms other transient CRISPR-Cas9 approaches. Alt-R CRISPR HPRT Control crRNAs for human, mouse, or rat were complexed with Alt-R CRISPR tracrRNA. The resulting complexes were transfected with Cas9 expression plasmid, *Cas9* mRNA, or as part of a Cas9 ribonucleoprotein (containing Alt-R™ S.p. Cas9 Nuclease 3NLS, pre-complexed with the crRNA and tracrRNA) into human (HEK293), mouse (Hepa1-6), or rat (RG2) cell lines. The Cas9 RNP outperformed the other transient Cas9 expression approaches, and performed similar to reference HEK293-Cas9 cells that stably express *S. pyogenes* Cas9.

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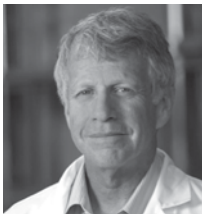

The son of a pharmacist, **Lawrence Steinman** says it was only natural that he ended up pursuing a career in medicine. After earning a BA in physics from Dartmouth College in 1968, Steinman decided to devote his career to medical research and patient care. He enrolled at Harvard Medical School, where he developed a particular interest in the brain. "It's always been the most intriguing of the organ systems," he says, "one of the big mysteries that needs to be understood." With a specialization in immunology, Steinman says it was another natural decision shortly after graduating in 1973 to begin work on multiple sclerosis (MS)—"the most prevalent immunological disease of the brain"—and signed on for a postdoc at the Weizmann Institute of Science in Israel. A member of Stanford University's neurology faculty since 1980, Steinman holds numerous patents on MS treatment methods, works on the commercial development of therapeutics, and has expanded his research to consider other autoimmune diseases including diabetes. "Although my main interest has been in brain diseases, I want to solve this problem," he says.

Steinman examines potential therapies to train the body's defenses to tackle autoimmune disease in "Taming Autoimmunity" on page 32.

Richard Muscat's interest in DNA biotechnology took hold shortly after he had completed a master's in physics at the U.K.'s University of Nottingham in 2006. "I was really fascinated by the way you can use biological components to construct things at the nanoscale," he says. Pursing this interest through a PhD at the University of Oxford, Muscat built nanomachines from DNA molecules, while also starting to focus on "the next level" of biological engineering—synthetic biology. "Not only could you program bits of DNA to stick together," he explains, "but you could program entire cells." Graduating in 2011, Muscat moved to Seattle for a postdoc with Georg Seelig at the University of Washington to work on translating DNA nanotechnologies to the cell. Then, in 2015, following a brief stint working in the research division of Oxford Nanopore Technologies, he took up a position at Cancer Research UK. "I wanted to try something a bit different, but stay in science," he says. Now working on the charity's multidisciplinary funding scheme—set up to support collaborations between cancer researchers and other scientists, mathematicians, and engineers—Muscat says that he has enjoyed learning a new set of skills. "The fun part," he says, "is that you're constantly hearing about exciting new research."

In "The Imitation Game" (page 38), Muscat discusses the biological insights afforded by synthetic gene circuits.

J.D. Trout credits a schoolteacher with instilling a love of philosophy at an early age. "He was teaching a lot of the classics," Trout recalls. "Plato, Anselm's ontological argument, existentialism—a lot of the big topics that sweep up teenagers." After obtaining a BA in philosophy and history from Bucknell University in 1982, Trout started a PhD in the philosophy department at Cornell University. However, he soon began investigating psychology to complement his understanding of the philosophy of mind. "I couldn't see how you could adjudicate any of the philosophical disputes without scientific knowledge of how the mind actually works," he explains. So alongside his PhD work, Trout carried out coursework and research on speech perception in the psychology department. After graduating from Cornell in 1988, Trout spent time in several departments across the U.S. before taking up a position as a member of the philosophy faculty at Loyola University, Chicago, in 1992, where he has remained since. He has authored several books, including *The Empathy Gap* (2009), exploring psychological biases that influence human decisions and policy making, and continues to draw on his expertise in both philosophy and psychology. "The amount of stimulation I got in both fields just from thinking about the independent issues has been really worth it," he says.

Trout explores the forces driving scientific progress—and the influence of "unusual accidents"—in "False Climbs" (page 64), an essay based on his latest book *Wondrous Truths: The Improbable Triumph of Modern Science*.

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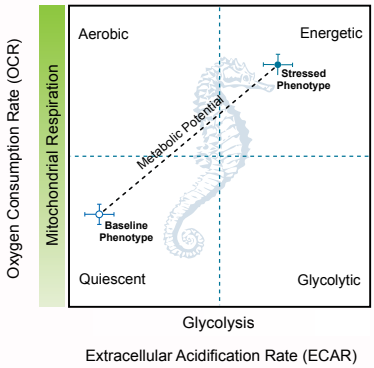

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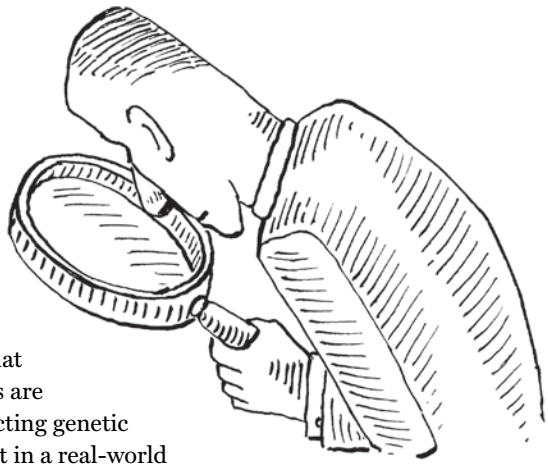
Small But Mighty

From whole cells to genes,
closer examination continues to surprise.

BY MARY BETH ABERLIN



ANDRZEJ KRAUZE

Little things mean a lot. To any biologist, this time-worn maxim is old news. But it's worth revisiting. As several articles in this issue of *The Scientist* illustrate, how researchers define and examine the "little things" does mean a lot.

Consider this month's cover story, "Cryptic Codes" (page 26), by TS correspondent Ruth Williams. Combing the human genome for open reading frames (ORFs), sequences bracketed by start and stop codons, yielded a protein-coding count somewhere in the neighborhood of 24,000. That left a lot of the genome relegated to the category of junk—or, later, to the tens of thousands of mostly mysterious long noncoding RNAs (lncRNAs). But because they had only been looking for ORFs that were 300 nucleotides or longer (i.e., coding for proteins at least 100 amino acids long), genome probers missed so-called short ORFs (sORFs), which encode small peptides. "Their diminutive size may have caused these peptides to be overlooked, their sORFs to be buried in statistical noise, and their RNAs to be miscategorized, but it does not prevent them from serving important, often essential functions, as the micropeptides characterized to date demonstrate," writes Williams.

How little things work definitely informs another field of life science research: synthetic biology. As the functions of genes and gene networks are sussed out, bioengineers are using the information to design small, synthetic gene circuits that enable them to better understand natural networks. In "The Imitation Game" (page 38), Richard Muscat summarizes the strides made by synthetic biologists over the last 15 years and offers an optimistic view of how such networks may be put to use in the future. And to prove him right, just as we go to press, a collaborative group led by one of syn bio's founding fathers, MIT's James Collins, has devised a paper-based test for Zika virus exposure that relies on a freeze-dried synthetic gene circuit that changes color upon detection of RNAs in the viral genome. The results are ready in a matter of hours, not the days or weeks current testing takes, and the test can distinguish Zika from dengue virus. "What's really exciting here is you can leverage all this expertise that synthetic biologists are gaining in constructing genetic networks and use it in a real-world application that is important and can potentially transform how we do diagnostics," commented one researcher about the test.

Moving around little things is the name of the game when it comes to delivering a package of drugs to a specific target or to operating on minuscule individual cells. Mini-scale delivery of biocompatible drug payloads often needs some kind of boost to overcome fluid forces or size restrictions that interfere with fine-scale manipulation. To that end, ingenious solutions that motorize delivery by harnessing osmotic changes, magnets, ultrasound, and even bacterial flagella are reviewed in "Driving Tests" (page 50).

While technological advances continue to home in on the little (and little-known), doing so can also raise thorny ethical issues. In the same week that saw publication of the syn bio–inspired Zika test, two research groups reported the surprise result that human embryos can be cultured in vitro beyond the stage (seven days) at which this tiny mass of cells burrows into the wall of the uterus. Both research groups terminated the experiments before the embryos reached 14 days of development, the legal limit for study of human embryos in the lab. (For an MRI view of a 13-day embedded embryo, see "Speaking of Science," page 14.) Because the early stages of human embryonic development are essentially a black box, revisiting the ethical implications of this rule is under intense discussion.

Little things really do mean a lot. ■

Editor-in-Chief
eic@the-scientist.com

Speaking of Science

The 14-day rule was never intended to be a bright line denoting the onset of moral status in human embryos. Rather, it is a public-policy tool designed to carve out a space for scientific inquiry and simultaneously show respect for the diverse views on human-embryo research.

—**Insoo Hyun** et al., in a *Nature* commentary questioning the internationally agreed 14-day limit on human embryo research after two groups reported growing human embryos in vitro for 12–13 days (May 4)

What if scientists could culture the human embryo for longer than 14 days? Perhaps we could begin to understand the consequences of fetal alcohol syndrome, study the potential causes of autism and find out why some environmental chemicals can affect development. Perhaps we might, for example, be able to more quickly understand what the Zika virus does to embryos to cause major problems with brain development. There could be major benefits for society, but if the 14-day line is crossed then society has to fully understand the science and come to an informed decision about the use of the technology.

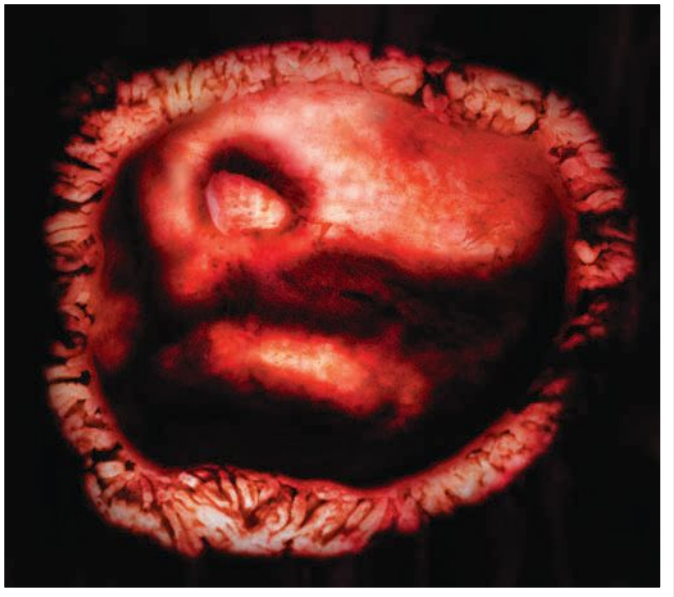
—**Peter Donovan**, biologist at the University of California, Irvine, in response to the recent *Nature* commentary questioning the internationally agreed 14-day limit on human embryo research (May 4)

I am rock solid about my research. I know it is very good. But this wastebook targeted a short paper that was the first paper in my young graduate student's career.

—Duke University biologist **Sheila Patek,** at an April event in Washington, DC, discussing the ramifications of her mentee's mantis shrimp research being included in Senator Jeff Flake's (R-AZ) "wastebook," which is designed to highlight what he deems to be unnecessary federally funded science (April 13)

This has been enlightening, and we want to make sure we are accurate. It is a learning process.

—Senator **Jeff Flake** (R-AZ), sharing his thoughts on an April meeting where several of the researchers targeted by his "wastebook" gathered to discuss their maligned projects and educate Congressional staffers on the utility of their federally funded science (April 13)



TESTING THE LIMITS: An artificially colored MRI of a 13-day-old human blastocyst implanted in the uterine wall

We can't save every species, of course. The planet is losing its biodiversity at an alarming rate, and there are too many species circling the drain. Conservation professionals acknowledge that we will need to perform a sort of conservation triage, a painful process of deciding which species to try to rescue and which to let go.

—Journalist **Hillary Rosner,** in a *New York Times* opinion piece about the efforts of biotechnologists to conserve Earth's biodiversity (April 16)

Many of my most talented graduate students that I've trained with PhDs in molecular biology and biochemistry have gone on to be lawyers, venture capitalists, you name it. I think that's great! Because they never forget their scientific training, and to me that's worth every effort. So that's why I say, no, we need more PhDs, not fewer.

—**Robert Tjian,** president of the Howard Hughes Medical Institute, on the need for increased scientific training in the U.S. (April 28)

Notebook

JUNE 2016

COURTESY OF MASASHI TANAKA

TWEET, TWEET: The zebra finch (*Taeniopygia guttata*) is fast becoming an important model for the study of neurodegenerative diseases.

Bird Brains to the Rescue

Richard Mooney's grandfather, a mechanical engineer, couldn't imagine why birds would be useful for understanding the human brain. "The same way that taking apart a one-cylinder lawn mower can prepare you for how a supercharged V8 in a Formula One racer works," explained Mooney, a neurobiologist at Duke University Medical Center. In fact, striking similarities between songbird and human brains are now driving bird neurobiology research in a new direction: the study of human neurodegenerative diseases.

Songbirds and people share the rare ability to learn vocal patterns through imitation, a skill driven by similar brain areas that have functionally converged over evolutionary time. This is true even at the genetic level, as Mooney's Duke neurobiology colleague Erich Jarvis demonstrated in 2014. Songbird gene expression in these brain regions more closely resembled that of humans' than that of chickens', and human gene expression was closer to that of songbirds than to that of a fellow primate, the macaque (*Science*, 346:1256846).

One of these regions, known as Area X in birds, is a basal ganglia subarea dedicated to song learning. In the most common laboratory songbird, the zebra finch (*Taeniopygia guttata*), Jarvis and others have shown that dopamine sent to Area X from the midbrain plays a key role in song variability. When not in the presence of females, male finches sing "undirected songs," perhaps to practice, that are more variable than the songs they use to attract mates. The less variable, female-directed songs are accompanied by an influx of the neurotransmitter dopamine into male basal ganglia neurons (*Eur J Neurosci*, 25:3406-16, 2007). That finding is intriguing for researchers interested in speech defects associated with the neurodegenerative disorder Parkinson's disease, which is marked by both a loss of dopaminergic neurons and basal ganglia damage.

Scientists have only recently begun to pin down the effect of a Parkinson's-like loss of dopamine in songbirds. Sam

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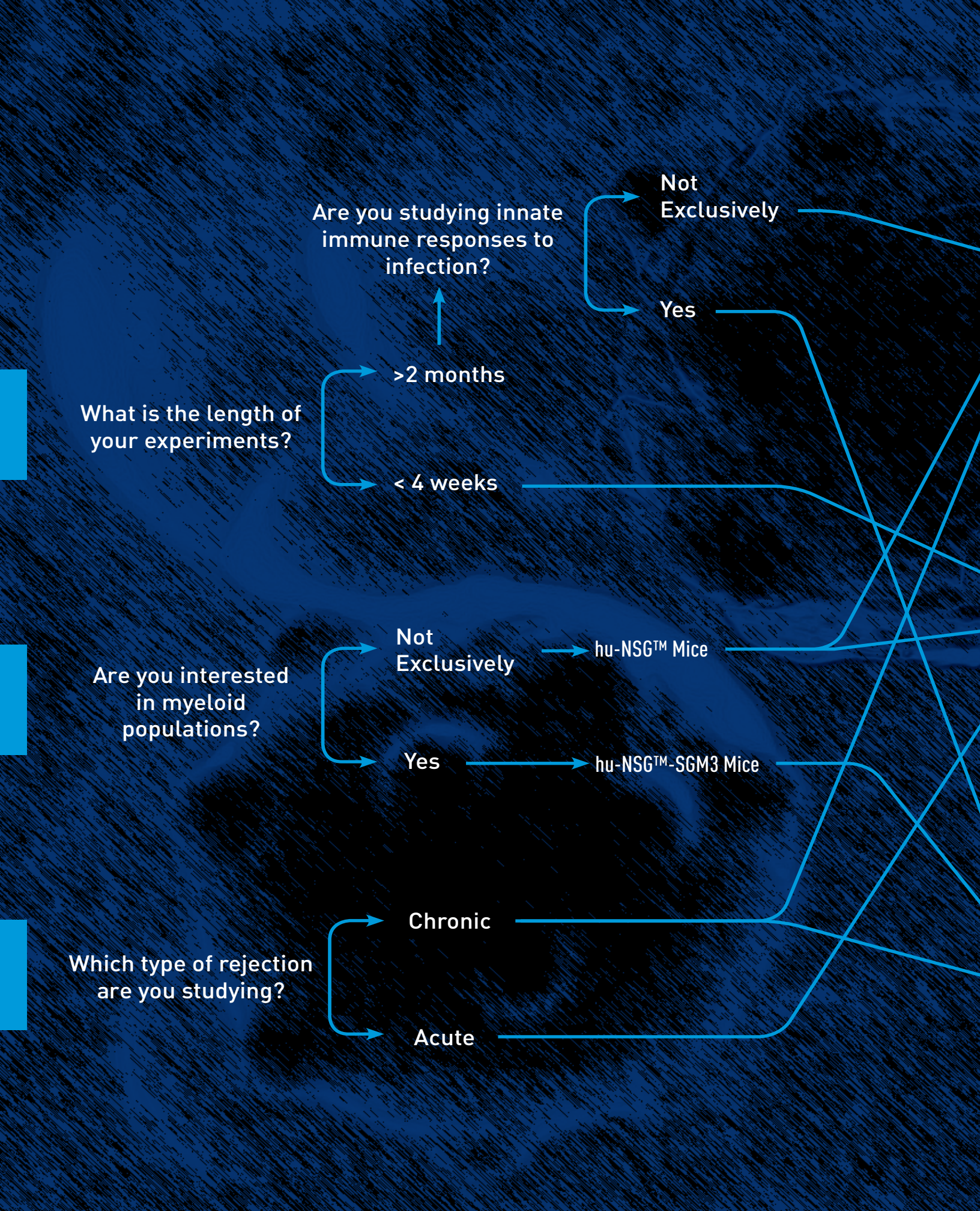
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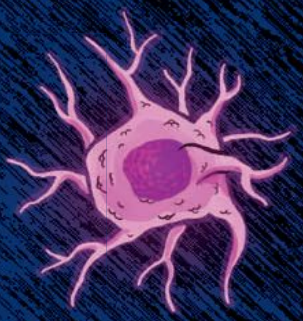
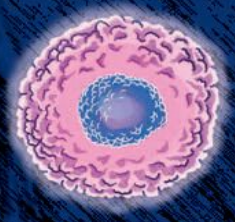


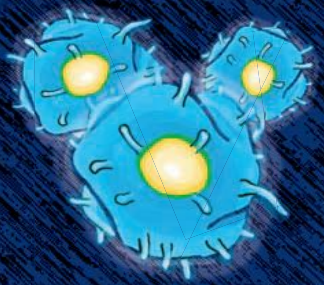
Dendritic cells

T cells (HLA-restricted CD4 and CD8)



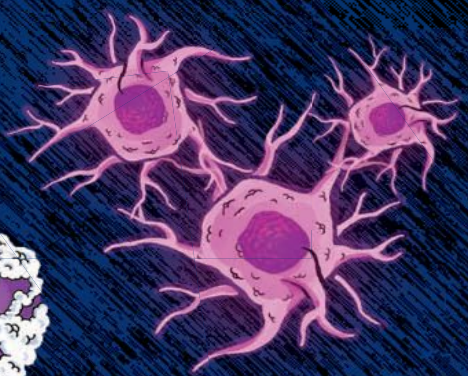
Natural Killer cells

T cells (MHC-restricted CD4 and CD8)



Monocytes

Macrophages



Dendritic cells

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TYPE	MODEL ID	TUMOR MARKERS
BLADDER	TM00015	PIK3CA H1047R
	TM00020	TP53 E336*; KDR Q472H; PTEN T321fs
BREAST	TM00089	TNBC ER-/PR-/HER2-; BRCA1 V757fs
	TM00090	TNBC ER-/PR-/HER2-
	TM00096	TNBC ER-/PR-/HER2-
	TM00098	TNBC ER-/PR-/HER2-
	TM00284	ER+/PR+/HER2-
	TM00386	ER+/PR+/HER2-
COLON	TM00179	BRAF V600E/PIK3CA G1049R
LUNG	TM00302	KRAS G12D/KDR Q472H/TP53 R158L
	TM00784	EGFR L858R
OVARY	TM00335	CA125 & MUC16 mRNA elevated
	TM00916	ER+/PR+
PROSTATE	J000079754	Castration-resistant
	TM00298	TP53 R273C/PTEN R233*/PTEN L265fs, AR+
SKIN	TM00702	BRAF V600V
	TM01149	BRAF V600V
BREAST	TM00095**	ER-,PR-, HER2+
LUNG	TM00199**	EGFR L858R
	TM00206**	ELM4-ALK fusion

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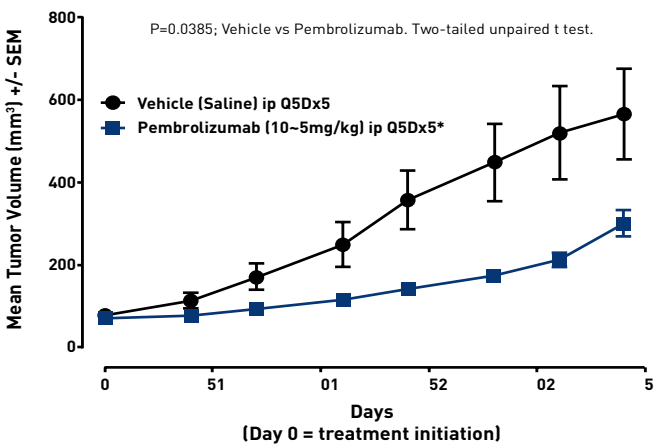
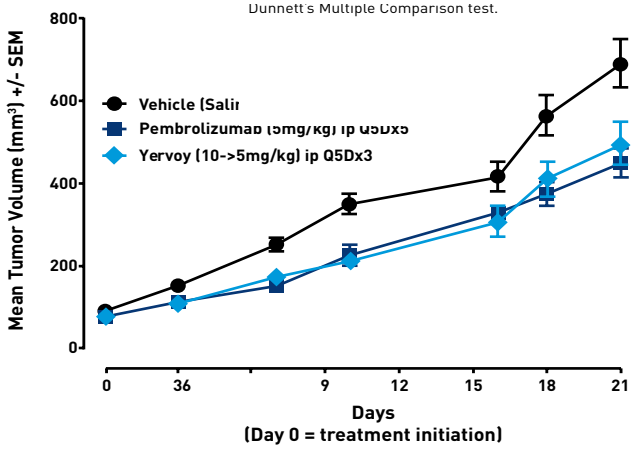
Mean Tumor Volume of TM00098 Breast Tumor (ER-/PR-/HER2-) in Onco-Hu™ Mouse



Mean Tumor Volume of TM00302 Lung Tumor (KRAS G12D) in Onco-Hu™ Mouse



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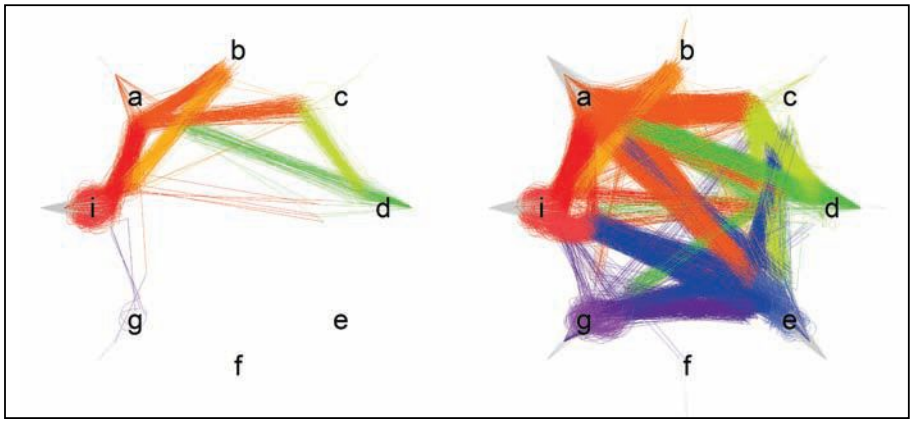
Sober and his colleagues at Emory University in Atlanta found that treating the basal ganglia of adult Bengalese finches (*Lonchura striata*) with the neurotoxin 6-hydroxydopamine, which destroys dopaminergic neurons, causes defects in vocal learning (*J Neurosci*, 36:2176-89, 2016). University of Washington neurobiologist David Perkel hails the study as the "first direct evidence for dopamine in learning." Previously, neurobiologists Stephanie White of the University of California, Los Angeles, and Julie Miller, now at the University of Arizona, found that 6-hydroxydopamine reduced vocal variability during undirected song, when the finches should have produced more-variable songs (*Physiol Rep*, 3:e12599, 2015). People with Parkinson's also speak in a more monotonous tone, but how dopamine affects this symptom is unknown.

Current finch models of Parkinson's rely on neurotoxin-induced lesions in specific brain areas, not genetic mutations associated with the disease in people. However, Parkinson's affects the entire brain—not just the basal ganglia—as well as peripheral nerves, muscles, and gut, says neuroscientist Michelle Ciucci of the University of Wisconsin School of Medicine and Public Health. Her work on speech and swallowing defects in Parkinson's patients and in rodent models of the disease has shown that dopamine replacement doesn't noticeably improve speech quality in Parkinson's patients, suggesting that a dearth of the neurotransmitter is not the sole cause of these deficits (*Semin Speech Lang*, 34:185-202, 2013). Because genetic models can better recapitulate the progression and wider effects of Parkinson's, Ciucci advocates using a combination of these models—including both rodents and finches—to understand the biology of the disease.

Finch researchers have already begun to probe genetic models in another neurodegenerative disorder: Huntington's disease. Last year, scientists characterized a transgenic songbird with the mutant *huntingtin* gene that exhibited both tremors and repetitive, "stuttering" song patterns similar to deficits seen in the human disease (*Nature Neurosci*, 18:1617-22, 2015).



SONG LINES: The stable trajectories of learned vocal sequences of a zebra finch (left) were destabilized after viral expression of a causal gene of Huntington's disease in a vocalization-related region of the basal ganglia (right).

Singing, like speech, is a complex voluntary motor skill of exactly the kind that becomes overactive and uncontrolled in Huntington's, and the well-established connections between brain circuits and singing behavior make the finch an ideal system to examine how the mutant gene disrupts these patterns. Mooney's group found that the expression of mutant *huntingtin* in the basal ganglia alone was sufficient to make birds sing more abundantly, but with the stereotypical syllables of the song mixed up (*PNAS*, 113:E1720-27, 2016). "It's really nice work and is very suggestive that this could be a great model for understanding the pathogenesis of Huntington's disease," says Perkel.

Intriguingly, Mooney found that some of the finches were eventually able to recover some of the correct order to the syllables in their songs, which may be due to their lifelong ability to generate new neurons in this part of the brain. This result agrees with an earlier finding from the Jarvis lab that finches could partially recover from a neurotoxin-triggered lesion in Area X by forging new neurons, but were left with a stutter in their song (*Sci Rep*, 4:6590, 2014). The mechanism and purpose of adult neurogenesis in these areas—which doesn't appear to occur in people—is still poorly understood, but uncovering its basis might eventually lead to therapies for humans with neurological damage, Jarvis suggests.

To turn that dream into reality, however, a combination of animal models will likely be necessary. "More money needs to be invested in comparing birds and mammals, or comparing birds and humans," Jarvis says.

Stephanie White agrees. "For a uniquely human disorder, you're never going to be able to find a perfect animal model," she says. "My hope would be that groups of researchers who have expertise across different models could collaborate."

—Jenny Rood

Sample Thyself

Nicole Haggerty carefully floated wax paper on the water in her dorm room toilet, then sat down to do her business. Once she was finished, the University of Michigan freshman used a small plastic scoop to sample a teaspoon of her feces and placed the scoop in a tube filled with a solution that removed any excess oxygen from the excrement. She then placed the tube in a little baggie and put it aside. The next day she would analyze the bacteria within.

"I remember telling my mom, 'I have to poop for class,'" Haggerty said. "It was the funniest thing. 'Anything to get an A,'" she'd joked.

Haggerty had enrolled in the research section of Biology 173 upon the recommendation of other undergraduates who had taken the new experimental biology

COURTESY OF MASASHI TANAKA

course. Microbial physiologist and ecologist Tom Schmidt had recently joined the University of Michigan faculty to study the human microbiome and found himself in need of research subjects. So when he saw a call for research proposals from the Howard Hughes Medical Institute (HHMI) for programs designed to integrate research with undergraduate education, he saw the perfect opportunity. "That's what HHMI brought to the table: it gave us a human cohort—healthy undergraduates."

With a five-year, $1.5 million grant from HHMI, Schmidt launched the program in the winter semester of 2015, accepting 40 students into the research arm of Biology 173. This year, he has accepted 80 each semester. (The HHMI grant also supports a parallel program in the chemistry department, with projects focused on solar cell technology and snow chemistry.) So far, the microbiome project has focused on the effects of dietary supplements. During the first semester,

It's been insanely cool to look at the all the different species that make up our individual microbiomes.

—Nicole Haggerty, University of Michigan

students randomly selected to the treatment group mixed 40 grams of Bob's Red Mill potato starch in 8 ounces of water and drank it down every day for two weeks. In the fall of 2015, the class tried different kinds of fiber, and in the winter of 2016, students, including Haggerty, ingested psyllium, another form of dietary fiber. "Each semester I view as a new experiment," Schmidt says.

The students, including those assigned to the control group who did not ingest any supplement, collected samples—breath, skin, and fecal—before and during the treatment period, and brought them to the lab for analysis. For quality-control reasons, Schmidt says, his lab does the official processing of the samples, which includes analyzing the metabolites produced by gut bacteria and sequencing 16S ribosomal RNA genes to identify microbiome composition, but the students also bring in samples that they analyze themselves, learning the techniques in the classroom lab, and Schmidt shares the project data with them once the real samples have been processed. In the first semester, the group found that ingesting resistant starch—a type of compound found in fruits and vegetables that cannot be broken down by human enzymes and thus requires the help of the microbiome for digestion—increased microbial production of butyrate, which has been suggested to have a variety of positive effects on human health. Schmidt and his colleagues currently have a paper reporting these results under review and are still mining the data from the second

semester. "The educational goals and the research goals overlap," says Schmidt, "so it's worked out well for everybody."

A few years ago, Noah Fierer at the University of Colorado at Boulder organized a similar project to involve students in microbiome research. Teaming up with Greg Caporaso at Northern Arizona University and Rob Dunn at North Carolina State, Fierer recruited undergraduates at all three universities to sample their poop once a week for an entire semester to look for temporal variation in individual microbiomes. At UC Boulder, the project was tied to a general microbiology lab course, in which students (both those who volunteered their samples and those who did not) got to do sample analysis and play around with the data Fierer and his colleagues were generating. The team published its results in 2014 in *Genome Biology* (15:531). "We were not just doing this for fun; we were trying to do some science," Fierer says. "I think there are a lot of opportunities for coursework where the students actually generate usable data."

One challenge in organizing student projects such as those at the University of Michigan and the multi-institution project led by Fierer and his colleagues is obtaining approval from the institutional review boards (IRBs) for doing research on human subjects. But there are plenty of other ways to involve students in research that avoid this logistical hurdle. Caporaso, for example, would like to continue to bring research into his bioinformatics classes at Northern Arizona University, especially now that public online repositories provide easy access to large data sets. "We might as well have [students] working on assignments with real data rather than having them do contrived homework," he says.

Fierer is also moving away from human-subjects research while sticking with the student research model. Next fall, he is teaching a course on microbial ecology in which he plans to have students collect samples from the environment (soil, leaves, insects, etc.) and analyze the associated bacteria. "They're actually going to do all the lab work, all the way from DNA extraction to sequencing," he says. "And that's something I think we couldn't have done a couple of years ago. The sequencing is getting fast enough and cheap enough that you can start bringing it into the undergraduate classroom."

Such student research projects carry many benefits, Fierer notes. In addition to teaching students about the scientific process, taking a project from beginning to end introduces them to a variety of research fields. For his 2016 fall class, for example, Fierer plans to hit on microbiology, molecular biology, genetics, ecology, and statistics. "It's a great way to learn about a lot of different things that's not a boring lecture," he says. Involving students in the research is also helpful for keeping them engaged all semester long, Fierer adds. "For the students it's always more fun to be involved in real scientific research instead of just hearing about research that was done."

Haggerty couldn't agree more. "I've loved it," she says. "I've felt like I was an actual part of this research. . . . It's been insanely cool to look at the all the different species that [make up] our individual microbiomes."

—Jef Akst

ANDRZEJ KRAUZE

Reef on a Chip

Crucial habitats in underwater ecosystems, and harbingers of the damage inflicted by global warming on the world's oceans, coral reefs epitomize the beauty—and the fragility—of marine life. But they're also notoriously difficult to study.

"There are all these fascinating, basic biology questions that we don't know the answer to," says Virginia Weis, a marine physiologist at Oregon State University. How do corals respond mechanistically to bacterial infections, for example? What exactly happens during coral bleaching? "They're hard questions to

study, because we haven't had the cell biology and imaging capabilities."

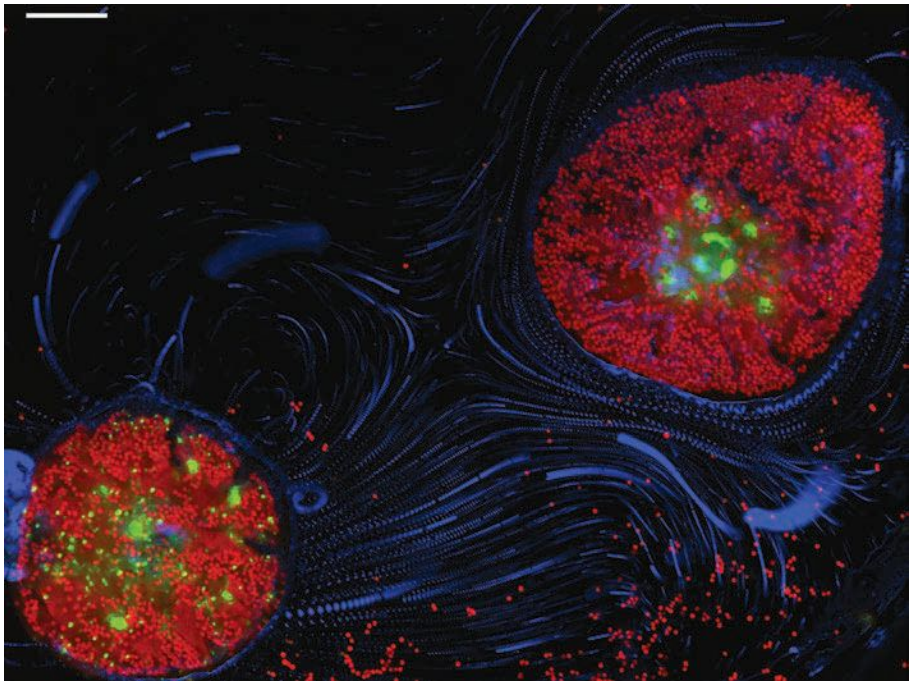
Getting to the bottom of these questions is becoming increasingly urgent. Reports of the worst coral bleaching ever seen have peppered scientific journals this year; Justin Marshall of the University of Queensland told *The New York Times* in April that the destruction of the world's reefs represented a "huge, looming planetary crisis."

But attempts to study corals in vivo have traditionally been hindered by the animals' resistance to life in the lab, and cell or tissue cultures taken from whole coral colonies rarely survive more than a few weeks. Meanwhile, owing to their bulk, reef-building corals are impractical to image at a fine scale in situ or in aquaria, meaning colony-level studies lack insight into the mechanisms underlying the animals' biology. "It's a black box, in terms of what actual microscale processes are happening there," says Assaf Vardi, a marine microbiologist at Israel's Weizmann Institute of Science.

Looking at this animal, normally you just see a rock. A very beautiful and very colorful rock, but basically a rock. Seeing it at this scale, it suddenly becomes alive. It opens up a whole new way of looking into these fascinating organisms.

—Orr Shapiro, Weizmann Institute of Science

A few years ago, Vardi and his colleagues decided to take a different approach. A reef-building coral colony is made up of hundreds of tiny cnidarian polyps, each harboring an algal symbiont, and capable of laying down calcium carbonate to build a skeleton. What if, the Weizmann researchers wondered, you could capture individual polyps; recreate a miniature, experimental environment for them to grow in; and then observe the creatures, in vivo, with a microscope? Vardi and an international team of researchers set to work to develop a micro aquarium, fit to house the world's tiniest reef.



CORAL HUG: Two polyps of the reef-building coral *Pocillopora damicornis* imaged with fluorescence microscopy, showing the coral's GFP, the chlorophyll of its symbiotic algae (red) and cilia-driven motion of microscopic particles (blue)

Starting the project was relatively straightforward. In collaboration with microfluidics expert Roman Stocker of ETH in Switzerland, Vardi's team designed a tiny chamber, consisting of a silicone elastomer channel on top of a microscope slide, with a 1-mm hole at either end of the channel to allow a constant flow of water. Then, fellow Weizmann researcher Orr Shapiro exploited a natural response to high salinity called polyp bailout—the mass ejection of polyps under stress—in order to obtain individual animals from the reef-builder *Pocillopora damicornis*.

The original plan was to pop a polyp into the chamber, let it settle, and slip it over a microscope lens. But that proved harder than expected. "They kept moving," Shapiro says. "It was kind of counterintuitive. Corals are basically stationary, but these kept turning around, doing like 60 rpm. You couldn't get any good pictures." The polyps were beating tiny hairs called cilia, causing the animals to spin at great speed in the chamber—a finding that led to a paper on how corals influence their microenvironment via ciliary currents (*PNAS*, 111:13391-96, 2014).

The spinning, the researchers realized, came down to housing preferences—the polyps wouldn't settle for just anything. "We tried a whole array of different substrates," Shapiro says. "It turned out that they wanted specific, cheap, Chinese microscope slides. The expensive German ones didn't work." The team also had to optimize various environmental parameters, including the salinity, acidity, and flow rate of the water in the chamber—a process that took a considerable amount of trial and error, Shapiro says.

But the payoff was worth it. Once a polyp had settled, it could be imaged through the microscope for hours on end, using different wavelengths to view different parts of the system (*Nat Commun*, 7:10860, 2016). "It looks fabulous," says Vardi. "With the red channel, you quantify the [algal] symbiont. With the green channel, you quantify the coral's health—because of natural GFP . . . you can characterize the state that the coral is in."

ORR SHAPIRO AND ASSAF VARDI, WEIZMANN INSTITUTE OF SCIENCE

To demonstrate the setup's potential, the team made observations of bleaching (what happens when corals expel their algal symbionts under stress), calcification (the deposition of calcium carbonate), and even interactions between two polyps. "You can see how much these polyps connect," Shapiro says. "They start prodding each other and sending out filaments, looking around. It's just amazing to be able to see these kinds of details."

Of course, the system is not without drawbacks. For starters, the technique can only be used to study the early stages of coral growth: too much skeleton, and the structure becomes too opaque to image. And the conditions used to trigger polyp bailout are necessarily stressful, notes coral biologist Chris Langdon of the University of Miami. "You worry a little bit that [the polyps are] hungover from that kind of stress and might not be behaving normally," he says. "There's a lot of validation that needs to be done, I would say, to see how long you need for them to recover."

Nonetheless, Weis and Langdon agree, it's an exciting new tool. "You have to have a playground where you can fiddle around with things," says Weis. "You can apply what you learn in those contexts to the broader, non-model real world. When you go into nature and look for signatures that you've seen in your playground, then you can start to understand what exactly might be happening."

Vardi and Shapiro acknowledge that they're only just scratching the surface with this technique. Now, their work focuses on addressing some of those physiological unknowns, such as how corals respond to infection—a process they can observe in real time by tracking labeled pathogens as they invade the polyps inside the chamber. But they also hope that other labs will take advantage of the coral-on-a-chip method to study other aspects of corals' complex physiology and behavior—including responses to environmental stressors affecting global marine life—in unprecedented detail.

"[Looking at] this animal, normally you just see a rock," says Shapiro. "A very beautiful and very colorful rock, but basically a rock. Seeing it at this scale, it suddenly becomes alive. It opens up a whole new way of looking into these fascinating organisms."

—Catherine Offord

Wired for Dexterity

Eighteenth-century German polymath Matthias Buchinger possessed myriad talents, from magic to music to micrography, an art form in which impossibly tiny words are arranged to form larger images, often portraits. Among his most spectacular works is a self-portrait in which his

voluminous curls—covering just a few square centimeters on the page—spell out passages from the Bible and the Lord's Prayer.

Buchinger's drawings are a feat of precision and dexterity, made all the more noteworthy by the fact that the father of 14 completely lacked hands and feet. He was born in 1674 in Germany with stumps at the ends of his arms and above where his knees would have been.

Ricky Jay, a well known sleight-of-hand artist, has been fascinated with Buchinger for decades. As a boy he read books about the history of magic that included portraits of Buchinger. "I thought it was extraordinary that this fellow did magic," says Jay.

Buchinger was skilled at the trick called cups and balls, in which a ball is hidden beneath one of three cups. At the finale, Jay says, Buchinger would lift the cups, revealing fruit, grain, and even live birds. Because he couldn't lift the cup with one hand and insert the ball with another, the routine required a mechanical device—of Buchinger's invention—that demanded special skill to obscure it from the audience. "It's speculative in terms of specifics," says Jay, "but quite wonderful to think about his ability."

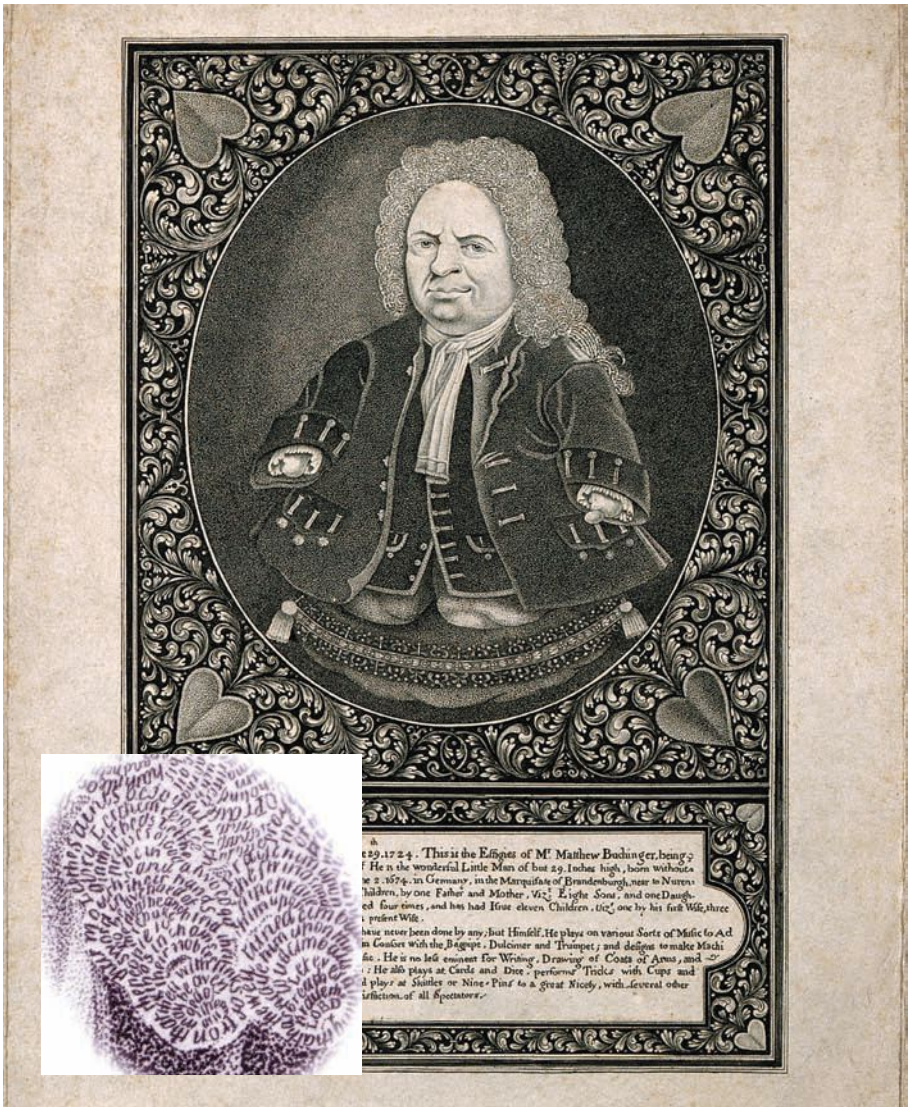
The way the brain organizes control of our body parts may be less about the anatomy—hand, mouth, foot—and more about the job—manipulating objects, eating, kicking.

Over the years, Jay has amassed a large collection of Buchinger's works, about 30 of which were featured in a show earlier this year at the Metropolitan Museum of Art in New York City. Eric Altschuler of Temple University, who studies the role cognitive neuroscience plays in rehabilitation, read a review of the show and decided to check it out.

Altschuler was struck by Buchinger's micrography. "He's got like nanocalligraphy, not micro. It's very hard to see," he says. "What's more interesting is not how you see it, but how does he draw it?"

Jay says Buchinger did not use his mouth to create his drawings, but his stumps alone, which were not perfectly smooth, but had some contours. What gave him such dexterity with his stumps, Altschuler proposed in a recent *Current Biology* essay, was (in addition to talent and most likely a good mentor) a rejiggering of his cortical mapping—a reassignment of brain areas normally given to motor control of the hands and feet (20:R228, 2016). "The stump can go take possession of that territory," says Alschuler. "And since it's from birth, it will happen early and automatically. That must be his secret sauce."

"This has been the case in the literature for so long," says the University of Oxford's Tamar Makin, who studies brain reorganization among people who are missing body parts. "Cortical neighbors take over when you have a missing input."



ABLE ARTIST: Accomplished micrographer Matthias Buchinger hid infinitesimally small script in his artwork, such as this self portrait, which contains seven biblical psalms and the Lord's Prayer in the curls of his hair (inset).

The phenomenon might not be limited to just adjacent cortical regions, such as those controlling the arm and the hand, but could include body parts more distantly represented in the brain. The motor cortex is a strip of brain tissue running from the top of the head down toward the ear. The mouth, for instance, is controlled by an area more lateral (further down the side of the head) than the hand region, and the two are separated by control centers for other body parts. Yet the mouth's control area may also take over the hand region if the person uses the mouth to compensate for the missing hand.

Using fMRI, Cathrin Buetefisch, a neurologist at Emory University School of Medicine, has examined the cortical mapping of people born without hands who use their feet with exceptional skill. She found the "foot" areas of the brain both in the typical foot spot of the cortex—located near the top of the head and deeper into the brain, away from the skull—and also several centimeters (and body parts) away in what is usually the hand area (*PNAS*, 106:2395-2400, 2009).

Disrupting the hand area of the brain with transcranial magnetic stimulation made those same subjects slower to wiggle their dominant toe in response to a visual signal. "That for me is really nice evidence that there is a causal relationship between how you process information in the hand area and how good you are with your feet," says Makin.

Makin points out that the idea of the cortical map as one governed by body parts may be outdated. Rather, it's the function of that body part that defines a particular cortical area. "It seems to me the explanation for all of this is: the hand area is not the area that represents five fingers, knuckles, and nails. It's the area that represents body parts that we're using in order to interact with objects in our environment," Makin says. In other words, the way the brain organizes control of our body parts is less about the anatomy—hand, mouth, foot—and more about the job—manipulating objects, eating, kicking.

The idea is still speculative, but Makin and her colleagues have shown that how a person compensates for a missing body part is reflected in his brain. For instance, people who are born without one hand and who use the handless arm in everyday tasks have more symmetry across hemispheres in the functional connectivity of their cortical hand regions than those who favor their unaffected arm and hand (*eLife*, 4:e04605, 2015). "What we find is this reorganization scales with usage," Makin says.

Makin is recruiting people in the U.K. who paint using a brush placed in their feet or mouths for a study of their cortical organization and how various body parts and functions are represented in their brains. From the evidence to date, it's fair to expect their cortical maps to look different from those of people with normally functioning hands. Buetefisch says it's likely Buchinger also did not have a normal cortical map, but "may have had a different representation of his legs and arms because of . . . being born without these target organs."

Such an adaptive cortical map probably formed from birth, rather than by breaking down and reconstructing the wiring of a typical cortical landscape. "It's not reorganization, it's organization," Buetefisch says. —Kerry Grens

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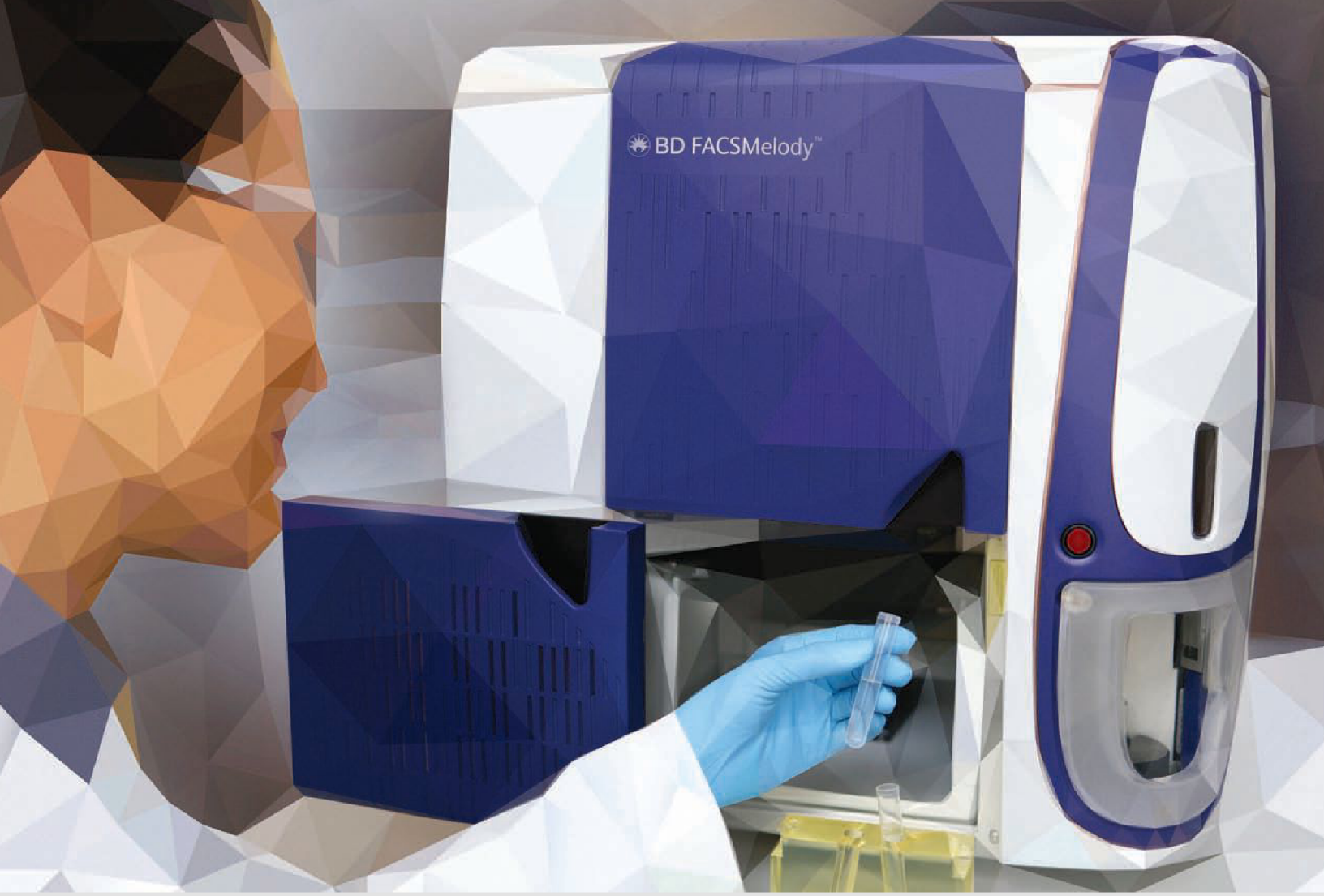

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Trojan Bacteria for Insect RNAi

Scientists employ "friendly" gut bacteria to suppress gene expression in insect hosts.

BY RUTH WILLIAMS

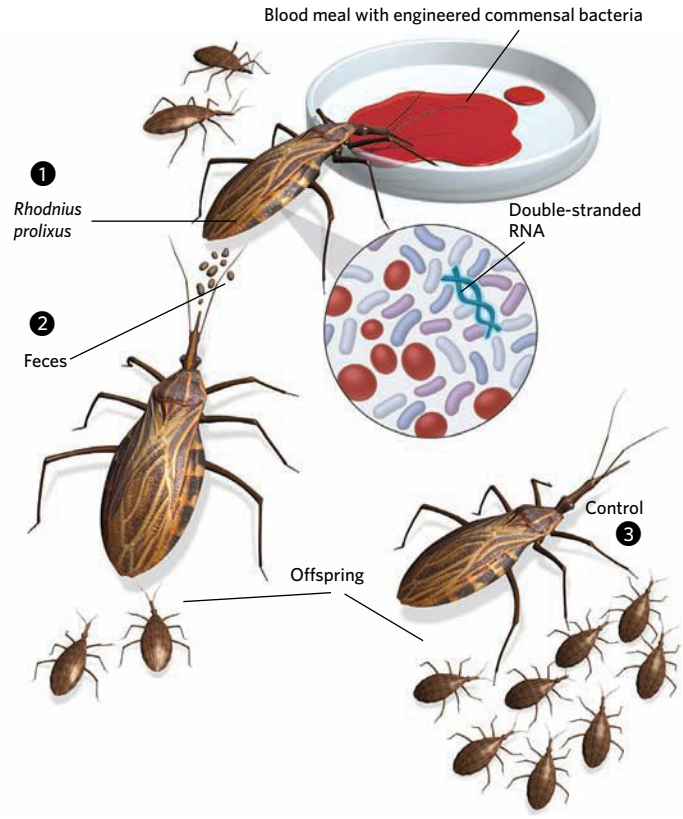
RNA interference (RNAi)—the use of a double-stranded RNA (dsRNA) to trigger the destruction of a corresponding gene's messenger RNA—is a popular method for ascertaining gene function. But for entomologists there's a problem. "We have a major challenge with insects in terms of getting an effective and efficient delivery mechanism," says Owain Edwards of the Commonwealth Scientific and Industrial Research Organisation (CSIRO) in Canberra, Australia.

Current dsRNA delivery methods include ingestion and injection, but the primary issue with both of these is the transient availability of dsRNA once inside the insects—thus necessitating a continuous supply. Injection can also cause trauma, especially to smaller insects.

To prolong RNAi's effects in insects and to develop a gentler mode of delivery, Paul Dyson, Miranda Whitten, and colleagues at Swansea University in the U.K. enlisted the help of the animals' own symbiotic gut bacteria. They engineered a microbial species that resides in western flower thrips (*Frankliniella occidentalis*), a widespread crop pest, to express custom dsRNAs, then mixed the bacteria with the insect's food.

Once the microbes colonized the hosts' guts, the dsRNA released from the bacteria suppressed a gene essential for thrip survival, and many insects died. The researchers used the same approach to successfully block a key fecundity gene in a major Chagas disease vector, *Rhodnius prolixus*, and the number of offspring subsequently declined. In the latter example, the bacteria—different from those deployed in thrips—stably expressed the dsRNA, persisted in the insects' guts for many months, and could suppress the target gene in other *R. prolixus* members by spreading to individuals via coprophagy, eating one another's poop.

Such a sustained RNAi effect could be valuable for insect-control strategies, says Dyson. Angela Douglas of Cornell University says the technique "enormously expands the potential for RNAi in insects." (*Proc R Soc B*, doi:10.1098/rspb.2016.0042, 2016)



EATING UP INTERFERENCE: To control reproduction in *Rhodnius prolixus*, which spreads Chagas disease, researchers introduce double-stranded RNA via bacteria mixed into the insect's food. The blood meal contains commensal gut microbes that have been genetically engineered to express the RNA, which blocks the expression of a gene involved in producing offspring ❶. Such RNA interference can spread to other individuals who eat the treated insect's feces ❷. Control animals produced roughly 70 percent more offspring than treated ones ❸.

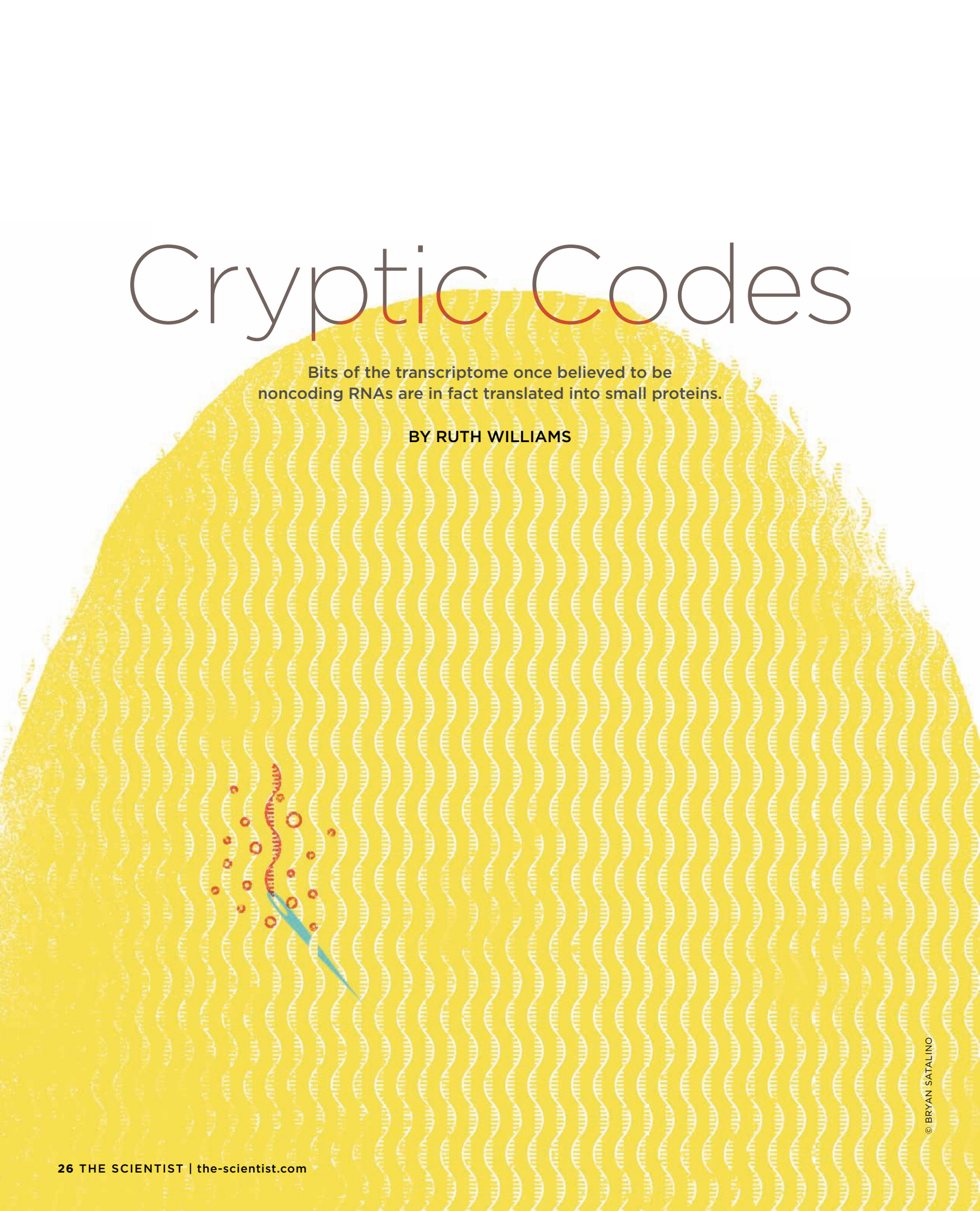
AT A GLANCE

dsRNA DELIVERY TECHNIQUE	HOW IT WORKS	ADVANTAGES	LIMITATIONS
Ingestion	The dsRNA of interest is mixed with the insect's food, and once in the gut it is absorbed into the tissues.	No genetic engineering of vector bacteria is required.	RNAi effect ceases without a constant supply of the dsRNA.
Genetically engineered symbiotic gut bacteria	A commensal bacterium's genome is engineered to express a dsRNA of interest. The microbes are then introduced to the insect's gut via food. The bacteria reside in the gut long-term, continuously supplying dsRNA.	If bacteria stably express the dsRNA, then the RNAi effect lasts as long as the bacteria are present in gut. Depending on the insect and bacterial species, horizontal transfer of engineered bacteria may be possible, thus targeting many insects at once.	A commensal bacterium amenable to laboratory culture and genetic manipulation may not be available for a particular insect species of interest.

Cryptic Codes

Bits of the transcriptome once believed to be noncoding RNAs are in fact translated into small proteins.

BY RUTH WILLIAMS



In 2002, a group of plant researchers studying legumes at the Max Planck Institute for Plant Breeding Research in Cologne, Germany, discovered that a 679-nucleotide RNA believed to function in a noncoding capacity was in fact a protein-coding messenger RNA (mRNA).[1] It had been classified as a long (or large) noncoding RNA (lncRNA) by virtue of being more than 200 nucleotides in length. The RNA, transcribed from a gene called *early nodulin 40* (*ENOD40*), contained short open reading frames (ORFs)—putative protein-coding sequences bookended by start and stop codons—but the ORFs were so short that they had previously been overlooked. When the Cologne collaborators examined the RNA more closely, however, they found that two of the ORFs did indeed encode tiny peptides: one of 12 and one of 24 amino acids. Sampling the legumes confirmed that these micropeptides were made in the plant, where they interacted with a sucrose-synthesizing enzyme.

Five years later, another ORF-containing mRNA that had been posing as a lncRNA was discovered in *Drosophila*.[2,3] After performing a screen of fly embryos to find lncRNAs, Yuji Kageyama, then of the National Institute for Basic Biology in Okazaki, Japan, suppressed each transcript's expression. "Only one showed a clear phenotype," says Kageyama, now at Kobe University. Because embryos missing this particular RNA lacked certain cuticle features, giving them the appearance of smooth rice grains, the researchers named the RNA "polished rice" (*pri*).

Turning his attention to how the RNA functioned, Kageyama thought he should first rule out the possibility that it encoded proteins. But he couldn't. "We actually found it was a protein-coding gene," he says. "It was an accident—we are RNA people!" The *pri* gene turned out to encode four tiny peptides—three of 11 amino acids and one of 32—that Kageyama and colleagues showed are important for activating a key developmental transcription factor.[4]

Since then, a handful of other lncRNAs have switched to the mRNA ranks after being found to harbor micropeptide-encoding short ORFs (sORFs)—those less than 300 nucleotides in length. And given the vast number of documented lncRNAs—most of which have no known function—the chance of finding others that contain micropeptide codes seems high.

The hunt for these tiny treasures is now on, but it's a challenging quest. After all, there are good reasons why these itty-bitty peptides and their codes went unnoticed for so long.

As researchers take a deeper dive into the function of the thousands of noncoding RNAs believed to exist in genomes, they continue to uncover surprise micropeptides.

Overlooked ORFs

From the late 1990s into the 21st century, as species after species had their genomes sequenced and deposited in databases, the search for novel genes and their associated mRNAs duly followed. With millions or even billions of nucleotides to sift through, researchers devised computational shortcuts to hunt for canonical gene and mRNA features, such as promoter regions, exon/intron splice sites, and, of course, ORFs.

ORFs can exist in practically any stretch of RNA sequence by chance, but many do not encode actual proteins. Because the chance that an ORF encodes a protein increases with its length, most ORF-finding algorithms had a size cut-off of 300 nucleotides—translating to 100 amino acids. This allowed researchers to "filter out garbage—that is, meaningless ORFs that exist randomly in RNAs," says Eric Olson of the University of Texas Southwestern Medical Center in Dallas.

Of course, by excluding all ORFs less than 300 nucleotides in length, such algorithms inevitably missed those encoding genuine small peptides. "I'm sure that the people who came up with [the cut-off] understood that this rule would have to miss anything that was shorter than 100 amino acids," says Nicholas Ingolia of the University of California, Berkeley. "As people applied this rule more and more, they sort of lost track of that caveat." Essentially, sORFs were thrown out with the computational trash and forgotten.

Aside from statistical practicality and human oversight, there were also technical reasons that contributed to sORFs and their encoded micropeptides being missed. Because of their small size, sORFs in model organisms such as mice, flies, and fish are less likely to be hit in random mutagenesis screens than larger ORFs, meaning their functions are less likely to be revealed. Also, many important proteins are identified based on their conservation across species, says Andrea Pauli of the Research Institute of Molecular Pathology in Vienna, but "the shorter [the ORF], the harder it gets to find and align this region to other genomes and to know that this is actually conserved."

As for the proteins themselves, the standard practice of using electrophoresis to separate peptides by size often meant micropeptides would be lost, notes Doug Anderson, a postdoc in Olson's lab. "A lot of times we run the smaller things off the

bottom of our gels," he says. Standard protein mass spectrometry was also problematic for identifying small peptides, says Gerben Menschaert of Ghent University in Belgium, because "there is a washout step in the protocol so that only larger proteins are retained."

But as researchers take a deeper dive into the function of the thousands of lncRNAs believed to exist in genomes, they continue to uncover surprise micropeptides. In February 2014, for example, Pauli, then a postdoc in Alex Schier's lab at Harvard University, discovered a hidden code in a zebrafish lncRNA. She had been hunting for lncRNAs involved in zebrafish development because "we hadn't really anticipated that there would be any coding regions out there that had not been discovered—at least not something that is essential," she says. But one lncRNA she identified actually encoded a 58-amino-acid micropeptide, which she called Toddler, that functioned as a signaling protein necessary for cell movements that shape the early embryo.[5]

Then, last year, Anderson and his colleagues reported another. Since joining Olson's lab in 2010, Anderson had been searching for lncRNAs expressed in the heart and skeletal muscles of mouse embryos. He discovered a number of candidates, but one stood out for its high level of sequence conservation—suggesting to Anderson that it might have an important function. He was right, the RNA was important, but for a reason that neither Anderson nor Olson had considered: it was in fact an mRNA encoding a 46-amino-acid-long micropeptide.[6]

"When we zeroed in on the conserved region [of the gene], Doug found that it began with an ATG [start] codon and it terminated with a stop codon," Olson says. "That's when he looked at whether it might encode a peptide and found that indeed it did." The researchers dubbed the peptide myoregulin, and found that it functioned as a critical calcium pump regulator for muscle relaxation.

With more and more overlooked peptides now being revealed, the big question is how many are left to be discovered. "Were there going to be dozens of [micropeptides]? Were there going to be hundreds, like there are hundreds of microRNAs?" says Ingolia. "We just didn't know."

Olson suspects the number is quite large. The fact that "myoregulin went below the radar screen for all these years . . . really told us that there's likely to be a gold mine of undiscovered micropeptides out there," he says. "So we are aggressively mining that right now."

Hunting for hidden peptides

In the mid-2000s, Menschaert was working on mass spectrometry protocols to enrich small peptides, which at that time were believed to be cleaved from larger proteins, when he read the papers about the polished rice sORFs. If there is one example of sORF-encoded micropeptides, he thought, there are bound to be others.

To find out if his hunch was correct, Menschaert performed a lot of RNA sequencing to identify sORFs, and a lot of mass spectrometry to find the putative peptides. But it was a slow and painstaking endeavor, as he could only survey a small number of sORFs at a time. Then, in 2009, researchers developed a new, rapid, genome-wide approach called ribosome profiling, which enabled the translation of all ORFs, large and small, to be assessed en masse using next-generation sequencing of ribosome-associated RNA.

The technique was an update of another method called ribosome footprinting, in which researchers would isolate ribosome-associated RNAs, digest

ALGORITHMS FOR ASSESSING sORF CODING POTENTIAL

Genomes contain countless sORFs, but most do not produce functional proteins. To help identify the true protein-coding needles in the nonsense haystacks, scientists have devised methods and metrics to calculate sORFs' coding potential based on their sequences and ribosome profiling characteristics.

Ribosome Release Score (RSS): After a ribosome reaches the stop codon of a true protein-coding mRNA, the ribosome's association with the transcript ceases. The distribution of ribosome-bound fragments for those RNAs would thus show a dramatic reduction following the putative stop codon. (*Cell,* 154:240-51, 2013)

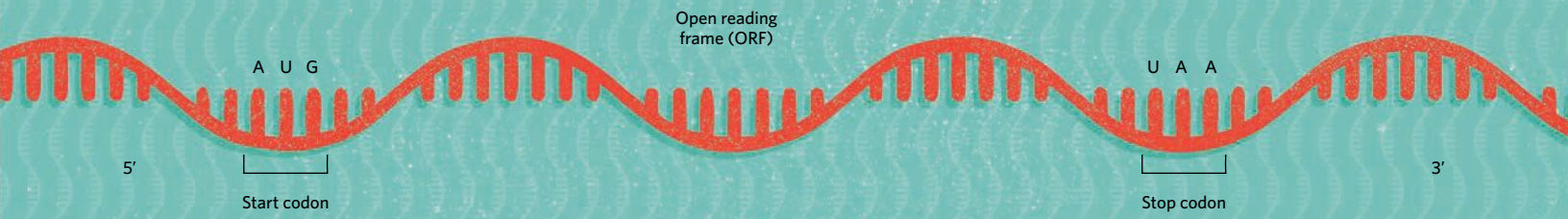
Fragment Length Organization Similarity Score (FLOSS): This metric distinguishes RNAs that have ribosome profiling fragment sizes clustered tightly in the 30–32 nucleotide range—the size protected by a eukaryotic ribosome—from those that have more varied fragment sizes, which might indicate protection by contaminating nonribosomal proteins. (*Cell Rep,* 8:1365-79, 2014)

ORF Regression Algorithm for Translation Evaluation of RPFs (ribosome-protected mRNA fragments) (ORF-RATER): This algorithm determines the likelihood that an ORF is translated based on its similarity to known protein-coding ORFs in terms of ribosome-occupancy pattern—that is, the distribution of ribosome profiling fragments across the ORF. For example, true protein-coding ORFs tend to exhibit peaks in the number of fragments at the start and stop codons where ribosomes are built and dismantled, and their fragments show a three-nucleotide periodicity in the expected reading frame—the ribosome appears to jump along three nucleotides (one codon) at a time. (*Mol Cell,* 60:816-27, 2015)

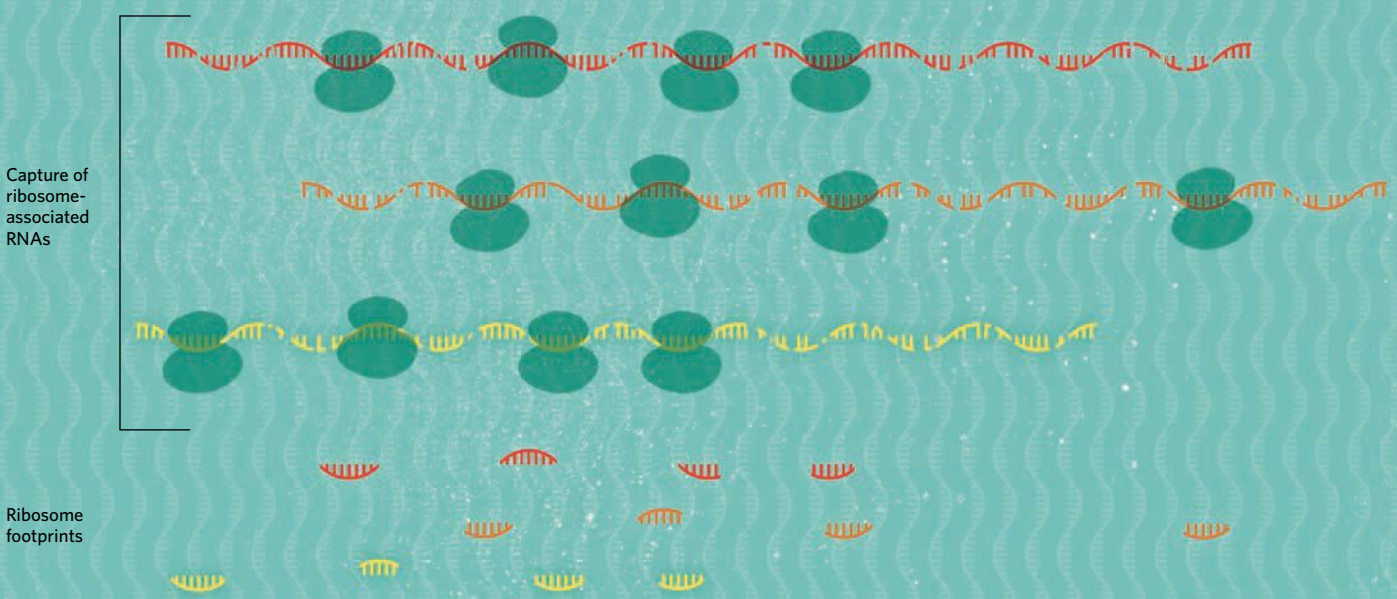
Phylogenetic Conservation Score of a sORF (PhyloCSF): This metric examines conservation of a sORF across species. (*Bioinformatics,* 27:i275-i282, 2011)

FOLLOWING THE CODE

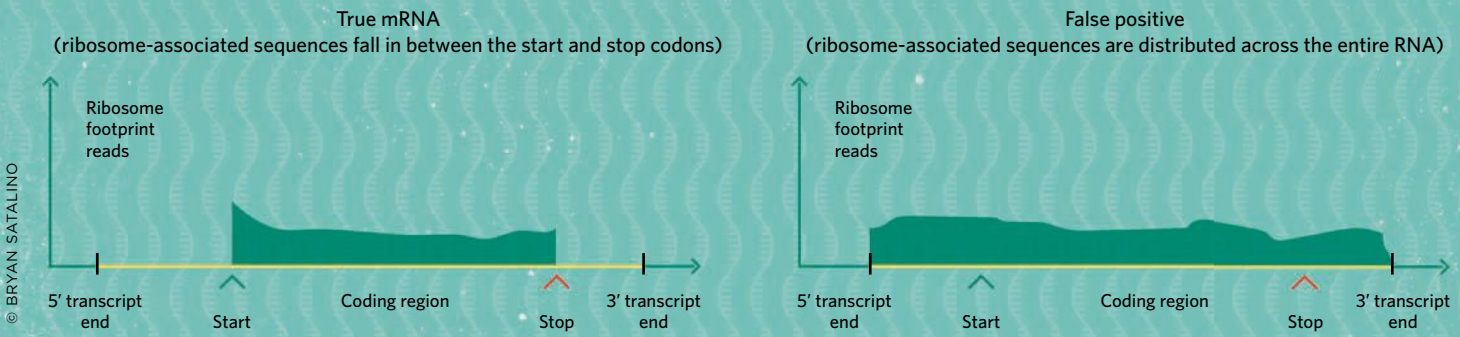
With the advent of genome sequencing technologies, researchers began combing genomes for open reading frames (ORFs). To enrich for genuine protein-coding ORFs and to eliminate those random sequences that by chance were bookended by start and stop codons, most ORF-finding algorithms ignored any stretches shorter than 300 nucleotides. Unfortunately, this also meant that many short ORFs encoding micropeptides were missed. Now, new techniques are helping scientists identify tiny ORFs within what were presumed to be long noncoding RNAs.



To search for coding RNAs directly, rather than through the genome, researchers turned their attention to translation and implemented a technique known as ribosome footprinting, which involves isolating and digesting ribosome-associated RNAs to leave only those fragments that are protected by the bound ribosomes. Advances in next-generation sequencing technology have allowed researchers to make this process high-throughput, capturing likely translation events across a cell's entire transcriptome.



Not all ribosome-associated RNAs are truly protein coding, however. To identify true protein-coding mRNAs, researchers are now devising analytical techniques such as the ribosome release score (RSS), which assesses the distribution of ribosome-bound fragments along the whole RNA molecule. True mRNAs should have more ribosome-associated regions within the ORF than after the stop codon.



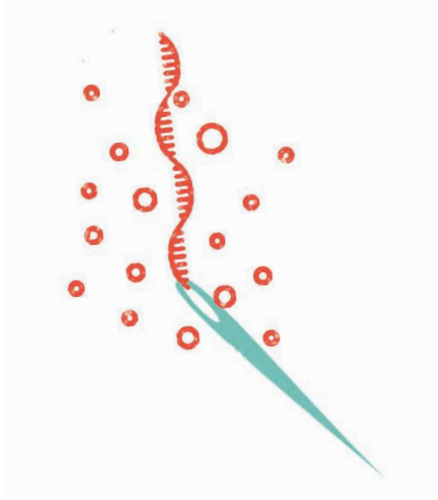
them with a nuclease, and then recover and sequence the short fragments of RNA protected from digestion by the bound ribosomes. Mass spec was still required to confirm that the proteins generated from these RNAs actually existed in the cell; even truly noncoding RNAs can sometimes associate with ribosomes by chance. But ribosome footprinting was a straightforward way to identify RNAs that, at the very least, associated with the translation machinery.

Until the past decade of advances in sequencing technology, however, this too was a time-consuming process, says Ingolia. "People had used ribosome footprinting on single, specific messages, but you couldn't apply it to everything that was going on in a cell." Then next-gen sequencing was developed, giving researchers the power to "read hundreds of millions of these footprints at once," says Jonathan Weissman of the University of California, San Francisco.

So he, Ingolia—then a postdoc in his lab—and their colleagues turned ribosome footprinting into ribosome profiling to obtain a global snapshot of translation events across the entire transcriptome. In 2011, the researchers reported that in mouse embryonic stem cells, the majority of lncRNAs transcribed from apparently noncoding regions of the genome were in fact associated with ribosomes.[7] "Very early on . . . we could see that we were getting signals outside of the canonical open reading frames," says Weissman.

"That paper was really a milestone in terms of showing that there is a lot of translation outside of [known] coding regions," says Pauli.

But just how much is still unclear. While Ingolia and Weissman's findings could have pointed to a transcriptome littered with micropeptide-encoding sORFs, they also found some fully characterized lncRNAs with well-known nuclear functions to be associated with ribosomes in their analysis. Classical noncoding RNAs such as telomerase RNA, which acts as a template for telomeric DNA replication, for example, and small nuclear RNAs known to be involved in splicing "come up as very highly translated" in ribosome



Their diminutive size may have caused micropeptides to be overlooked, but it does not prevent them from serving important, often essential functions.

profiling assays, says Caltech's Mitch Guttman. "That's what originally clued us in to the fact that . . . this ribosome-occupancy measure is not [always] indicative of real translation."

Some ORFs may associate with ribosomes as part of translation regulatory mechanisms, Guttman says, or simply as random interactions—these latter associations might even produce small nonfunctional peptides that, it's thought, would be unstable and thus rapidly degraded. To distinguish ribosome profiles that reflect true translation from those that don't, Guttman joined forces with Ingolia and Weissman to create a metric, called the ribosome release score, based on the distribution of ribosome-bound fragments recovered from a particular mRNA. When ribosomes translating a genuine ORF come to the stop codon, they are released from the mRNA. Truly translated RNAs, then, should display a greater proportion of ribosome footprint fragments from their coding region than from the downstream untranslated region. "For bona fide peptides, you see a very clear drop [after the stop codon]," says Guttman, "while for classic noncoding RNAs you do not." (See illustration on previous page.)

Applying this metric to Ingolia and Weissman's 2011 mouse embryonic stem cell data, the researchers found that the vast majority of intergenic lncRNAs can still be considered noncoding.[8] But not all of them. About five percent of supposedly lncRNAs have ribosome release scores akin to protein-coding transcripts, says Guttman. "Five percent is a huge number if you think about the fact that there are tens of thousands of lncRNAs," he says. "[It] still creates a huge number of possible micropeptides. So that's very interesting and worthy of exploration."

To aid in the verification of sORF translation and the identification of the micropeptides produced, new metrics and algorithms—based on ribosome footprint patterns, sequence conservation, synonymous mutation frequency and other features—continue to be developed.[9,10] (See table on opposite page.) And in a study published online last November, Menschaert and colleagues established a searchable sORF database called sORFs.org with the aim of accumulating and centralizing data on sORFs and their translation potential.[11]

For now, the researchers have included all sORFs identified during ribosome profiling studies in mice, *Drosophila,* and humans—"with no filtering whatsoever," says Menschaert. "The idea was to include everything." All told, the database currently contains a whopping 266,342 sORFs, but screening with assorted metrics can narrow down this vast list. Stringent filtering of the human sORFs, for example, reduces the list to about 400 or so strong candidates, says Menschaert, who is systematically performing mass spectrometry experiments to determine whether the putative peptides are actually expressed in cells.

Once a new micropeptide is identified, it's back to the molecular biology bench to interrogate its function. "That's the slow bit," says Menschaert. But several of the scientists interviewed for this article indicated that they have new micropeptides in their sights. In January of this year, for example, Olson and his colleagues reported their discovery of a second lncRNA-concealed muscle-specific micropeptide—a 34-amino-acid peptide they named dwarf open reading frame

NEW PEPTIDES ON THE BLOCK

Species	Gene name	Size of encoded proteins (number of amino acids)	Function	Reference
Soybean (*Glycine max*)	*ENOD40-1*	12 and 24	Associate with a subunit of sucrose synthase in root nodules	1
Fruit fly (*Drosophila melanogaster*)	Polished rice (*pri*), also known as tarsal-less (*tal*)	Three of 11; one of 32	Activate an essential transcription factor, driving formation of cuticle structures during embryo development	2,3,4
Fruit fly	*pgc*	71	Prevents phosphorylation of RNA polymerase II in germline progenitor cells	14
Fruit fly	*SclA* and *SclB*	28 and 29	Involved in calcium handling in muscle cells	15
Red flour beetle (*Tribolium castaneum*)	*mlpt*	10, 11, 15, 23	Ortholog of *pri* involved in the development of abdominal segments	16
Zebrafish (*Danio rerio*)	*Toddler*	58	Activates a G protein–coupled receptor to promote migration of mesendodermal cells in the developing embryo	6
Mouse (*Mus musculus*)	*myoregulin*	46	Interacts with and inhibits a calcium pump in muscle cells, interfering with muscle relaxation	5
Mouse	*DWORF*	34	Interacts with and enhances calcium pump activity in muscle cells	12

(DWORF).[12] The team found evidence that DWORF acts as a regulator of muscle contractility, is abundantly expressed in the mouse heart, and is suppressed in ischemic human heart tissue, suggesting a possible link with heart failure.

Other such small peptides might be immunogenic, says Weissman, who has found that micropeptides encoded by sORFs in a human-infecting cytomegalovirus lncRNA were capable of producing T-cell responses in previously infected patients' cells.[10] "I'm sure there will be some that are important for certain diseases," agrees Pauli.

And as researchers continue to more carefully comb small snippets of genomes, it's likely that even more cellular roles for micropeptides will be uncovered. Their diminutive size may have caused these peptides to be overlooked, their sORFs to be buried in statistical noise, and their RNAs to be miscategorized, but it does not prevent them from serving important, often essential functions, as the micropeptides characterized to date demonstrate.

In short, size isn't everything. Indeed, says Pauli, the only reason researchers haven't identified more peptide-encoding sORFs to date is "because one just didn't know that these things existed." ■

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» AUTOIMMUNE TARGETS: This false-color transmission electron micrograph (TEM) displays a slice through a mammalian pancreatic islet cell, the target of an aberrant immune response in type 1 diabetes patients. The red spots in white spaces are membrane-bound secretory granules containing insulin and other hormones to be excreted into the blood. (Nucleus, upper right.)

Taming Autoimmunity

Therapies that train the immune system to cease fire on targeted native tissues could improve outcomes for autoimmune patients, but clinical progress has been slow.

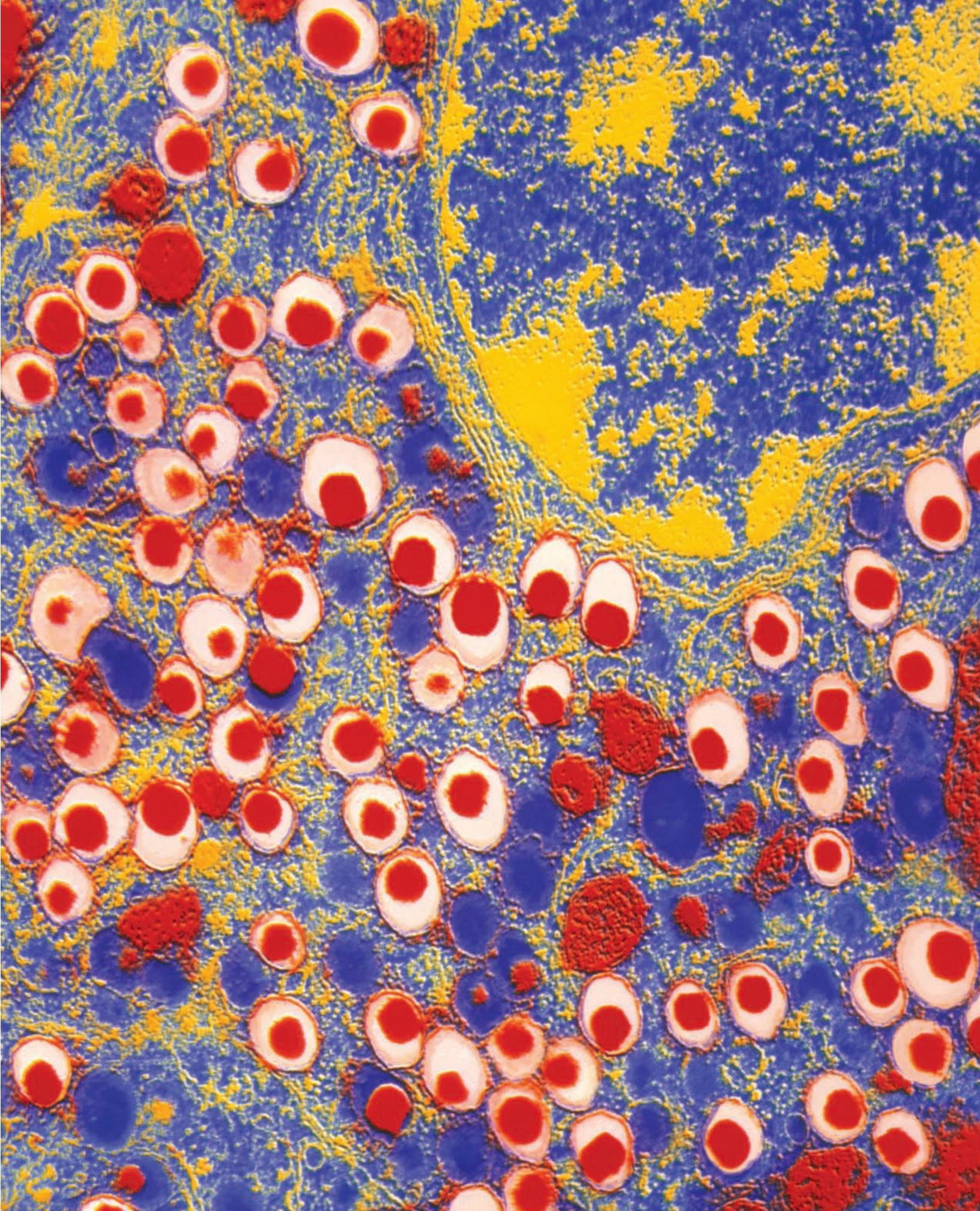
BY LAWRENCE STEINMAN

History often repeats itself. More than 100 years ago, one of the world's leading immunologists, Nobel Laureate Paul Ehrlich, doubted the very existence of autoimmunity, in which the immune system begins to attack healthy tissues. Envisioning a nightmare scenario where the body turns against itself, Ehrlich reasoned that it would be quite improbable. His skepticism regarding this phenomenon, which he termed "horror autotoxicus" (literally, "the horror of self-toxicity"), delayed the acceptance of this concept for another half century—even in the face of compelling clinical examples of immunity gone haywire.

A century of basic research later, scientists now accept that autoimmunity does in fact exist, with devastating consequences. More than 20 autoimmune diseases have been identified; these disorders affect every organ in the body and afflict some 50 million people in North America and Europe. But autoimmunity researchers now face resistance once again—this time from the pharmaceutical industry, which is loathe to invest in a more-targeted approach to treating these millions of patients.

Most autoimmune disorders are characterized by a focused attack on a particular organ system: the brain and spinal cord become inflamed in multiple sclerosis, the skin is attacked in psoriasis, the joints are pummeled in rheumatoid arthritis, and the intestines are injured in Crohn's disease and ulcerative colitis. In some cases, autoimmunity strikes only a single cell type in one organ. In type 1 diabetes, the insulin-producing beta cells located in the islets of Langerhans of the pancreas are destroyed or severely injured. This specificity of the autoimmune response is the result of immune reactivity to particular self-antigens. If researchers can identify and target those antigens, they can theoretically develop therapies to strike at the heart of such disorders.

Such a precision approach would overcome a major problem with today's autoimmune disease treatments, which generally target critical components of our immune systems, making patients susceptible to infections that their own immune responses would normally contain. In a recent Phase 3 clinical trial of a Janus-kinase inhibitor called baricitinib, which works by blocking key biochemical pathways involved in the production of inflammatory molecules, treatment reduced symptoms of rheumatoid arthritis, but patients experienced a



dose-dependent two- to fourfold increase in the incidence of shingles compared to placebo.[1] Similarly, an antibody to a Velcro-like adhesion molecule called α4 integrin involved in lymphocyte homing to the brain has proven effective in treating relapses in multiple sclerosis. But this approach carries the risk of an often-fatal brain infection known as progressive multifocal leukoencephalopathy (PML). Thus far there have been more than 500 cases of PML in patients taking the α4 integrin antibody, called natalizumab (Tysabri), with an overall rate of about 1 in 250 and the risk increasing with monthly usage beyond a year.[2] Therapies that target only those immune cells that are attacking the affected tissue in a particular autoimmune disease would leave the remainder of the immune system, including the portions that fight infection, free to do its job.

This approach, called antigen-specific therapy, has yet to gain traction, however. Part of the problem is that for many autoimmune conditions we simply do not know the identities of the antigens that are recognized and attacked by the immune system. Although researchers have identified the targeted molecules in a few autoimmune diseases, the pharmaceutical industry has been reluctant to invest in antigen-specific therapies. So far no one has succeeded with this approach, and such attempts are thus deemed "nonvalidated" by industry. Lack of support from the pharmaceutical industry, however, has slowed progress toward validating these targeted therapies. Antigen-specific therapy has only rarely been tested in humans, and failures of clinical trials testing antigen-specific therapies in multiple sclerosis, where we do not yet have a convincing understanding of the key antigens driving disease progression, have cast further shadows over our ability to target any autoimmune disease at its roots.

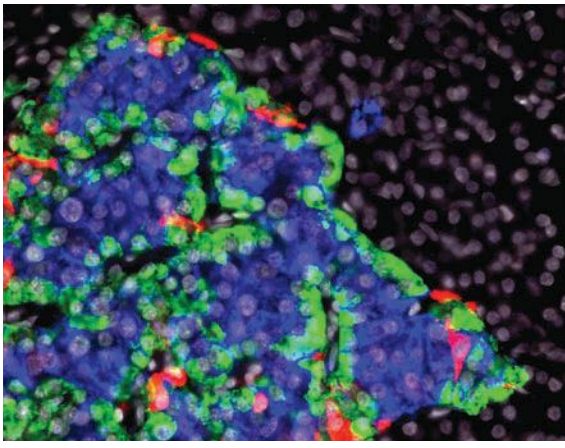
This mind-set has hindered effective drug development for autoimmune diseases compared with therapeutic advances for many other disorders, where the standards of care now include treatments that attack specific mediators of pathogenesis. In the field of allergy, for example, immune desensitization to offending allergens is accepted medical practice. Even in as complex a disease as certain cancers, researchers have successfully targeted the root cause of cells' prolific growth. Imatinib (Gleevec) is an exemplary drug. It attacks chronic myelogenous leukemia directly at the point of the fundamental mutation that causes the deadly cancer.

Such highly targeted therapeutics can be considered what Ehrlich called "magic bullets." In autoimmunity we have no such magic bullet. Development of a therapy that turns off the fundamental disease-causing immune pathways languishes behind other approaches that attack critical components of the normal immune response. Indeed, there are no therapies on the market aimed at blocking those immune responses at the core of the disease process, and only a few, for type 1 diabetes, have even progressed to early-stage clinical trials.

Targeting diabetes

Type 1 diabetes is one major autoimmune disease that is ready for a magic-bullet approach. Currently, the only treatment option is insulin, a peptide hormone whose role in maintaining glucose homeostasis was discovered a century ago. Although it has been a lifesaving therapy for type 1 diabetics, insulin does not prevent the long-term, insidious aspects of the disease such as heart attack, stroke, neuropathy, kidney complications, and retinal disease. But the immune responses underlying this widespread disease have been well characterized: both antibodies and cytotoxic "killer" T cells direct attacks on molecules that are produced exclusively in pancreatic beta cells, where the body's insulin is synthesized. Now, two research teams, including one that I direct, are staging a full frontal assault on these aberrant immune responses, with promising results in early clinical trials.

One beta-cell antigen (called an autoantigen) targeted in the treatment of type 1 diabetes is proinsulin, the polypeptide precursor of insulin.[3] In a 2004 study, researchers followed children from birth and found that the appearance of high-affinity antibodies to proinsulin correlates with the onset of type 1 diabetes. To tolerize patients to their own proinsulin and to quell the inappropriate immune response, my colleagues and I engineered a DNA plasmid encoding proinsulin. The noncoding DNA in the backbone of the plasmid contains naturally occurring hexanucleotide motifs that are immune stimulatory, and are termed CpG sequences. We replaced CpG sequences with a suppressive hexanucleotide sequence, called GpG sequences, that are known to suppress an immune response. An intron was also incorporated into the plasmid to enhance the expression of proinsulin after plasmid injection into muscle.



GLOWING ISLET: This composite light micrograph of a pancreatic islet shows beta cells (blue), alpha cells (green), and delta cells (red).

Preclinical studies in nonobese diabetic (NOD) mice, which spontaneously develop type 1 diabetes, showed that the engineered plasmid successfully stemmed the autoimmune response and restored normal glucose metabolism.[5] The plasmid did so by inducing the expression of proinsulin by antigen-presenting cells (APCs) such as macrophages and muscle cells without the normal costimulation of T cells. To activate T cells and generate an immune response, an antigen like proinsulin must be presented to a T cell by an antigen-presenting cell in the presence of an array of other molecules called costimulatory molecules, such as CD80 and CD86. By expressing proinsulin without costimulation, the engineered plasmid leads to immune tolerance. (See illustration on page 36-37.) After intramuscular injections of the tolerizing plasmid, the NOD mice experienced an attenuation

of the immune response to proinsulin. This resulted in restoration of glucose homeostasis, reduction in islet inflammation, and reduction in antibodies directed against islet cells.

With these preclinical results in hand, we founded a biotechnology company, Tolerion, to sponsor an 80-patient, placebo-controlled clinical trial in the U.S., Australia, and New Zealand in which participants received 12 weekly injections of either the tolerizing plasmid encoding proinsulin or a placebo. The primary endpoint of the trial, which was completed in 2012, was to measure C-peptide, a 31-amino-acid-long section of the proinsulin molecule that is cleaved before secretion from the pancreas as insulin. The level of C-peptide serves as a measure of how well the pancreas is functioning, and C-peptide levels typically decline over time in patients with type 1 diabetes. At the end of the injection period, we observed an increase in the production of C-peptide in the treated individuals, and a drop in C-peptide in the placebo group, compared with C-peptide levels at the start of the trial. We suspect this indicates an improvement in beta cell function in the treated patients. Beta cells that were injured and not yet destroyed may recover function when there is a cease-fire and attenuation of the immune attack. In addition, as C-peptide levels increased, there was a corresponding decrease in cytotoxic T cells that recognized proinsulin.[6]

Importantly, no T cells that respond to other antigens were affected. This suggests that the plasmid's effect was antigen specific; tolerance to proinsulin was achieved without altering immunity to viral antigens or to other islet antigens. We are currently planning a follow-up trial in which children with type 1 diabetes will receive weekly dosing of the tolerizing DNA plasmid for one year. Preclinical research has demonstrated that this same plasmid-based approach can provide tolerization to other islet antigens, including glutamic acid decarboxylase, islet-specific glucose-6-phosphatase, and a zinc transporter.[5,7] Although the main attack in type 1 diabetes targets proinsulin, these other islet cell molecules may also play a role in the disease, and they too can be targeted.

Another exciting autoimmunity treatment strategy aims at inducing regulatory cells that might suppress unwanted immune activity. First posited by Ehrlich as one way the body prevents autoimmunity from wreaking havoc, regulatory T cells (Tregs) are now known to play an important role in winding down immune responses. Ramping up the production of such regulatory immune cells could provide another avenue for quelling the autoimmune response of type 1 diabetes.

In our preclinical experiments with the proinsulin plasmid, for example, the NOD mice began to produce a regulatory T cell that secreted the immune-suppressive cytokine known as interleukin-10 when stimulated with proinsulin. To harness such natural immune control, Jeffrey Bluestone of the University of California, San Francisco, and colleagues extracted Tregs from 14 type 1 diabetics, expanded the cells in the lab, then reinfused the cells back into the patients. There were no infusion reactions or significant adverse effects, and some of the recipients experienced a slowed decline in their C-peptide levels that lasted up to two years following the treatment.[8]

A PRECISION APPROACH TO AUTOIMMUNITY WOULD OVERCOME A MAJOR PROBLEM WITH TODAY'S TREATMENTS, WHICH GENERALLY MAKE PATIENTS SUSCEPTIBLE TO INFECTIONS THAT THEIR OWN IMMUNE RESPONSES WOULD NORMALLY CONTAIN.

Other therapies in clinical trials for type 1 diabetes aim to increase the number of Tregs in the pancreas. But so far, all of these attempts have focused on Tregs that are not generated in response to a specific antigen. Thus, like existing treatments for autoimmune disorders, such therapies might increase the risk of opportunistic infection by affecting a broad swathe of immune cells. Researchers are now working to refine this approach to target a regulatory cell governing only responses to the pancreatic beta cell.

Earlier this year, for example, the University of Calgary's Pere Santamaria and colleagues tested such a targeted therapy in a variety of mouse models of autoimmunity. The researchers used engineered nanoparticles coated with relevant autoimmune peptides and portions of the major histocompatibility complex, which normally help activate cytotoxic T cells. Without additional costimulatory signals, these nanoparticles instead triggered the in vivo differentiation of self-reactive T cells into antigen-specific Tregs. In animal models, the nanoparticles have resolved diverse autoimmune phenotypes, including diabetes.[9] If these results hold up in humans, therapies that aim to boost levels of relevant Tregs could serve as another type of antigen-specific approach to quelling the aberrant immune responses that plague patients with type 1 diabetes.

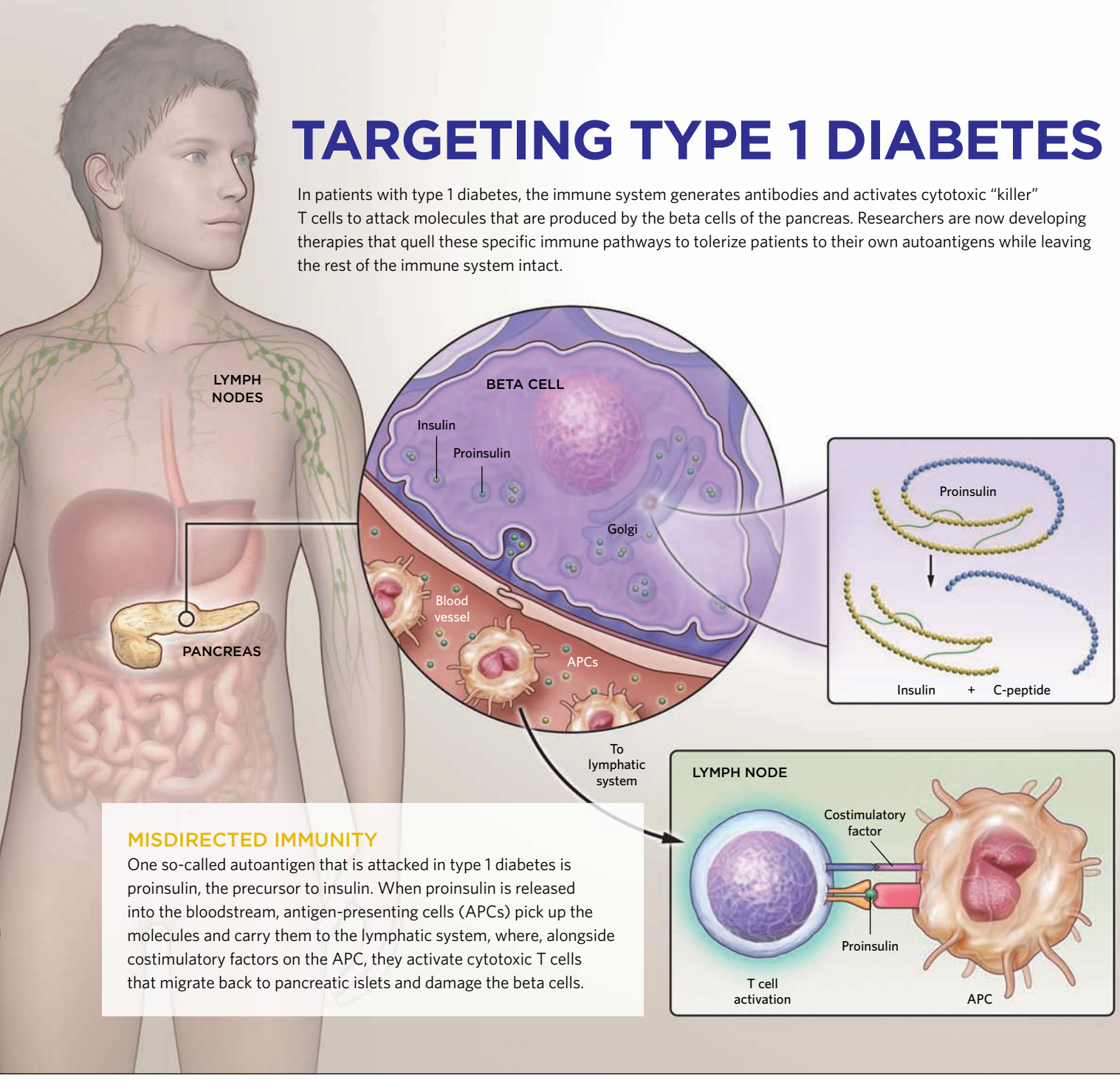
Treating other autoimmune diseases

Such progress in type 1 diabetes gives hope that an antigen-specific approach might succeed in treating other autoimmune diseases for which the underlying immune pathways are known. And, thanks to many years of bench work and advances in our understanding of human immunity, there are now a handful of disorders for which these immune details are coming to light.

In the vast majority of individuals with myasthenia gravis, for example, the immune system generates antibodies that attack the receptor for acetylcholine on the muscle side of the synapse between motor neurons and muscle cells. Such antibodies are highly pathogenic; a pregnant mother with myasthenia gravis can deliver a myasthenic newborn due to the transfer of such anti-acetylcholine receptor antibodies across the placenta. Presenting the body with acetylcholine receptor (AChR) without the necessary costimulatory factors to trigger an immune response, then, may help tolerize the immune system to this autoantigen, and reduce or stop the pro-

TARGETING TYPE 1 DIABETES

In patients with type 1 diabetes, the immune system generates antibodies and activates cytotoxic "killer" T cells to attack molecules that are produced by the beta cells of the pancreas. Researchers are now developing therapies that quell these specific immune pathways to tolerize patients to their own autoantigens while leaving the rest of the immune system intact.



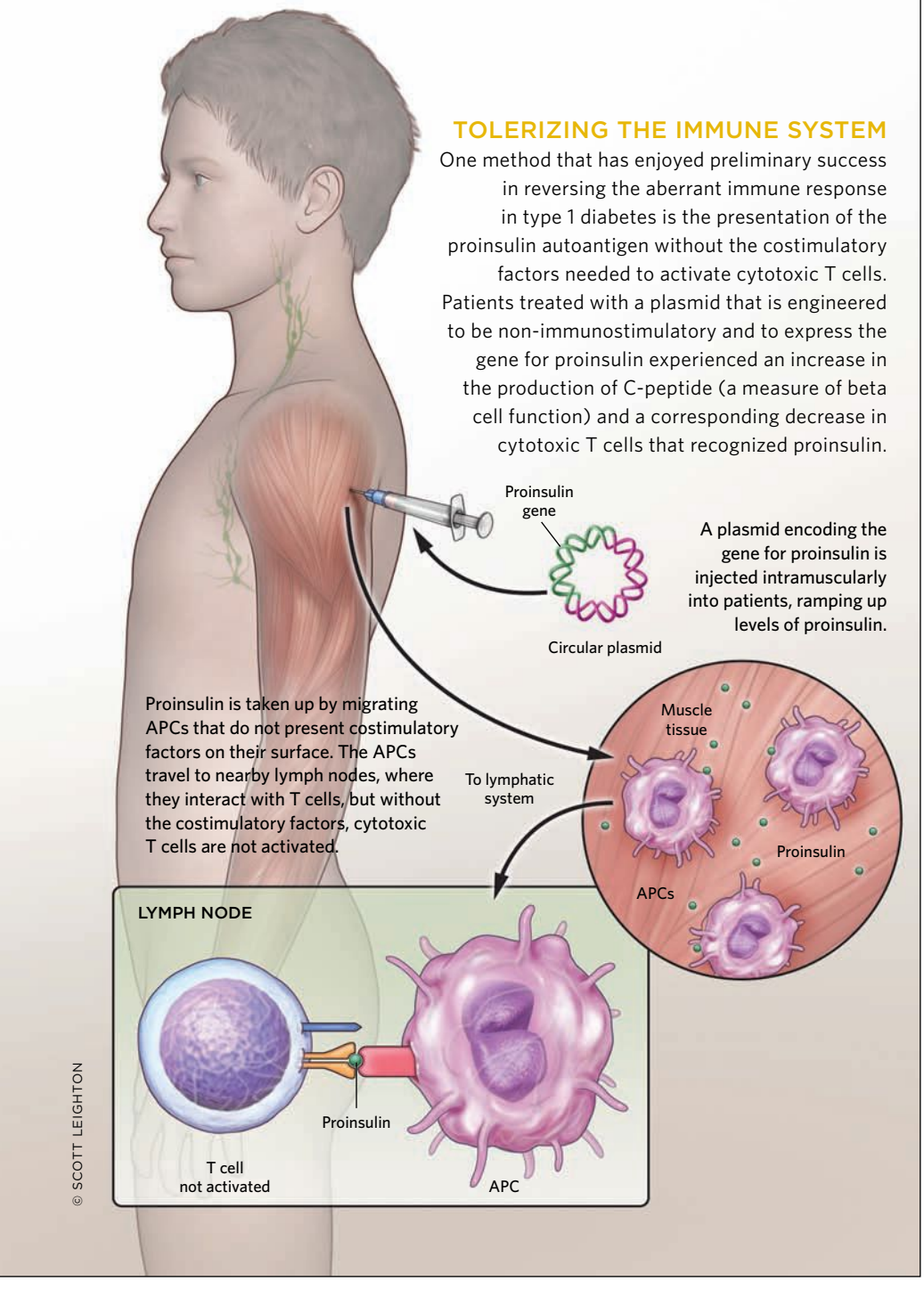
MISDIRECTED IMMUNITY

One so-called autoantigen that is attacked in type 1 diabetes is proinsulin, the precursor to insulin. When proinsulin is released into the bloodstream, antigen-presenting cells (APCs) pick up the molecules and carry them to the lymphatic system, where, alongside costimulatory factors on the APC, they activate cytotoxic T cells that migrate back to pancreatic islets and damage the beta cells.

duction of the disease-causing antibodies against acetylcholine receptors. Indeed, injection of an AChR-encoding plasmid, developed by Tolerion, reduced disease severity in an animal model of myasthenia. Other approaches involving nasal application of peptide fragments of AChR have also shown efficacy in animal models.[10] Despite such promising results, no therapies have yet entered clinical trials.

There have been a few trials of antigen-specific therapy in multiple sclerosis (MS). Specifically, my group and others have designed therapies to tolerize MS patients to various proteins of the myelin sheath that surrounds nerve axons in the brain and spinal cord and is attacked by the immune system. However, we simply do not know which of the dozen myelin proteins and several dozen lipids are the targets of the autoimmune affront.[11] Perhaps it should not be surprising that early clinical trials have yielded disappointing results. In a 267-patient, Phase 2 trial of a plasmid encoding myelin basic protein—the major protein of the brain's white matter—the reduction in brain lesions was much less than that seen with the already approved drugs for MS that quell the immune system more broadly.[12] Clearly, if one is to succeed with antigen-specific tolerance to treat autoimmune disease, one must have a solid understanding of the antigenic targets involved in the pathogenesis of the disease.

Unfortunately, these recent failures have likely added to prolonging the phar-



maceutical industry's resistance to the development of antigen-specific treatments for autoimmunity. Once again, we may take a lesson from history. Resistance to the development of Gleevec in the 1990s was fierce, and progress was slow, delaying trials for approximately five years. The drug's developer, Ciba Geigy (now Novartis), was initially reluctant to advance the drug to the clinic, with concerns ranging from Gleevec's potential toxicities to the small size of the market. These days, similar worries plague the pharmaceutical industry when presented with developing antigen-specific therapies for autoimmune diseases.

With Gleevec, its academic developers Brian Druker and Charles Sawyers fought this resistance and convinced a reluctant pharmaceutical company to proceed. Results in the clinic were stunning, with chronic myelogenous leukemia patients surviving and experiencing long-term remissions of a previously fatal disease. Once the key antigen or antigens that are the targets of autoimmunity in a particular disease have been discovered, scientists should take a similar chance on developing antigen-specific therapies for those diseases. Perhaps then they would achieve similarly stunning results. ■

Lawrence Steinman is a professor of pediatrics and neurological sciences at Stanford University. He is a founder and member of the board of directors of Tolerion, and serves on the board of directors for Atreca and the scientific advisory boards for Transparency Life Sciences, Receptos (Celgene), TG Therapeutics, Raptor, and Teva.

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The Imitation Game

Synthetic biologists create novel genetic circuits that give insight into, and are inspired by, naturally occurring gene circuits.

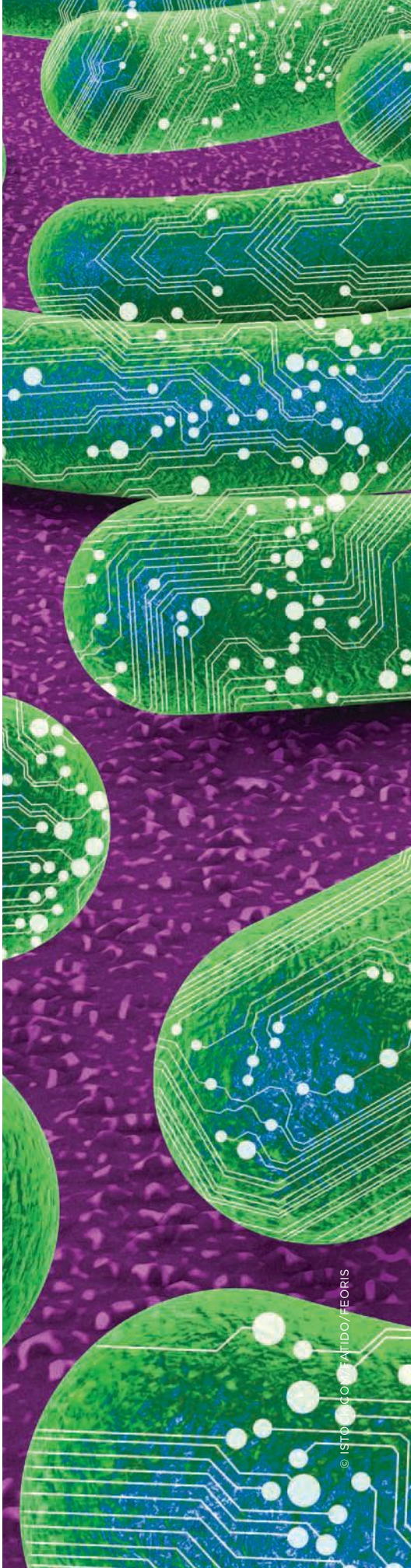
BY RICHARD A. MUSCAT

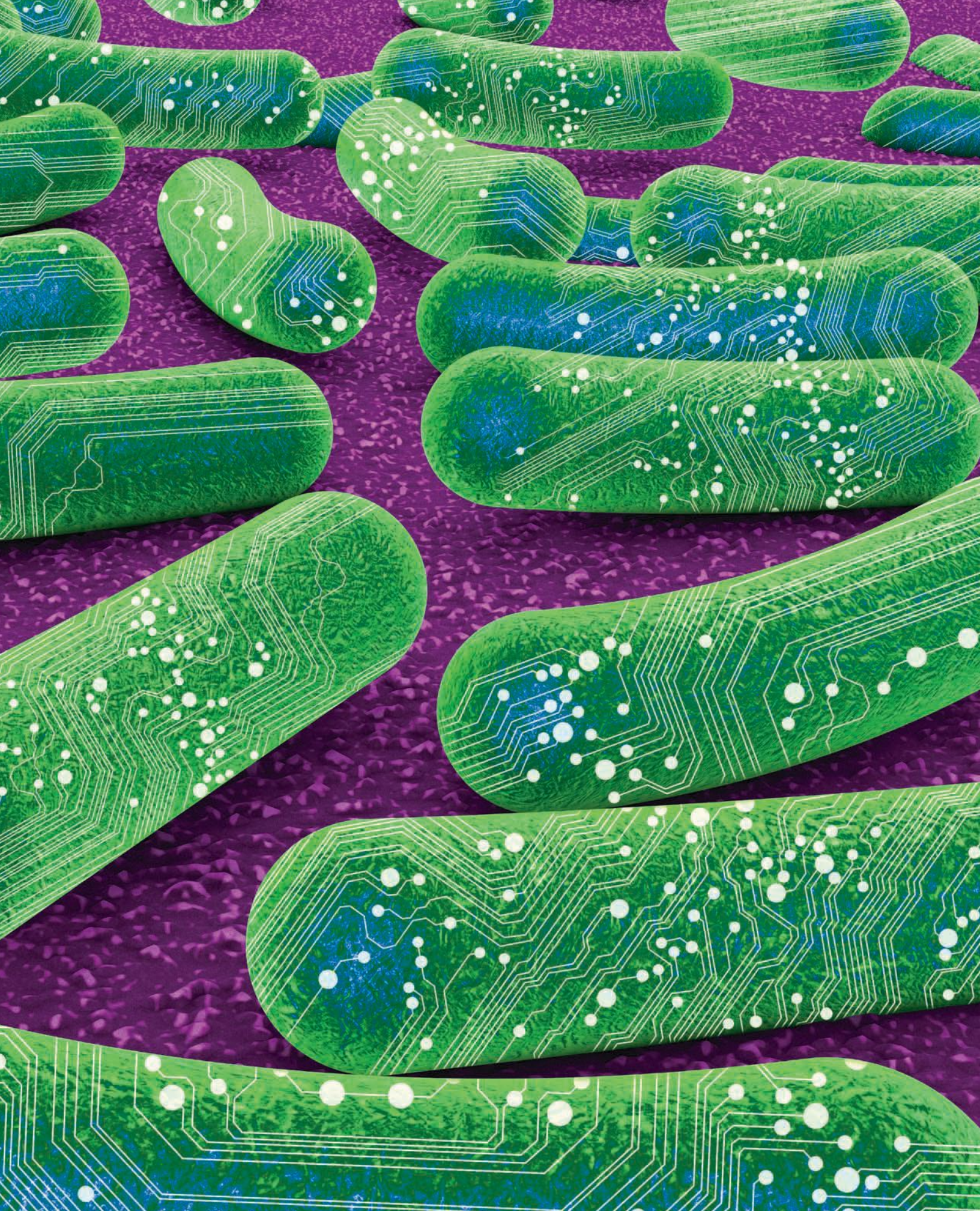
Every two hours in Matthew Bennett's Rice University lab, cyan and yellow lights flashed in synchronization. Bennett and his team had engineered 12 components to generate the coordinated oscillations. This circuit wasn't electronic, however; it was biological. Two populations of *E. coli,* each carrying a synthetic gene circuit, cycled in synchronous pulses every 14 hours.

Bennett's work, published last year in *Science,*[1] is a key application of modern synthetic biology: taking biological components and linking them together to form novel functional circuits. Instead of a program coded in Java and executed by a computer's working memory, commands were written in DNA and carried out by the microbes' cellular machinery. LEDs were replaced with fluorescent proteins, and molecular signaling cascades served as the system's wires.

Stripped back to its most basic components, a synthetic or natural biological network consists of a gene that either switches another gene on (activation) or turns it off (repression). By assembling networks from components found in nature, and harnessing these simple activation and repression interactions, synthetic biologists can engineer oscillators, sensors, counters, and computations of Boolean logic that can be expressed in bacteria, yeast, or mammalian cells, with applications ranging from biological computation to medical diagnostics and biofuel production.

The first synthetic networks were created in 2000, when researchers built an oscillator and others constructed a bistable switch in *E. coli*. In an oscilla-

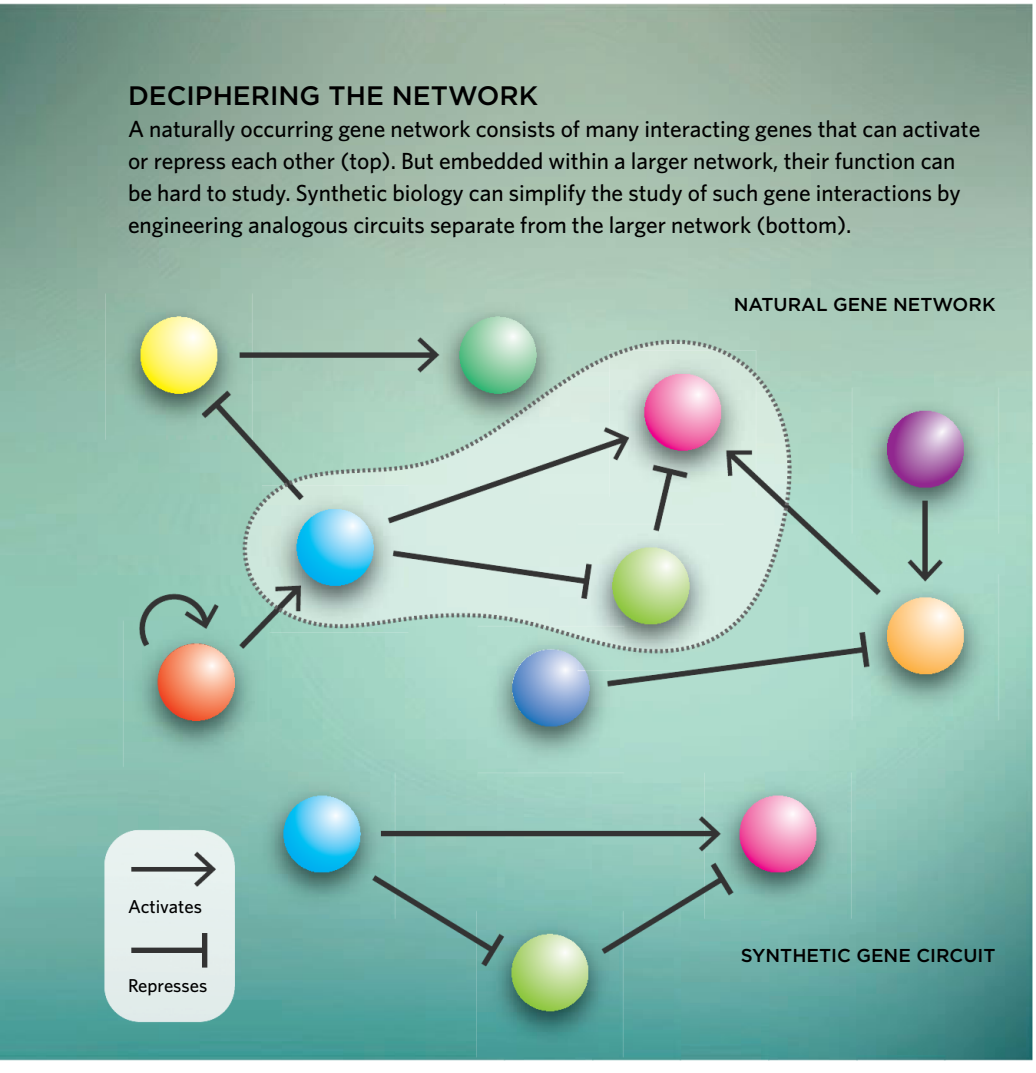




tor circuit, three genes form a cascade, in which each gene triggers the inactivation of the next gene. In the case of the landmark oscillator constructed by Rockefeller University's Stanislas Leibler, then at Princeton, and his graduate student Michael Elowitz, one of the three repressor genes was also linked to green fluorescent protein (GFP), resulting in visible pulses of light.[2] A bistable switch, on the other hand, consists of just two genes that inactivate each other. When one gene is on, the other is off. Due to variation in the expression of the active gene, the inactive gene occasionally gets the chance to switch on and suppress the expression of the first gene. Like Leibler and Elowitz, MIT bioengineer James Collins and his grad student Tim Gardner, then at Boston University, linked one of the genes with a sequence encoding GFP, and they were able to see the cells switch between states.[3] (See "Tinkering With Life," *The Scientist*, October 2011.)

Since these early studies, engineers, computer scientists, mathematicians, and physicists have been applying their expertise to engineer synthetic gene networks. In addition to supporting the creation of novel functions, synthetic networks can also give insight into how naturally occurring ones work. As physicist Richard Feynman once wrote on his office blackboard, "What I cannot create, I do not understand." Studying gene circuits in their natural context is complicated by the complex cellular environment in which they function; reconstructing and tuning gene interactions in vitro can provide a simplified model for how equivalent networks behave in nature. (See illustration on this page.)

"Naturally occurring gene oscillators, especially the circadian oscillator that regulates our daily rhythms, are hard to study," says Bennett. "We can easily make changes and fine-tune synthetic gene circuits in ways that are difficult in natural systems. Though our synthetic circuits are inherently different from their natural counterparts, we can use them to study some of the basic principles of how genes dynamically regulate each other."



Stripped back to its most basic components, a synthetic or natural biological network consists of a gene that either switches another gene on or turns it off.

Researchers at the J. Craig Venter Institute (JCVI) in San Diego have even gone so far as to create the smallest functional genome to date, a mycoplasma bacterium consisting of just 473 genes.[4] This stripped-down cell can now provide insights into what each of the genes and their respective proteins are doing to keep the organism alive.

Building man-made circuits can also lead to something entirely new, Bennett adds. "I try to find ways to engineer a new synthetic circuit that can mimic the unexplained phenomenon, even if my solution is not the same as nature's. Sometimes this leads to new insights into the natural circuit and sometimes not. Either way it's exciting."

Genetic networking

In the early 2000s, Uri Alon of the Weizmann Institute of Science in Israel and colleagues studied the connections between genes in *E. coli*, discovering common motifs, or patterns of gene connec-

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tivity. Importantly, the researchers found that these motifs occurred more often than could be expected if you took the same number of genes and randomly connected them, suggesting that biological networks have evolved these patterns.[5] After early studies demonstrated researchers' ability to create novel gene circuits, many synthetic biologists began making synthetic replicas of these natural motifs.

By isolating a subset of genes and inserting them into a new cell, synthetic biologists can assemble a motif that has little interaction with the molecular machinery of that cell; the genes are considered orthogonal. For example, viral promoters—sequences of DNA that drive gene expression—can be used to express GFP, a gene taken from jellyfish, inside mammalian cells. Modifications to promoters can allow them to be controlled by signaling molecules, not only allowing novel genes to be expressed, but giving synthetic biologists the ability to switch them on and off.

One of the simplest signaling motifs involves a gene that either activates or represses *itself*. Positive autoregulation is when a protein triggers its own expression. At first, due to the absence or very low concentration of protein, its expression is very low. After a while, however, an intermediate level of the protein builds up, speeding up the rise in expression levels. The overall effect of positive autoregulation is thus a delay before the gene reaches normal expression rates.[6] Conversely, negative regulation, when a gene inhibits its own expression, allows the fast activation of a gene upon exposure to a signaling molecule, but then slows its own production once it reaches a critical level, allowing it to rapidly reach a steady state.[7] (See illustration at right.)

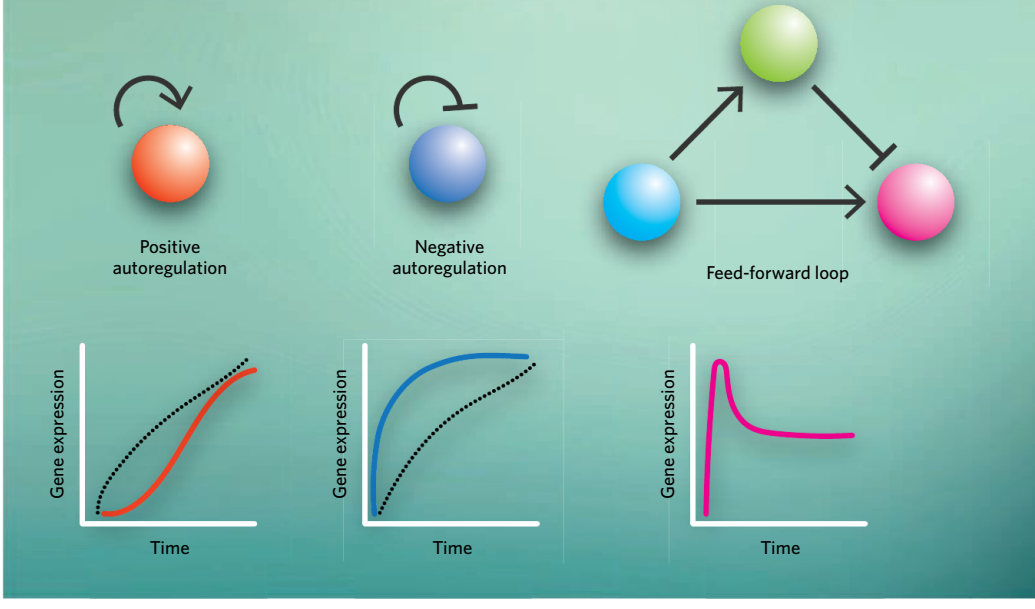
Another common motif includes the interaction of several genes forming feed-forward loops, in which one gene activates or represses the expression of another only under certain conditions. Synthetic implementation of one particular feed-forward loop has been shown to produce a pulse of gene activation—a large peak of gene expression followed by steady state expression.[8]

Autoregulation and feed-forward loops highlight how synthetic biology can create direct replicas of naturally occurring circuits to understand their function. However, synthetic biologists also have engineered a number of novel behaviors in cells: for example, different types of computation. One of the choices a synthetic biologist might make when constructing a synthetic circuit is whether to make it digital (i.e., on/off) or analog (varying levels of output). Researchers have constructed digital circuits implementing Boolean computations such as AND/OR and NOT/NOR logic with up to 10 regulators and 55 component parts in *E. coli*.[9] Of course, many genes are not expressed in a digital manner; neither completely on nor completely off, they are, rather, expressed dynamically over a range of levels. As a result, synthetic biologists are increasingly taking inspiration from nature and designing computational circuits in analog, implementing functions such as addition, subtraction, and division.[10]

More than 15 years of constructing such biological gene networks has made waves in a wide variety of scientific fields. For example, Mary Dunlop of the University of Vermont is taking advantage of feedback circuits in the design of biofuel-producing bacteria. Her group has modi-

DYNAMIC GENE EXPRESSION

A number of motifs that appear in naturally occurring networks have been reconstructed in synthetic circuits. Positive autoregulation (left) occurs when a gene is activated by its own product; this results in delayed activation. (The black dotted line provides a comparison to gene activation with no autoregulation.) Conversely, negative autoregulation occurs when a gene represses its own expression (middle), allowing its rapid activation until it reaches a steady state, and then preventing overexpression. Finally, a combination of several genes can form a motif known as a feed-forward loop (right). Depending on the way the genes are connected, activating a single gene triggers the simultaneous activation and repression of another gene, causing a pulse in expression followed by a lower steady state.



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fied *E. coli* to express biofuels such as alcohols, diesels, or jet fuels that are exported from the cell by efflux pumps. Too much biofuel accumulating in a cell is toxic, and expression of too many efflux pumps places a strain on the cell. Either of these problems can prevent cell growth and the production of more biofuel. Through mathematical simulation, Dunlop has demonstrated that a negative-feedback sensor could control the balance by delaying pump expression until it is needed, when there is enough biofuel inside the cell to necessitate pumping it out.[11]

Synthetic circuits are also showing potential as valuable tools for diagnosing and treating disease. In one study, researchers created synthetic circuits designed to detect combinations of microRNAs associated with a particular case of cervical cancer, and inserted the circuits into cancer and noncancer cell lines. Using a combination of AND and OR logic allowed the detection of specific combinations of different microRNA species only present in HeLa cells. If the right microRNA combination was detected, the synthetic circuit expressed a gene that caused the cells to die.[12]

While many technical challenges stand in the way of applying synthetic biology techniques in treating patients, a more near-term application may come in the form of paper-based diagnostics. In 2014, Collins and his colleagues at Harvard and Boston Universities developed synthetic gene circuits that function outside of cells and can be embedded in paper, which changes color through the expression of fluorescent proteins if certain markers are present in the sample. In this proof-of-concept study, the researchers showed that such paper-based diagnostics could be designed for a diverse range of applications, from glucose detection to the identification of different strains of Ebola virus, with outputs that can be seen by eye or a cheap microscope.[13] This year, the team updated the test to detect 24 RNA sequences found in the Zika genome; when a target RNA is present, a series of interactions turns the paper purple.[14] Paper-based diagnostics are easy to store through freeze-drying and to move to low-resource settings out of the lab, and researchers are now working to design such diagnostics for use in the field.

Working in tandem

The examples described so far have been of genetic circuits operating in isolation inside many identical cells or outside cells altogether. In nature, however, cells don't exist in a vacuum; rather, small signaling molecules that can be easily transmitted across cell membranes allow cells to communicate with their neighbors.

In the case of the oscillator created in 2000, each individual cell in a population of bacteria would act in isolation, with one cell oscillating out of phase from its neighbors. Ten years later, University of California, San Diego's Jeff Hasty and his colleagues used small signaling molecules that could pass out of one cell and into another to regulate the gene network within that cell. As a result, the oscillations of entire populations of bacteria were linked together and cycled in unison.[15]

Bennett's group at Rice University took this idea one step further when they made use of two interacting populations of *E. coli* carrying different genetic circuits to coordinate the long-term, stable oscillations of fluorescent protein expression. One population of bacteria acted as an activator strain while the other population acted as a repressor strain. The activator strain produced a signaling molecule that activated even more of its own signal production, triggering activation of the repressor strain. When activated, the repressor strain produced another signaling molecule that repressed both itself and the activator strain. Each strain also produced an enzyme that degraded the signaling molecules in the system, preventing the buildup of leftover signal.

"We took the circuitry of a single strain oscillator and reconfigured it so that two strains must work together to achieve the oscillations," Bennett explains. "It's similar to taking a book and giving the even pages to one person and the odd pages to another. To either individual, their portion of the book is useless. But if the two can communicate and work together, the book will make sense."

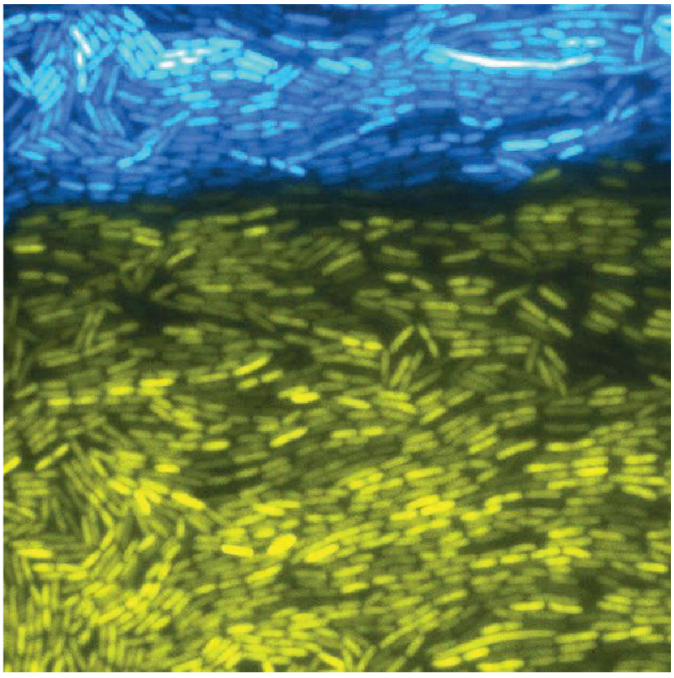
Implementing synthetic biological circuits in mixed cell populations that have coordinated behavior might illustrate ways in which complex synthetic tissues and organs could be engineered.

As each strain is activated, a fluorescent molecule is produced: cyan in the activator strain and yellow in the repressor strain. When mixed together, both populations are activated and repressed in unison, causing fluorescent oscillations over the entire cell population. When one strain is grown in isolation, no oscillations are observed.

Applying principles of basic gene motifs such as feedback loops with cell population biology can thus expand the repertoire of synthetic biologists looking to create novel genetic circuits. Likewise, implementing synthetic biological circuits in mixed cell populations that have coordinated behavior might illustrate ways in which complex synthetic tissues and organs could be engineered.

The decreasing cost of DNA synthesis and sequencing, the ability to share plasmids, the creation of databases describing genetic components, and the development of novel techniques to easily assemble and edit genomes have greatly accelerated progress in this area. As researchers engineer new genetic components, the relatively new field of synthetic biology could soon begin to bear actionable fruit, with applications that include compound synthesis, diagnostics, and even medical treatments. In addition, the design and study of synthetic systems will continue

SCIENCE, 349:986-89, 2015, COURTESY OF MATTHEW BENNETT; COURTESY OF RICHARD MUSCAT



BACTERIAL MOSAIC: Two populations of *E. coli* fluoresce yellow and cyan in unison as they activate or repress the other's expression as well as their own. (See illustration below.)

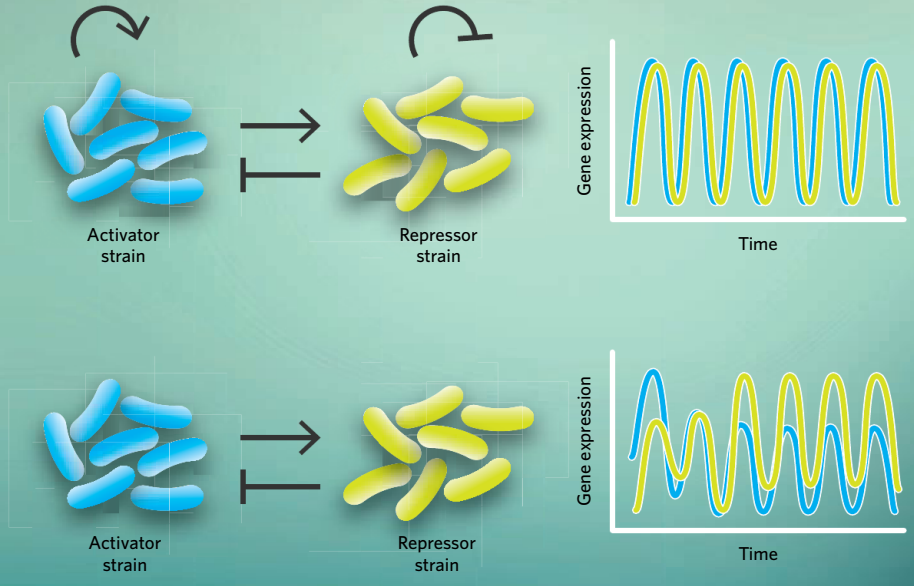
to give us a deeper understanding of the biology that exists around us.

"I take a great deal of inspiration from nature," says Bennett. "Sometimes I see a circuit that is well-characterized and wonder if we can build it just as well as nature. Other times, I look at a phenomenon in nature that is unexplained. Then I get really excited." ■

Richard A. Muscat works at the London-based Cancer Research UK, bringing together multidisciplinary teams of researchers using engineering and physical sciences to find new ways to tackle cancer.

COORDINATED OSCILLATIONS

Two populations of bacteria interact via signaling molecules to coordinate expression of fluorescent proteins. When using positive and negative autoregulation (top), the oscillations are robust as the two populations grow. The negative feedback loop of the repressor strain and the positive feedback loop of the activator strain thus reinforce oscillations; when feedback is removed from the circuit (bottom), oscillations are less coordinated and prone to failure. "You can think of feedback loops as self-correction mechanisms," says Bennett. "They are constantly assessing the current performance of the circuit and make changes if necessary."



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The Literature

CELL & MOLECULAR BIOLOGY

Alternative Energy

THE PAPER

G. Aubert et al., "The failing heart relies on ketone bodies as a fuel," *Circulation,* 133:698-705, 2016.

As organs go, the heart is an energy hog. To keep it fueled, mitochondria within cardiomyocytes (heart muscle cells) constantly churn out ATP as a product of the citric acid cycle. In the heart, most of the cycle's substrates come from the metabolism of fatty acids, but the organ can also make use of other compounds such as lactate or ketones.

When Daniel Kelly of Sanford Burnham Prebys Medical Discovery Institute in Orlando, Florida, learned that some rare genetic disorders both cause dysfunction of the heart muscle and simultaneously disrupt fatty acid oxidation and increase ketone metabolism, he wondered if ketones might play a role in heart failure. "It was kind of a genetic proof of concept that these fuel changes might really be important, rather than just innocent bystanders," he says.

During starvation, the liver makes extra ketones, allowing the brain to switch to ketones for fuel when glucose is low. To find out whether a similar process occurs early in heart failure, Kelly's team mimicked two common causes of human heart failure—heart attack and hypertension caused by aorta constriction—in mice, and performed proteomic analyses four weeks later.

They observed lowered levels of proteins that process fatty acids for energy and increased levels of a ketone-metabolizing enzyme called βOHB dehydrogenase 1 (BDH1). They also found elevated levels of three ketone metabolites, and when the team perfused excised hearts with a radioactively labeled version of the ketone βOHB, hypertrophied hearts ate up more of the ketone to produce substrates for the citric acid cycle than healthy hearts did.

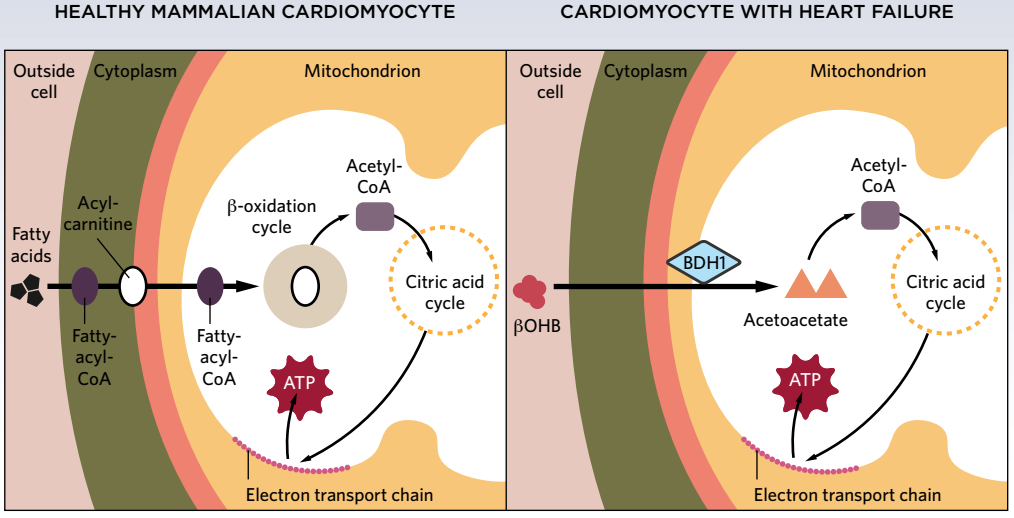
Kenneth Margulies and colleagues at the University of Pennsylvania saw similar metabolic changes in failing human hearts sampled during surgery or removed during cardiac transplantation (*Circulation,* 133:706-16, 2016). Margulies's team observed that the expression of the genes for BDH1 and other ketone-metabolizing enzymes ramped up in failing heart tissue. Although failing hearts and healthy controls (from organ donors) displayed no differences in the abundance of proteins involved in fatty acid oxidation, failing ones had lower levels of fatty acid intermediates called acylcarnitines, suggesting the hearts were not using their normal fuel source.

Both mice and humans with heart failure had increased concentrations of ketones in their serum, hinting that ketone production in the liver was increased. "This was really fascinating because that means there is a liver-heart connection," Kelly says. To find out if that's the case, he plans to study mice lacking an enzyme required for ketone production in the liver.

These mice will also be useful in discerning whether the switch to ketones is a good thing. "We don't know if it's adaptation or maladaptation," says Heinrich Taegtmeyer, a cardiologist at the University of Texas Health Science Center in Houston. Figuring that out could potentially inform ways to protect hearts in bad situations.

—Amanda B. Keener

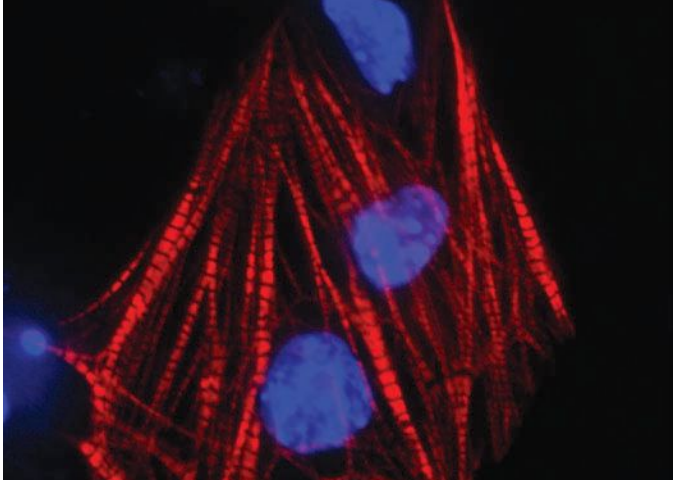
THE SCIENTIST STAFF



CHANGE-UP: Healthy cardiomyocytes (left panel) mainly use fatty acids as their energy source. To produce ATP, fatty acids are first converted into acylcarnitines, which are converted back to fatty-acyl-CoA and enter the β-oxidation cycle inside mitochondria. The resulting acetyl-CoAs then enter the citric acid cycle.

In a mouse model of heart failure and in failing human hearts (right panel), cardiomyocytes depend more on ketones for energy. The ketone βOHB enters the mitochondrion where the enzyme BDH1 converts it into acetoacetate, whose products serve as substrates for the citric acid cycle.

Both mice and humans with heart failure experience increased serum levels of βOHB, but in human cells βOHB and acylcarnitine levels go down. In mice, heart failure reduces the abundance of proteins involved in fatty acid oxidation. Both alterations suggest ketones are preferred over fatty acids in failing hearts.



TRANSFORMED: Mouse heart muscle cells derived from induced cardiovascular progenitor cells

CELL & MOLECULAR BIOLOGY

Heart Seeds

THE PAPER

Y. Zhang et al., "Expandable cardiovascular progenitor cells reprogrammed from fibroblasts," *Cell Stem Cell*, 18:368-81, 2016.

THE TRIALS

To repair cardiac damage after a heart attack, numerous clinical studies have experimented with injecting a variety of potentially therapeutic cells into patients, but very little of the introduced material sticks around. It's thought these cells act indirectly—via paracrine mechanisms—to regrow heart muscle, and the benefits have been modest at best. So Sheng Ding of the Gladstone Institute of Cardiovascular Disease and the University of California, San Francisco, has been working on another idea: produce progenitor cells that will grow into new heart tissue.

PRECURSORS

Ding's team succeeded in generating easy-to-grow progenitor cells—either from induced pluripotent stem cells or directly from fibroblasts—that could become any of three lineages in the heart: cardiomyocytes, smooth muscle, or endothelium. Injecting these cardiac precursors into mice with infarcted hearts staved off declines in heart function.

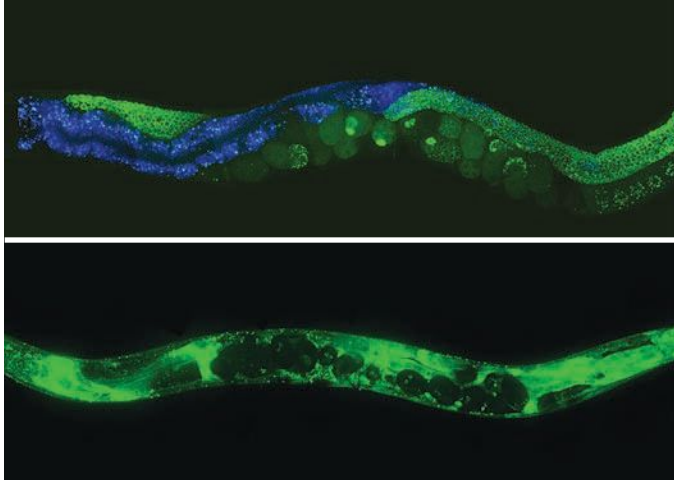
RECIPES

Lab-grown cardiac precursors have been produced before, says Christine Mummery of Leiden University Medical Center, but they've been difficult to expand or have required an activated oncogene to grow. In Ding's study, a cocktail of transcription factors did the job, and the cells proliferated readily. "It's amazing they were able to get so many [cardiomyocyte] cells" from the precursors, says Stanford University's Joseph Wu. "I think people will be encouraged there are independent ways of making cardiac progenitors that can expand," Mummery says.

NEXT STEPS

Wu calls the findings "exciting," especially the production of precursors directly from fibroblasts. He adds that more research is needed to replicate the results and see how the cells behave in humans. Ding says his group is now focused on optimizing the protocol for human cells.

—Kerry Grens



NORMAL LIFE: Top: Somatic (blue) and germline (green) cells of *C. elegans* Bottom: Mutant worms with germline factors in somatic cells are not longer lived.

CELL & MOLECULAR BIOLOGY

Past Life (Span)

THE PAPER

A.K. Knutson et al., "Reevaluation of whether a soma-to-germ-line transformation extends lifespan in *Caenorhabditis elegans*," *PNAS*, 113:3591-96, 2016.

OLDIES

Germline cells are considered immortal, because, unlike somatic cells, they can theoretically replicate indefinitely. In 2009, a study by Gary Ruvkun's lab at Harvard Medical School reported that an insulin-deficient, long-lived *Caenorhabditis elegans* mutant, *daf-2*, misexpressed genes coding for germline factors called P-granules in its somatic cells—a result the team linked back to the mutants' longevity (*Nature*, 459:1079-84).

TAKE TWO

The findings piqued the curiosity of *C. elegans* researcher and P-granule expert Susan Strome of the University of California, Santa Cruz. Interested in performing related research, she and her group set out to replicate the findings.

MISSING PIECES

Strome and colleagues searched for germline factors in *daf-2*'s somatic cells, but the researchers failed to find evidence of the proteins by immunostaining. They also didn't detect an upregulation of germline transcripts. "Certainly our RNA profiling didn't give us a sense that *daf-2* has a little bit of misexpression in somatic cells," Strome says. What's more, knocking down P-granules and other germline factors in young adult worms using RNAi showed no effect on *daf-2*'s life span.

NOT ALL WORMS ARE EQUAL

The expression results might not contradict his team's original findings, says Ruvkun; the discrepancy could have to do with life stage, given that part of the Harvard analysis focused on dauers (a spore-like developmental stage). "It's an apples and oranges thing," he says. "The induction we saw in these P-granule components was really quite striking." But as for life span, he agrees the new analysis suggests that "there isn't any evidence that germline granules are important for aging."

—Catherine Offord

YU ZHANG; SUSAN STROME, *PNAS*, 113:3591-96, 2016

PROFILE

Sweet Spot for Glycans

Seeking to discern their function, Carolyn Bertozzi opens visual windows onto complex sugars on and inside living cells.

BY ANNA AZVOLINSKY

I was one of three girls, and when we were asked in school what we wanted to be when we grew up, and other kids said doctor or fireman, we each said 'medium-energy nuclear physicist,'" laughs Carolyn Bertozzi, whose father was a professor of applied physics at MIT in Boston. "As early as I remember, he would talk about how, when we grew up, we were going to be nuclear physicists," recalls the Stanford University chemistry professor. As little kids, she and her sisters donned MIT T-shirts and attended summer day camp there. "That was the difference between us and the other kids, especially the girls. I was born in the mid-1960s at a time when women were catastrophically underrepresented in science and actively discouraged from higher education," she says. Bertozzi's own mother had put herself through secretarial school instead of attending college because "her parents were not supportive, thinking [college] was a waste of money." Bertozzi's parents sent her and her sisters—a younger sister is an occupational therapist, and an older sister, Andrea, is an applied math professor at UCLA—a clear message: "Go to college, get a PhD in science, have your own career, be independent," Bertozzi recounts.

> "I had no concept of social media until a year ago December. Every day, I am learning what you can do with that platform."

Bertozzi enjoyed high school biology, but was not particularly drawn to other sciences. She entered Harvard University in 1984, initially majoring in biology. She had been recruited to play soccer and originally wanted to major in music. "I played keyboard in jazz and heavy metal bands." But Bertozzi had also followed in her older sister's footsteps, joining math teams in junior high and high school. "I mostly did whatever my sister did, because I didn't have a better idea," she says.

Thinking she might go to medical school, Bertozzi took the required organic chemistry course as a sophomore and something clicked: "Organic chemistry turned out to be my thing. I loved how you could see the three-dimensional molecules—their shapes and behaviors—and that there were just simple, beautiful core principles that let you rationalize really complicated sequences of events. I loved that you could look at molecules and predict what would happen, and when your predictions were good, you could engineer and make new molecules no one had made before." Bertozzi switched to a major in chemistry and has never looked back.

Here, she talks about how to get over a fear of technology, how she made cold calls to learn biology from experts, and the gut feeling that helped her avoid bad career advice.

BERTOZZI BLOSSOMS

No fear. At Harvard, Bertozzi did undergraduate research in a physical chemistry lab, where she constructed a photoacoustic calorimeter—an instrument that measures heat deposition into a solution following the photoexcitation of molecules. She was accepted into a graduate research program for women at Bell Labs in New Jersey that included a pregraduate internship there and a PhD fellowship award to pursue a graduate degree at an institution of her choice. At Bell, Bertozzi studied the kinetics of electron transfer on various surfaces with Christopher Chidsey, now her colleague at Stanford. "I learned that you didn't need to be intimidated by instruments. I became fearless about taking a wrench to a back panel and digging in the electronics. I also learned the fundamental way instruments work, no matter the instrument: how you set it up to initiate a process, collect a readout, digitize it into data you can analyze," she says.

Going out west. Bertozzi grew up in Lexington, Massachusetts, and had never been on an airplane until her West Coast tour of schools—Caltech, Stanford, and the University of California, Berkeley—after she had already been accepted into all three graduate chemistry programs. The trip won her over to the Golden State: "It seemed so foreign to me: the landscape where you can see for miles, the smells, the weather, the architecture. My student host [at Berkeley] picked me up from my hotel on a motorcycle and we drove over the Bay Bridge to a club in San Francisco. That really wowed me." As she began her graduate career at Berkeley in 1988, Bertozzi finally got to pursue research in organic chemistry, the subject she had fallen in love with in college. The chemistry department at Berkeley was just beginning to develop the field that is now called chemical biology, then known as bio-organic chemistry.

She chose to work with Mark Bednarski, who had recently set up his own lab, and began to synthesize stable analogs of carbohydrates that interacted with bacterial and viral receptors that promote immune attack. Because the chemistry department had little crosstalk with the life-sciences departments, Bertozzi and her labmates made cold calls to immunology labs to get advice on biology techniques and concepts. "This was before the Internet. You couldn't easily search for people on this big campus, so we would just go floor by floor in a building looking for immunologists." During her third year, her advisor was

LINDA A. CICERO/STANFORD NEWS SERVICE

CAROLYN BERTOZZI

Anne T. and Robert M. Bass Professor of Chemistry
Stanford University
Investigator, Howard Hughes Medical Institute

Greatest Hits

- Introduced the concept of metabolic engineering for imaging glycans in cells, zebrafish embryos, and mice without disturbing natural functions
- Founded the field of bio-orthogonal chemistry, which has led to many enabling technologies within and beyond the field of glycobiology, including methods for site-specific antibody-drug conjugation that have been translated to commercial settings
- Invented a nanoscale cell-injection system, using carbon nanotubes to deliver molecules into cells
- Developed a new method for point-of-care diagnosis of tuberculosis
- Discovered new avenues for cancer immune therapy that target tumor-specific glycosignatures

diagnosed with colon cancer, decided to leave his faculty position, and enrolled in medical school. Bertozzi and two other students "convinced the department that we should be left to finish our projects as we wished. There was money in the bank from Mark's grants and we were able to spend those resources down," she recalls. Most of Bertozzi's papers were completed during this time, including work showing that the galactosphingolipid analogs she had synthesized bound to the gp120 protein of HIV-1. "It was an unusual experience. At the time it was like, 'Poor us, our boss quit,' but now I think back and realize how much I developed during that period and what a perspective it gave me on what is important in life."

Biology 101. As she was wrapping up her PhD, Bertozzi reasoned that, to have an impact on the field of biology, she needed to be at the leading edge of biology, "not just reading about it from a distance." At the time, in 1992, researchers had just discovered the selectin family of adhesion receptors, molecules found on the surface of endothelial cells and lymphocytes that direct immune cells to sites of tissue damage to generate inflammation. The finding had pushed glycobiology from a small, niche research topic into the mainstream, says Bertozzi, as the selectins were host targets for developing anti-inflammatory drugs. Bertozzi seized the opportunity to sell her chemistry skills to a selectin researcher as an entrée into a biology laboratory. In the 1990s, chemists joining biology labs were still a rare phenomenon. "To have a carbohydrate synthesis chemist apply for a postdoctoral position in a cell biology lab was considered truly bizarre," she says. Bertozzi joined the University of California, San Francisco (UCSF), lab of Steven Rosen, one of the few biologists who even granted her an interview.

Bootleg research. Bertozzi proposed synthesizing putative glycans that could bind to L-selectin to help identify the molecule's natural ligands. Rosen's lab knew a few generalities about ligands that could bind to the receptor. Using radiolabeling, the lab had figured out that the L-selectin–interacting glycoprotein was sulfated, had sialic acid side chains, and also fucose. But the details of the carbohydrate structures were still beyond their reach and "it was clear that the carbohydrate was the business part of this glycoprotein," Bertozzi explains. It was a race to figure out what the exact sugar molecules were. "I didn't realize how competitive the field was." Rosen wanted her to get started right away, so she began to make potential carbohydrate ligands while still completing her PhD. In 1994, after joining Rosen's lab, Bertozzi published a paper identifying GlyCAM-1 as one of the ligands for L-selectin.

BUSY BERTOZZI

Quick transition. Only one year into her postdoc, Chidsey, Bertozzi's former Bell advisor who had recently joined the chemistry faculty at Stanford University, convinced her to apply there for a faculty position as a chemical biologist. She found out Berkeley was also looking to hire and applied there as well. "I think Steve [Rosen] thought I was out of my mind. In immunology, a postdoc is often a six year commitment and there I was, a year into it, with no papers, talking about faculty jobs! It was unusual for biology but not for chemistry," says Bertozzi, who received offers from both universities and chose to return to Berkeley.

Building new tools. At Berkeley, Bertozzi's research took many new directions while continuing to focus on enzymes that produce L-selectin ligands. But her lab became most known for figuring out how to image cell-surface glycans using what is now called bio-orthogonal chemistry—a term coined by Bertozzi to describe chemical reactions that neither interact nor interfere with the biological system under study. The idea to build tools to visualize cell-surface glycans came to her while in Rosen's lab. Studying glycosylation on the surface of cells, the lab had no way to know whether certain glycans were expressed or changed their abundance under different conditions. "Meanwhile, there were genetic reporter systems like GFP fusions that enabled imaging of proteins in cells. I remember thinking it would be so great to image sugars like that," says Bertozzi.

Bertozzi's lab went on to develop such methods. First her team demonstrated the ability to engineer sialic acids on cell-surface glycans with chemical handles that allowed the addition of affinity probes such as biotin or fluorescent probes for imaging, and also held promise for such applications as cancer-drug targeting. Then the lab developed the first truly bio-orthogonal reaction: the Staudinger ligation, which incorporated azides—functional groups that are biologically inert—into cells in living mice. Because the initial reaction was rather slow under physiological conditions, the lab then developed a biocompatible copper-free azide-alkyne cycloaddition chemistry, a reaction now often referred to as copper-free click chemistry. The inspiration for the reaction came to her while she was on a plane writing a lecture for a sophomore organic chemistry class, says Bertozzi. The lab has since used the technology to visualize the expression and trafficking of cell surface glycans in vivo during zebrafish embryonic development.

Clinical applications. Bertozzi's lab also works on *Mycobacterium tuberculosis*, and has shown that the bacterium produces sulfated molecules that modulate its interaction with human host cells. "This tuberculosis project crossed paths with a sugar-imaging study. We figured out that we could use metabolic labeling as a potential point-of-care diagnostic for detection of tuberculosis in patient sputum samples. One of my students is going to Africa to do field testing of the method we developed." The lab has also begun to study how glycans and glycoproteins are perturbed in tumors. In 2014, in collaboration with Valerie Weaver's lab at UCSF, Bertozzi's group showed that the glycoprotein and polysaccharide coating on epithelial cells—called the glycocalyx—increases in thickness and stiffness as a tumor evolves, and that this physical alteration enhances cancer cell fitness.

BERTOZZI BROADCASTS

A recent move. After 19 years at Berkeley, Bertozzi moved to Stanford in 2015 to help create an institute named ChEM-H which denotes Chemistry, Engineering, and Medicine for Human Health. Bertozzi is one of 20 new faculty who will ultimately be hired for the integrated, interdisciplinary center. "I am now equally comfortable at tuberculosis and immunology conferences as at chemistry ones. I love the human health–centered environment here," she says.

Boys club. "In the 1980s, the chemistry community, especially the organic chemistry community, was not particularly inclusive of women. Organic chemistry has its own unique culture and was dominated by large labs with influential professors who didn't think women could contribute in a meaningful way. That was the sense I got as an undergraduate. Graduate classes were about 10 percent women. But at Berkeley, as a student, I thought that I at least had a shot. It was a bigger department and graduate program, so even though women were no better represented as a proportion, at least the absolute numbers were higher."

Bad advice. "When deciding on a postdoc, I was getting advice from other senior professors, since my advisor was gone. Their message was, 'If you want to be a professor yourself or work in a top company, you will need brand-name faculty backing you up.' They gave me a list of people it would be prudent for me to work for. That was the counseling I got: If I wanted a faculty position working at the interface of chemistry and biology, I had better go work for someone who had a track record of placing people in top academic jobs. This gave me a queasy feeling in my stomach. I remember thinking, 'Why should some professor I have never met have that much power over my life? That doesn't seem right. Shouldn't I have that power over my life?' It seemed strange to choose an advisor on that basis rather than, say, based on how excited I was about their scientific interests. When I thought about the problems those suggested labs were working on, I just couldn't get excited about them, at least not as much as I was about the discovery of the selectins."

A voice on social media. Since becoming the editor in chief of the American Chemical Society's new journal *ACS Central Science*, Bertozzi has been active on Twitter with a goal of elevating the visibility of chemistry "beyond the boundaries of the chemistry community," she explains. "I had no concept of [social media] until a year ago December. Every day, I am learning what you can do with that platform. I started following other journals and was never so up to date on the literature in my life. I had to write an editorial for our April issue on the status of women faculty in chemistry departments and asked on Twitter what I should discuss. Tons of people sent suggestions, so now I am basically crowdsourcing my editorial!" ■

Thirumala-Devi Kanneganti: Immersed in Immunology

Department of Immunology, St. Jude Children's Research Hospital. Age: 43

BY KAREN ZUSI

Thirumala-Devi Kanneganti became fascinated by disease when she was a high school student in India. "I always used to think, 'Why do some people get diseases?'" she says. "In tropical countries, we are exposed to all of these bacterial and viral infections. Seeing these infectious diseases spreading, it was always: 'Why some, and why not all?'"

As an undergraduate at Kakatiya University in Warangal, Kanneganti triple-majored in zoology, botany, and chemistry, seeking answers. She went on to earn her master's and PhD in microbiology and immunology from Osmania University in Hyderabad, but the pace of research in India frustrated her. "I used to wait for antibodies for two months," she remembers. "I couldn't stand that."

Kanneganti seized the opportunity for a postdoc position in fungal genetics at the University of Wisconsin–Madison in 2001, and brought her husband and daughter to the United States. "It was my dream to do good science," she says. "That's the only reason we came."

In 2007, after she'd completed two more postdocs, a listing in *Science* for a position at St. Jude Children's Research Hospital in Memphis, Tennessee, caught her attention. When she saw Nobel Laureate Peter Doherty's name in the hospital's department of immunology, Kanneganti says, she had to check it out. After she interviewed, Doherty gave her a copy of his book *The Beginner's Guide to Winning the Nobel Prize*. "He signed it, saying 'You have to come to Memphis,'" she recalls.

"What impressed me so much was her real desire to get down and do the work," says Doherty. "I thought she was something special."

Within a month, St. Jude hired Kanneganti as a researcher, and she zeroed in on genetic mutations in the murine innate immune system. "She has made major discoveries in understanding how foreign DNA is sensed in the cell, and how this and other products of infection trigger inflammation," writes Douglas Green, chair of the department of immunology at St. Jude, in an email to *The Scientist*.

Kanneganti's team has generated multiple disease models in mice, probing the function and development of inflammasomes. In 2013, she demonstrated that the cytokines interleukin (IL)-1α and IL-1β regulate inflammation through different pathways, and that blocking a kinase that ramps up IL-1α production could prevent mice from developing a version of the skin disorder neutrophilic dermatosis.[1]

The following year, Kanneganti linked changes in the composition of intestinal bacteria in mice to the likelihood of genetically susceptible individuals developing an autoinflammatory bone disorder.[2] Dietary changes affected both gut community composition and production of IL-1β in neutrophils, providing a possible mechanistic explanation.

In 2015, Kanneganti and her colleagues published work exploring the role of the protein AIM2 in colorectal cancer. The protein was initially found to be a tumor suppressor in human melanoma cells, but Kanneganti's lab discovered that AIM2 protected mice from colon tumor formation, both by controlling intestinal stem cell populations and by helping maintain the gut microbiota.[3]

"Those basic questions that I used to have all the time, these are the questions that I'm still trying to solve," says Kanneganti.

"It's been a great pleasure to watch Thiru achieve what she has," says Doherty. "We all look with a certain amount of wonder to see what she's going to do next." ■

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Driving Tests

Researchers are developing potent ways to fuel and control the movement of micromotor devices.

BY JYOTI MADHUSOODANAN

In the 1966 film *Fantastic Voyage*, Czech scientist and defector Jan Benes discovers a way to miniaturize matter, enabling his colleagues to navigate a pint-size submarine through his blood vessels and into his own brain to destroy a lethal blood clot. Today, this sci-fi gem is edging closer to reality. With the help of microfabrication, researchers are beginning to learn how to deploy tiny, cellular-scale machines into biological systems.

Micromotors of all shapes and sizes are being developed to sense environmental toxins in air or water, deliver drugs to target tissues, and perform surgical procedures at the single-cell level. What complicates their use in living organisms or cell-culture systems, however, is that their tiny size leaves them struggling against fluid forces. While a large ship at sea can weather the waves, a smaller rowboat might struggle to stay on course. As micromotors grow smaller, blood or viscous cell-culture fluids make it difficult for the motors to control their movements. To counter the viscosity, many efforts begin with propulsion systems that prove toxic to living cells.

"It's easier to move micromotors in vitro because you can use any kind of fuel," says Joe Wang, a nanomaterials researcher at the University of California, San Diego. "In vivo, it's much more challenging."

Here, *The Scientist* explores four strategies for making micromotors biocompatible and getting them in shape for real-life voyages.

SPEEDY SHELLS

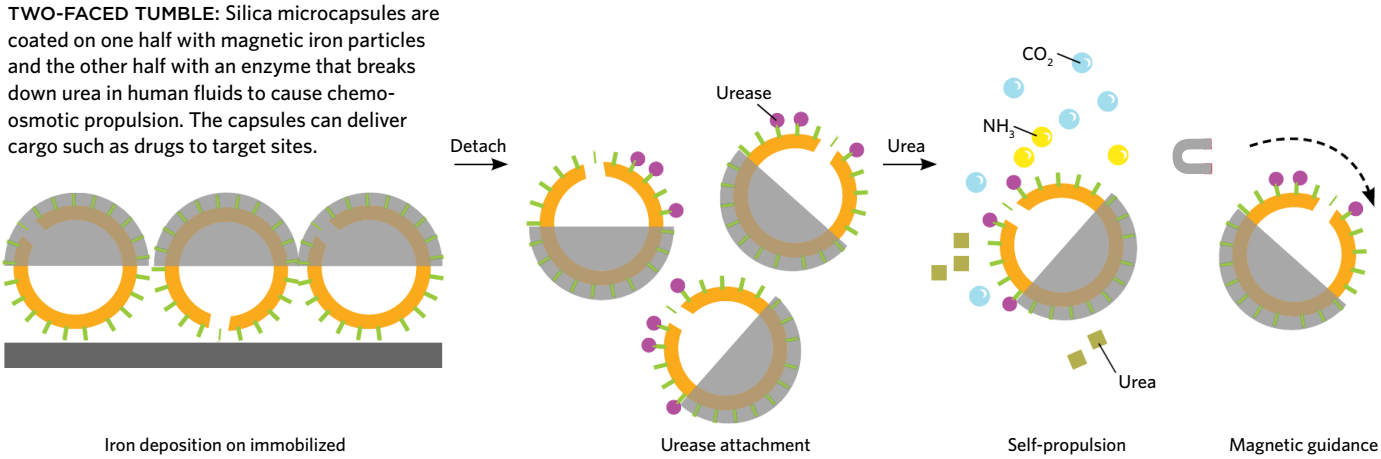
INVESTIGATOR: Samuel Sánchez, Group Leader, Smart Nano-Bio Devices, Max Planck Institute for Intelligent Systems, Germany

PROJECT: Urea-powered microcapsules for controllable drug delivery

PROBLEM: Micromotors need some sort of propellant; one example utilizes the enzyme catalase, which acts on hydrogen peroxide, to generate fast-flowing bubbles of oxygen that push the capsules forward. But catalase, and most other currently used forms of propulsion, are not safe for living cells. Sánchez and his colleagues wanted to devise a nontoxic propulsion system that could ultimately be used safely in vivo.

SOLUTION: The researchers set out to fuel the tiny machines with common metabolites such as glucose and urea. They synthesized the machine bodies—hollow silica balls 2.3 µm in diameter—and immobilized them in a monolayer on a solid surface to deposit iron onto one-half of their outer surfaces. Then they coated the other half of the balls with urease, an enzyme that decomposes urea present in blood or cell-culture liquids to produce carbon dioxide. The iron in the metal layer enabled the team to use magnets to steer these two-faced shells, commonly known as Janus microparticles. The enzyme-coated half powered chemo-osmotic propulsion by producing carbon dioxide and ammonia from urea. Adding urease activators or inhibitors turned the microparticles on or off, and could also control the rate of the enzymatic reaction to regulate the particles' speed. Human blood typically

TWO-FACED TUMBLE: Silica microcapsules are coated on one half with magnetic iron particles and the other half with an enzyme that breaks down urea in human fluids to cause chemo-osmotic propulsion. The capsules can deliver cargo such as drugs to target sites.



REDRAWN FROM SANCHEZ ET AL., *ACS NANO*, DOI:10.1021/ACSNANO.5B08067

contains a 2 mM to 7 mM concentration of urea. These microparticles move at an average velocity of 3 microns/sec at urea concentrations as low as 0.5 mM, making them likely to remain active in the bloodstream (*ACS Nano*, doi:10.1021/acsnano.5b08067, 2016).

The porous silica shells can carry small drug molecules in their surface pores, while their hollow interiors can be loaded with additional cargo such as therapeutic nanoparticles, says Sánchez. "In the last 5 to 10 years, many studies have used these kinds of structures for passive drug delivery," he adds. "Here we use it as an active drug-delivery system."

Since the shells have been shown to work with urea as a fuel source, scientists can now explore other options, says Sánchez. He aims to create a library of enzymes that work on different metabolites under physiological conditions, so researchers can pick particles based on the fuel sources available in different tissues or disease conditions.

POINTERS FROM PLANTS

INVESTIGATOR: Sarvesh Srivastava, postdoctoral researcher in materials chemistry, Institute for Integrative Nanosciences, IFW Dresden, Germany

Maneuvering a microstructure in liquid is not simple, and being able to drill [into a single cell] is another step forward.

—Sarvesh Srivastava, Institute for Integrative Nanosciences, IFW Dresden

PROJECT: Cellular surgery using plant-derived microneedles powered by a magnetic field

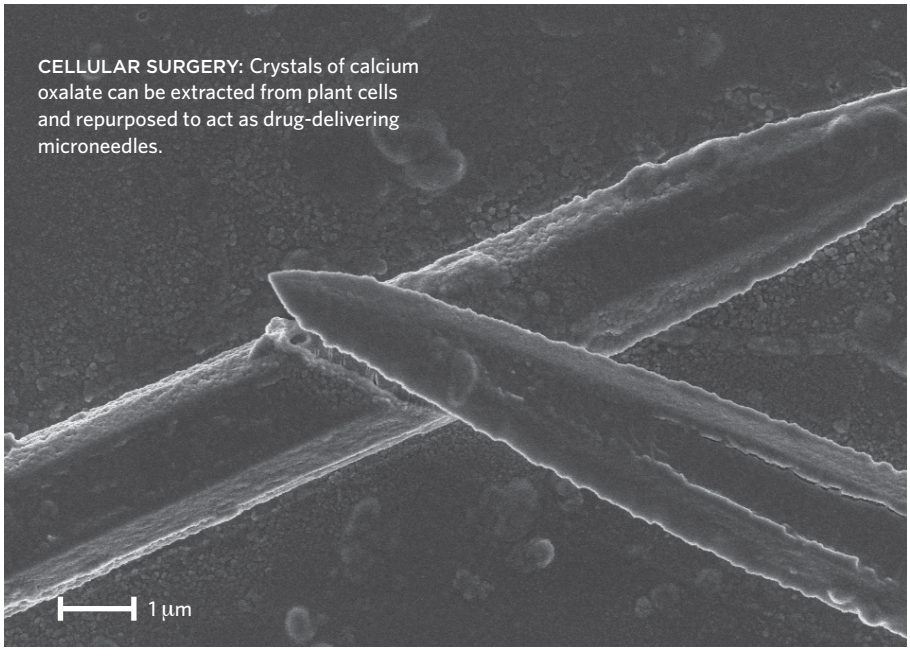
PROBLEM: Microscopic structures in plants, microbes, or animals often have properties such as low toxicity, adsorbent surface chemistry, or unique shapes that make them ideal materials for micromotors. But the precise three-dimensional nature of these structures, as well as their functions, have not yet been recreated in the lab. Rather than trying to imitate nature, Srivastava and his colleagues in Oliver Schmidt's lab wanted to use plant-derived materials to create maneuverable micromachines.

SOLUTION: The group extracted polygonal, needlelike crystals of calcium oxalate known as raphides from the leaves of *Dracaena marginata*, a common houseplant. These crystals, used primarily as a defense mechanism against herbivores, are abundant in many plants, where they reside within specialized plant cells known as idioblasts.

The researchers coated the 40–60-µm-long *Dracaena* raphides with an iron-titanium mix to make them magnetic, and then incubated the crystals with the anticancer drug camptothecin for three days. The needles' chalky material absorbed drug molecules through the iron-titanium, while their "broadsword-like" edges were able to penetrate cells. When incubated with HeLa cells under a rotating external magnetic field, the microneedles drilled through cells' plasma membranes and killed cells in culture. When drug-infused raphides were incubated with cells without the drilling action, the particles also released camptothecin to reduce proliferation and kill cancer cells (*Adv Mater*, 28:832-37, 2016). But the drilling action helps speed drug delivery, and the magnetic field can also help position the micromachines to regions where drugs can be more precisely diffused into tissues. "Maneuvering a microstructure in liquid is not simple, and being able to drill [into a single cell] is another step forward," Srivastava says.

The plant-derived microneedles have not been tested in animal models, but appear to be nontoxic in cell cultures. The coated raphides moved through cell culture media without the need for a surfactant to reduce surface tension.

Such structures have never been successfully synthesized in vitro, but they can be extracted from biological material in large amounts quickly and easily. A key step is removing the sticky gel-like matrix that surrounds the raphides inside plant cells. The enzyme cocktail used to do so must be customized to different plant species or sources of material, Srivastava says. For their study, the authors used a combination of commercial plant protoplast digest solutions and lab-made mixes.



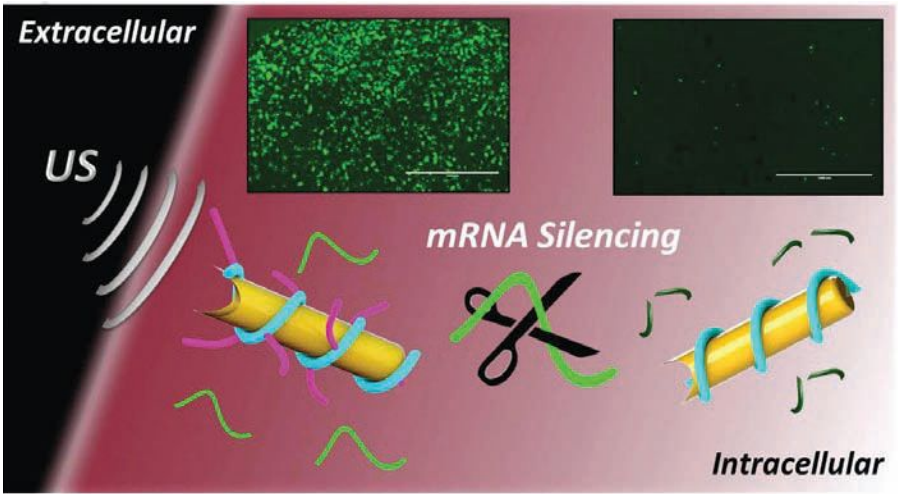
CELLULAR SURGERY: Crystals of calcium oxalate can be extracted from plant cells and repurposed to act as drug-delivering microneedles.

SCHMIDT ET AL., *ADV MATER*, DOI: 10.1002/ADMA.201504327

SHOOTING TO SILENCE

INVESTIGATOR: Yi Chen, assistant professor of nanoengineering, University of California, San Diego

PROJECT: Gold nanowires for injecting short interfering RNAs (siRNA) directly into target cells



SOUND STRATEGY: Gold nanowires encased in siRNA pierce cell membranes and, when activated by ultrasound, release siRNA to silence messenger RNA signals.

PROBLEM: In theory, siRNA targeted to selective stretches of mRNA can therapeutically repress protein levels. But siRNA knockdown is often inefficient and nonspecific, in part because of the lack of appropriate delivery systems to administer the molecules. "Right now the challenge is still how to get siRNA safely and effectively into targeted cells," Chen says.

SOLUTION: Most methods rely on cell membrane fusion or receptor-mediated uptake to transport siRNA into a cell, but Chen and his colleagues turned to nanomotors instead. They engineered a gold nanowire-based structure that pierces and enters the cell. In previous studies, his colleagues and others propelled similar nanowires inside cells to deliver plasmid DNA or to monitor the expression of microRNA (*ACS Nano*, 9:6756-64, 2015).

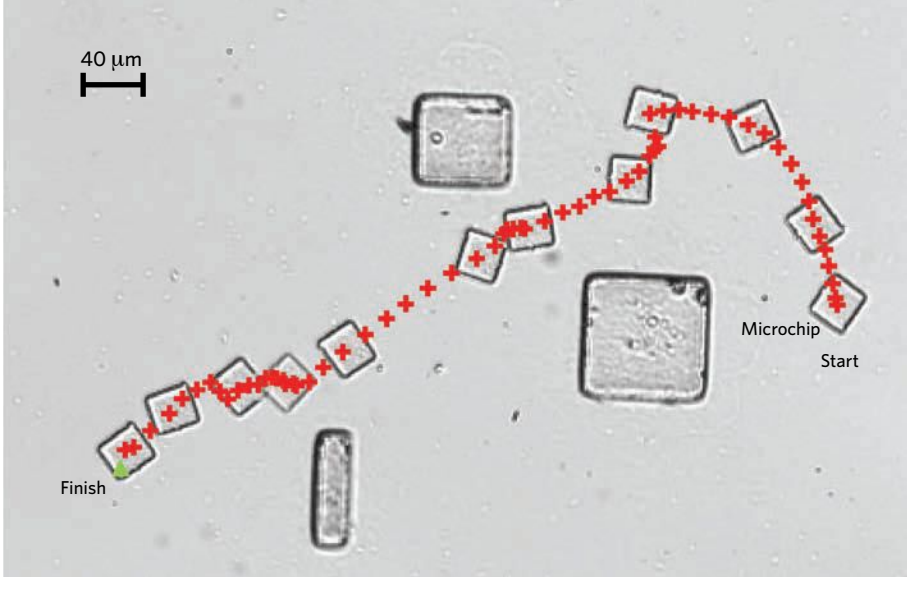
To turn the nanowires into siRNA delivery machines, Chen's group began by coiling a repetitive DNA template around 4-µm-long gold wires. This DNA strand was complementary to the DNA overhang on the siRNA it would be carrying, and because each overhang is just 20–22 nucleotides long, the DNA strand could anchor several copies of siRNA onto a single wire to increase the efficiency of uptake. These DNA-RNA coated nanowires, the researchers found, were 21 times more efficient than a solution of the same DNA-siRNA loops at knocking down green fluorescent protein expressed in mammalian cell lines in vitro. Using ultrasound to propel the nanowires made them even more effective: with a 2.66 MHz frequency applied for five minutes, the structures penetrated cells with an efficiency of 74 percent, compared with 20 percent when the nanowires were static. Most cells remained alive after the treatment. Ultrasound helped control the power and direction of nanomotor movement, Chen says.

Normally, siRNAs delivered via passive systems require a few hours just to enter cells because they must be absorbed via an active vesicular transport system. Then, each must be incorporated into a RNA-induced silencing complex with an Argonaute protein in order to exert its effects. But the ultrasound-propelled nanowires enter within minutes and reach peak activity within five hours. An overnight incubation leads to a more than fourfold enhancement in silencing, which Chen suspects might be due to the wires moving around within cells. "As a platform this can accommodate many conditions," Chen says. "We don't see any huge limitations."

Now, the team is working to further boost the nanowires' efficiency, testing how tweaks to their size and shape or the length of DNA coiled around them affect their performance. Results so far suggest that the nanowires work best when the DNA strand anchoring siRNAs is somewhat shorter than the ends of the nanowire itself, to ensure the entire payload is delivered to cells.

BACTERIAL BOOST

INVESTIGATOR: MinJun Kim, professor of mechanical engineering and mechanics, Drexel University



MICROBOT NAVIGATORS: In this time-lapse photo, a microchip, powered by bacterial flagella, relies on an algorithm to avoid obstacles and move to its destination

CHEN ET AL., *ACS NANO* DOI: 10.1021/ACSNANO.6B01415; KIM ET AL., DREXEL UNIVERSITY

PROJECT: Bacteria-powered microrobots to navigate lab-on-a-chip devices

PROBLEM: The viscous forces that impede the movement of synthetic micromotors are the same ones that motile bacteria rely on to propel themselves forward. Kim's previous work showed that it was possible to harness bacterial motility to power synthetic microbots, but he also had to devise a way to prevent the bacteria from bumping into obstacles as they swam through fluids so that they could more effectively drive the device.

SOLUTION: Kim and his colleagues coated microchips with *Serratia marcescens*, a species that uses whip-like flagella to move. Bacteria carry a negative charge on their surface, so the team could alter the microbes' direction or speed of movement by manipulating electric fields. But the system is also very susceptible to interference caused by nearby nonspecific external electric fields or insulators, so the researchers devised an algorithm that accounts for such distortions and allows the bacteria-powered microrobots to navigate a miniature obstacle course without crashing (*IEEE Trans Robot*, doi:10.1109/TRO.2015.2504370, 2016). The robots are scalable and can be adapted to use many different kinds of bacteria, such as *E. coli* or *Salmonella* species, Kim says. The species selected depends on the purpose of the microrobot, its size, and the properties of the fluid environment. Using magnetic particles instead of microchips would offer another way to control the robots.

The viscous forces that impede the movement of synthetic micromotors are the same ones that motile bacteria rely on to propel themselves forward.

Because the bacterial coating is likely to trigger immune reactions, these devices probably won't be used in vivo. But in vitro, their advantage over other forms of micromotor propulsion lies in the microbes' ability to act as sensors. Some bacterial species swarm toward light or specific chemicals, so they can be used to sense different phenotypes or cell types within lab-on-a-chip devices. "We can directly use these bacteria-powered microrobots for sensing cancer or other diseases," Kim says.

Such bacteria-driven machines have potential uses in many tasks that involve delivering targets from one point to another on the microscale, from maneuvering cells into specific positions within microfluidic devices to assembling and disassembling microscale machinery. ■

Pathogen Busters

Proteomics techniques enhance selection of antigens for vaccine development.

BY WUDAN YAN

STOPPING SCOURGES: Colored transmission electron micrographs of Zika virus (blue spheres, left) and Ebola virus (blue filaments and spheres, right)

COMPOSITE IMAGE: ISTOCK.COM/LOLON, NIAID, CDC/CYNTHIA GOLDSMITH

Ebola. Zika. Two foreign words with instant name recognition. As the extremely contagious—and highly deadly—hemorrhagic fever caused by the Ebola virus was ravaging West Africa, the more insidious Zika virus was beginning to infect people in Brazil, causing many infants whose mothers contracted the virus during pregnancy to be born with severe neurological damage. Even though the Ebola epidemic has waned, cases are still being reported. And the onset of summer in the Northern Hemisphere has brought fears of a widespread mosquito-borne Zika epidemic to a fever pitch.

These two epidemics underscore the pressing need for vaccines and other therapeutics to protect against and treat infections with viruses such as Ebola and Zika, as well as a host of other pathogens, some of which are increasingly antibiotic- and drug-resistant. But developing a vaccine against an infectious agent—be it a bacterium, a virus, or a parasite—is not simple: the human immune response itself is complex, and the more genes an infectious agent has and the more readily it mutates, the more challenging it can be for researchers to develop an effective vaccine against the pathogen.

When a disease-causing microorganism enters the human body, it first elicits an innate immune response, followed by the proliferation and differentiation of B cells that produce circulating antibodies directed at many different regions (epitopes) of the proteins on the surface of the pathogen. Later exposure to the same pathogen elicits antibody production by memory B cells. Most of the currently used vaccines work by provoking this immune reaction, known as the humoral arm of adaptive immunity.

The body also deals with intracellular pathogens by mounting a cell-mediated immune response choreographed by T cells. Unlike B cells, T cells only recognize surface antigens that have been processed and presented to them by antigen-presenting cells (APCs). Upon presentation of the antigen, T cells become cytotoxic to infected target cells, and can also release chemicals known as cytokines, which help recruit additional immune players to fight the microorganisms.

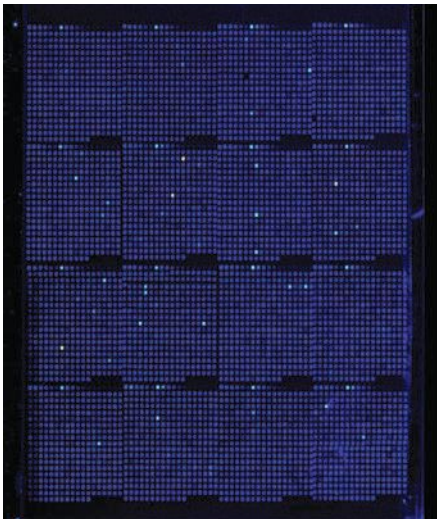
Due to the two-pronged approach that our adaptive immune system naturally takes against pathogens, researchers who want to develop vaccines or mine for vaccine targets need to investigate both the B- and T-cell responses to ensure that the vaccine will work against all the important antigens. With the recent advances in high-throughput sequencing, bioinformatics, and prediction algorithms, scientists can now determine potential vaccine antigen candidates more rapidly and efficiently.

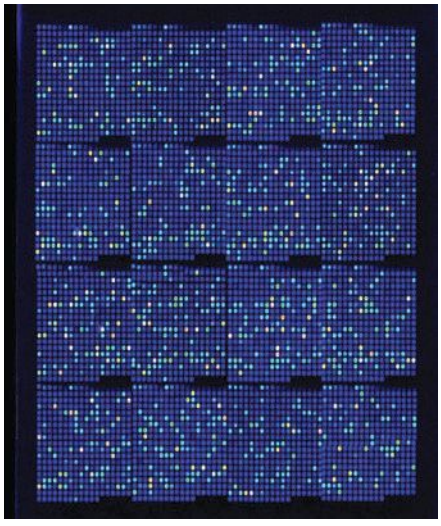
The Scientist spoke with investigators who use high-throughput proteomic approaches and proteome databases to inform antigen selection for the development of vaccines that can generate humoral or cell-mediated responses.

LARGE-SCALE PROTEOMIC SCREENING FOR ANTIGEN DISCOVERY

HOW IT WORKS: One approach to reduce laboratory time and cost with vaccine and antigen discovery uses a high-throughput method whereby each gene in an infectious agent's genome is amplified, cloned, and then translated into proteins using an *E. coli* cell-free transcription/translation system. This methodology was developed in 2005 at Antigen Discovery, Inc., a biotechnology company based in Irvine, California, and by researchers at the University of California, Irvine. It is helpful in elucidating the B-cell response to a particular pathogen.

Although this high-throughput approach was originally developed using the malaria parasite and the vaccinia virus, researchers have now employed the method to look for antigen targets for more than 30 human pathogens, including chlamydia, tuberculosis, HPV, HIV, and influenza (*PNAS*, 102:547-52, 2005). This approach can help scientists whittle down vaccine candidates from an organism that has thousands of proteins to a handful of potential antigens.





MINING FOR TARGETS: Sera from a US adult who had never been infected by malaria (left) and a naturally malaria-infected individual living in West Africa (right) were applied to microarrays expressing 4,500 proteins from the malaria parasite followed by exposure to labeled secondary antibodies. Bright spots on the array indicate antigens associated with protection.

PHILIP FELGNER, UNIVERSITY OF CALIFORNIA, IRVINE

After the synthesis of the pathogen's proteome, the proteins are printed onto a nitrocellulose array in a 384-well plate format. A small volume of serum (2 µL per patient) from appropriate patient cohorts can be applied to these arrays. After overnight incubation, a fluorescent secondary antibody that recognizes human antibodies is applied, and the fluorescent signals are read by a scanner (*Proteomics*, doi:10.1002/pmic.201500375, 2016).

This high-throughput approach can help scientists pin down what antigens are associated with protection. "If you have two populations of people—one group who is exposed and another who isn't—you can compare the antibody responses between those two groups and look for antibodies associated with protection," says Philip Felgner, an infectious disease specialist at UC Irvine and the founder of Antigen Discovery. (See photographs above.)

CONSIDERATIONS & GETTING STARTED: Arrays for a number of infectious diseases can be purchased through Antigen Discovery, and the cost varies based on the number of proteins printed. Many of the arrays need to returned to Antigen Discovery for scanning, because the machines used to scan these slides are no longer used in many research institutions, Felgner says.

If you'd like to try your own hand at creating an array for an infectious agent, you can do so by following the protocol in the original paper (*PNAS*, 102:547-52, 2005).

When working with proteins that are encoded by genes that are 3,000 base pairs or longer, Felgner advises splitting the gene up into overlapping segments to maintain the high-throughput approach in synthesizing proteins.

The proteins can be printed using a microarray printer from ArrayJet: these printers do not make direct contact, precluding any damage to the nitrocellulose plate.

Although many of the arrays manufactured by Antigen Discovery need to be scanned by the company, researchers can also adapt a battery-operated ArrayCAM imager from Grace Bio-Labs (*Proteomics*, doi:10.1002/pmic.201500375, 2016).

EPITOPE-BASED PREDICTION ALGORITHMS

HOW IT WORKS: A second high-throughput way to mine for vaccine targets is to look more specifically at the T-cell response. One approach researchers use is to print peptides based on the suggestions of epitope-prediction software. After these peptides are printed on an array, $CD8^+$ and $CD4^+$ T cells isolated from specific subsets of patients—for instance, those who are infected and those who are vaccinated—can be applied to the array. If the T cells are activated by a particular protein, that positive hit can be studied further for validation as a potential vaccine target. Currently, this technique is being used to mine for vaccine targets for diseases including tuberculosis, dengue, and allergies.

This T cell–based proteomic approach was first developed in 2003 using the parasite that causes malaria, *Plasmodium falciparum* (*PNAS*, 100:9952-57, 2003). Since then, the prediction algorithms for this technology have improved. "It's basically always a feedback loop: the more peptides we test, the more data we get," explains Daniela Weiskopf, an immunologist at the La Jolla Institute for Allergy and Immunology (LJIAI) in California. "This iterative loop feeds back to the bioinformaticians, who can better advance these algorithms."

The peptides identified by the algorithms are then printed and used in an enzyme-linked immunospot (ELISpot) assay, manufactured by the Swedish company Mabtech. The 24-hour ELISpot assays are a sensitive way to quantify cytokine-secreting T cells. Weiskopf and her colleagues at LJIAI look at interferon-gamma levels to measure T-cell activation. If the T cells bind to a particular epitope and respond to it by secreting a cytokine, then that printed peptide could be a potential vaccine candidate. "Each cell that makes a cytokine correlates to one spot on the membrane," explains Veronique Schulten, another LJIAI immunologist.

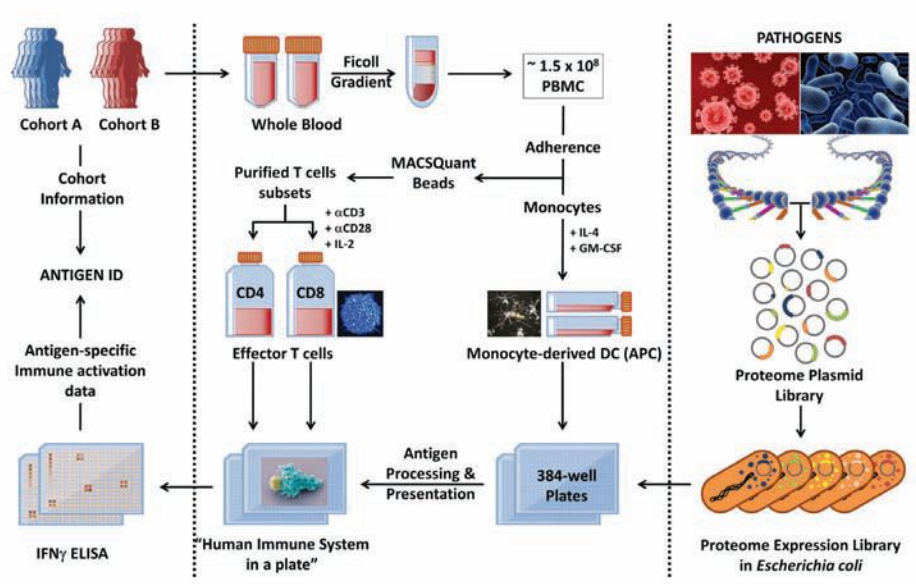
CONSIDERATIONS & GETTING STARTED: To generate your own array to identify T-cell targets for a particular infectious agent, start by searching the Immune Epitope Database (IEDB.org). This database can help predict if T cells might bind to specific epitopes of an infectious agent.

And because printing peptides can be expensive—synthesizing a crude peptide comprising 15 amino acids from scratch can cost anywhere from $25 to $40—you need to determine what you are interested in, Weiskopf advises. Purified peptides can run up to $300 a pop because of the additional purification steps involved. And the

larger the organism, the more expensive it will be to make the peptide pools, and the more blood will be required for your assay. "Everything becomes more complicated and your needs are greater," says Schulten.

Since the T cells used in this assay are derived from patients, make sure you are first comfortable with working with cryopreserved peripheral blood mononuclear cells (PBMC), a collection of cells from which T cells are isolated. "You need to know how to isolate, freeze, and thaw PBMCs efficiently and without killing them," says Cecilia Lindestam, an immunologist at LJIAI.

The researchers who spoke with *The Scientist* about this assay also advised taking into account the toxic effects that cryopreservants, such as dimethyl sulfoxide (DMSO), could have on PBMCs. For the ELISpot assay, peptides are usually resuspended in DMSO, so an important consideration is to balance the concentration of DMSO in each well to prevent the cryopreservant from killing the cells.



DOING WHAT COMES NATURALLY: The ATLAS technology mimics how T cells naturally respond to an infectious agent. A plasmid library prepared from the total proteome of a pathogen is expressed in *E. coli* and seeded into a microarray plate (right column). Whole blood from pathogen-exposed and nonexposed samples is separated into antigen-presenting cells (APCs) and T cells (middle column). APCs and T cells are applied sequentially and antigen targets are assayed using interferon gamma (IFNγ) as a measure of T-cell activation.

ATLAS (ANTIGEN LEAD ACQUISITION SYSTEM)

HOW IT WORKS: ATLAS is a rapid, high-throughput method researchers can use to comprehensively screen every single protein in a pathogen for vaccine target discovery. ATLAS also interrogates how T cells respond to an infectious agent. Developed based on research by immunologist Darren Higgins, now at Harvard Medical School, the technology is proprietary to Cambridge, Massachusetts–based Genocea Biosciences, a biotech company cofounded by Higgins to develop vaccines and immunotherapies that elicit protective immunity via T-cell responses. The ATLAS platform has helped identify T-cell vaccines for genital herpes and pneumococcus currently in clinical trials.

The premise of ATLAS is to mimic the natural human immune response to protein antigens, so the assay involves antigen processing and presentation by antigen-presenting cells (APCs) and immune activation of $CD4^+$ and $CD8^+$ T cells.

T-cell responses are typically polyclonal, meaning that a single antigenic protein can activate T cells directed at different epitopic regions of the protein.

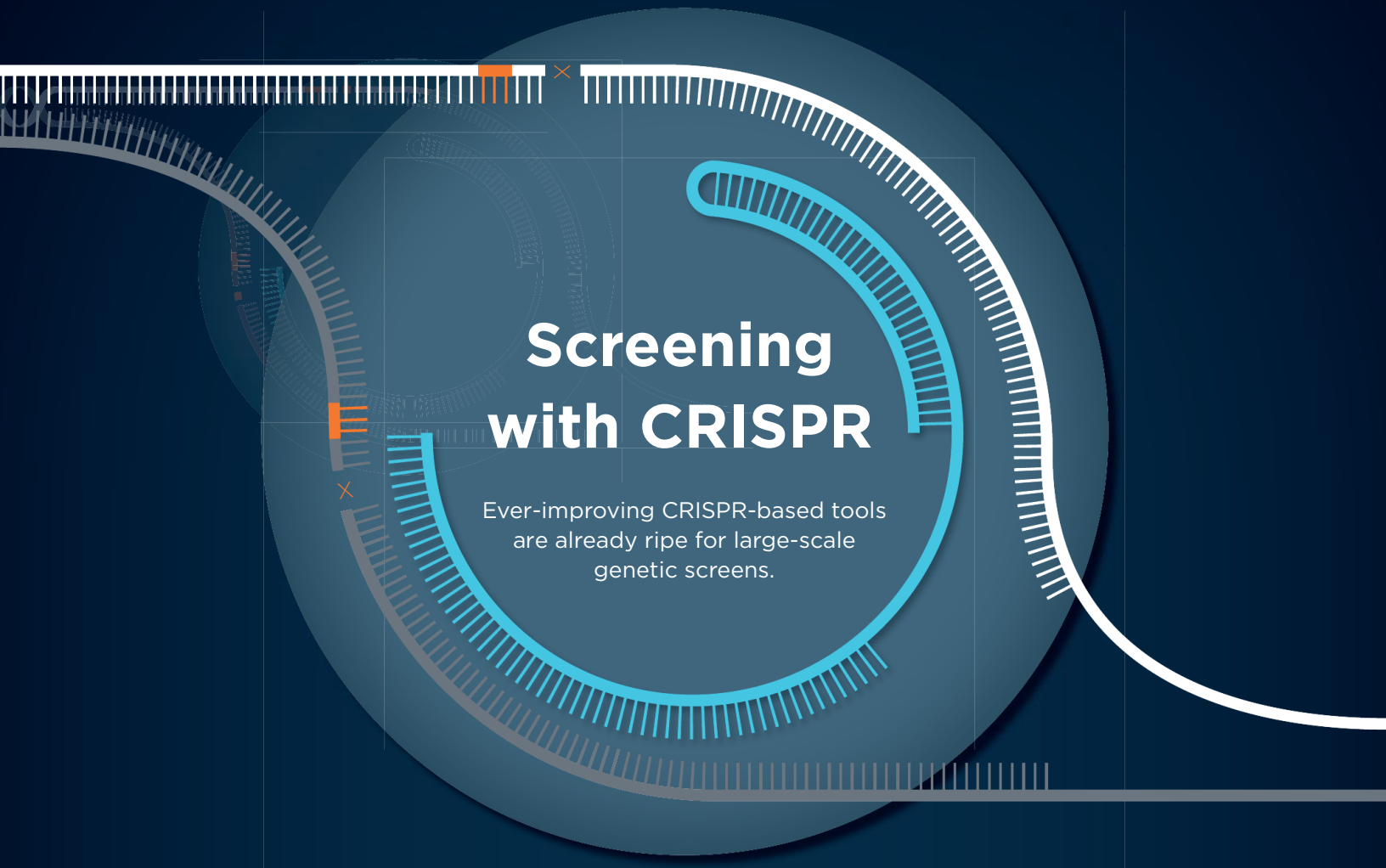
To identify T-cell targets using the ATLAS approach, each protein in the proteome of an infectious agent is expressed in *E. coli*. Each bacterial clone is then cultured in a separate well of a 384-well plate. From blood samples from human donors across a wide range of HLA types, researchers isolate nucleated cells, which contain all the immune cells the assay requires: the APCs and the T cells. After dividing the APCs and T cells into subsets, the APCs are applied to the plates with *E. coli*. After two hours—which gives the APCs enough time to process the bacteria—the T cells are added to the array. If a broad range of T-cell types are activated by a particular antigen expressed by the bacteria and presented to them by the APCs (a response level measured by production of interferon gamma (IFNγ) after an overnight incubation), that antigen may be a promising candidate for a vaccine.

"The core benefit of ATLAS is that we're taking advantage of the natural propensity of APCs to do their job, which is to eat bacteria," says Jessica Flechtner, an immunologist and chief scientific officer of Genocea.

GETTING STARTED: ATLAS is not a fee-for-service platform. Rather, the projects are set up as collaborations between researchers and the company, either through grants or as a material transfer. If researchers are interested in using this platform, they should ask themselves if T cells might be mediating an effect in the infectious disease they are studying.

Another question to consider is whether the pathogen proteome is large enough to do an unbiased screen. For instance, ATLAS might not be ideal for RNA viruses with genomes that only encode for a handful of genes. "There's not a magic number for how many expressed genes a pathogen should have," Flechtner says. "But when you get into tens and hundreds of expressed genes, that's where ATLAS should be used, because that's where our strengths lie: to be comprehensive and high-throughput for organisms that have larger proteomes." ■

GENOCEA BIOSCIENCES



Screening with CRISPR

Ever-improving CRISPR-based tools are already ripe for large-scale genetic screens.

With gene editing—and in particular, the CRISPR/Cas9 system—scientists are in some sense building a shiny new car at the same time they are taking it for a spin. And it's been a joyride. CRISPR/Cas9, as it was originally conceived for gene editing in late 2012, makes cuts at specific locations along DNA with help from a short stretch of guide RNA that takes the Cas9 endonuclease to a specific site.

Increasingly, groups are applying this technology in large-scale genetic screens—for example, to identify mutations that drive treatment resistance in cancer, or to rapidly assess drug targets. RNA interference doesn't come close to what CRISPR/Cas9 can do for genetic screens, both in specificity and in efficiency.

At the same time, researchers such as Traver Hart of the MD Anderson Cancer Center are working to further understand the power and the limitations of CRISPR/Cas9 and what it does in, for example, uncharacterized human cell lines. The CRISPR/Cas9 toolbox continues to expand. Disabled versions of Cas9, pioneered by the Broad Institute's Feng Zhang and by other groups, bind to the genome and either halt or enhance transcription, depending on the application. Zhang and others are engineering the Cas9 enzyme to make it more specific and are discovering new gene-editing proteins, too.

Hart and Zhang discuss their work on the frontlines of CRISPR/Cas9 development and application in "Genetic Screens: A Route to Rapid Progress in Disease Targeting and Drug Development," a webinar from *The Scientist*.

The following pages contain highlights from their presentations.

—Kelly Rae Chi

Screening with CRISPR

ADVANCES IN CRISPR TECHNOLOGY

Feng Zhang

MIT's Feng Zhang discussed engineering the *Streptococcus pyogenes* Cas9 protein to improve specificity (*Science,* 351:84-88, 2016). The Cas9 endonuclease can induce off-target mutations when pairing between DNA and the guide RNA is not perfect, which makes it less than ideal for precision editing of the genome in clinical settings.

DNA strands need to separate to accommodate the Cas9 protein. Structural analysis by Zhang's group revealed a positively charged groove on the Cas9 protein at the spot where the negatively charged non-target DNA fits. "We thought maybe if we [neutralized] some of these positive charges then we could weaken the stabilization and therefore make [Cas9] more specific," Zhang says.

They tried this approach using guide RNAs that have been known to land on particular off-target sites. The group created 32 single-point mutants of Cas9 and targeted each of them to the gene *EMX1* via its validated but error-prone guide RNA. Five of the Cas9 mutants were able to preserve the on-target editing of *EMX1* but reduce the cuts at off-target sites by tenfold. Then the team tried using the mutated Cas9s to cut another gene, *VEGFA* (whose guide is known to cut at two previously identified off-target sites). Although all of the mutated Cas9s reduced off-target effects, the group thought that they should combine mutations to make the Cas9 even more specific, Zhang says. They generated two different mutants with triple-point mutations and "found we were able to preserve the on-target activity and then even further reduce the off-target activity so that we no longer detect it," he says.

Zhang calls these enhanced-specificity Cas9 proteins "eSpCas9 variants." Testing numerous guide RNAs with one single-point and one triple-point mutant eSpCas9, the scientists found that both of them edited on-target sites with similar efficiency. Although there were some variations in efficiency with different guides, "on average, [the mutants] were on par with the wild-type Cas9," Zhang says.

By trying various mutations of guide RNAs in combination with the wild-type Cas9, Zhang's group has found that there is a so-called "seed region" at the 3' end of the guide that confers specificity. In contrast, the new eSpCas9 variants extend this seed region to include the entire guide. And the group is continuing to make the system even more specific, says Zhang.

Discovering unknown gene function using CRISPR

One exciting feature of the CRISPR system is how specific guide RNAs are, Zhang says. This makes it possible to generate a lentivirus library of CRISPR guides that target every gene or multiple sites within a given gene. These are then transduced into cell lines, resulting in pools of cells in which individual genes have been either inactivated (*Science,* 343:84-87, 2014; *Cell,* 160:1246-60, 2015) or activated (*Nature,* 517:583-88, 2015).

To show the power of these screens, Zhang's group addressed treatment resistance to melanoma. The BRAF V600E is a well-known cancer mutation that is treated by the US Food and Drug Administration–approved drug vemurafenib (Zelboraf). However, resistance arises in rapidly mutating cells, and by 24 weeks of treatment the tumors return.

"We thought this might be an opportunity for us to apply a genome-scale library to see what are the genes—when you either turn them on or turn them off—that would render the tumor cell resistant to vemurafenib," Zhang says.

Zhang's CRISPR knockout library uses guides that target all the conserved coding exons in the genome. "When designing these kinds of screening experiments, we always use multiple guides so that we have some redundancy and also to be able to know that the effect of any single guide is not due to an off-target modification," he adds. They then tried to validate their new candidates and compare their results to a previously conducted RNAi screen (*Cancer Discov*, 3:350-62, 2013), finding that they could confirm several of their top CRISPR hits confer vemurafenib resistance; in the RNAi screen, only the top hit was confirmed.

Gain-of-function CRISPR-based screens developed by Zhang's group have also enabled the study of vemurafenib resistance. Zhang calls this new CRISPR-based tool for activating genes the synergistic activation mediator, or SAM, which his team showed can activate 12 different genes that they had had trouble switching on using older methods. "And for many genes where the old system couldn't really activate, the new system is able to activate transcription by 100- or 1,000-fold," Zhang says.

Zhang's lab is working to further expand the CRISPR editing toolbox by identifying additional enzymes useful for genome editing. For example, last fall they discovered Cpf1, another DNA endonuclease that is equipped with different cutting actions that may make it more useful in some cases (*Cell*, 163:759-71, 2015). The group also described additional CRISPR/Cas systems C2c1, C2c2 and C2c3 (*Mol Cell*, 60:385-97, 2015), and is studying their mechanisms.

CANCER TARGETING USING GENETIC SCREENS IN HUMAN CELL LINES

Traver Hart

RNAi-based screens have made genome-scale perturbation screening in human cells possible. But an incomplete understanding of the biology of RNAi and of the data generated by such screens has led to some false starts in the field, says Traver Hart of MD Anderson Cancer Center, citing a 2012 *Science* perspective that expounded on these issues and argued for more-sophisticated approaches to studying mammalian gene function (337:421-22, 2012).

Working in Jason Moffat's lab at the University of Toronto, Hart himself helped define a set of gold standards that can be

used to evaluate the quality of both RNAi and CRISPR genetic screens (*Mol Syst Biol*, 10:733, 2014), just as the first two large-scale CRISPR knockout screens in human cell lines were published (*Science*, 343:84-87, 2014; *Science*, 343:80-84, 2014). Using the HCT116 cell line (derived from a human colon cancer) as an example, Hart showed that CRISPR screens are not only more sensitive but they also do not generate additional background errors.

How do CRISPR screens represent such an improvement over RNAi? CRISPR screens work across a "whole range of biologically meaningful gene expression [levels], whereas shRNA [short hairpin RNA] seems to work really well only at very high expression levels," Hart says. "This was information that we didn't have from the shRNA data until we had something better to compare it to. So, we didn't know what we didn't know. This is one way that we're quite sure that CRISPR has completely revolutionized this field of genetic screening."

Hart's group recently generated the Toronto KnockOut (TKO) library, a second-generation library of lentiviral-encoded guide RNAs that target 17,661 human protein-coding genes. They conducted fitness screens (also known as essentiality screens) in four cancer cell lines and one normal cell line. In their study, they defined a fitness or essential gene as one whose perturbation diminishes cell growth and proliferation. The team detected nearly 1,600 core fitness genes (*Cell*, 163:1515-26, 2015). "That number is astonishing to anyone who's done shRNA or RNAi work (where that number is in the low hundreds)," Hart says.

What Hart and his colleagues are primarily interested in, however, is what genes are *differentially required* across different cell lines or genetic backgrounds. Knowing the genotypes of the cell lines they've studied so far helps them make predictions. For example, the colon cancer–derived DLD1 and HCT116 cell lines both have *KRAS* driver mutations, suggesting that the downstream MAPK pathway is active, and this prediction bears out in their screens.

To better understand context-specific vulnerabilities in each cell line, Hart subtracted the core essentials and conducted functional enrichment tests on their remaining context-specific essentials. This revealed a unique signature of processes required for optimal fitness, he says.

These studies have revealed some surprises: cell lines with similar genetic profiles can differ in their fitness profiles. For instance, both DLD1 and HCT116 carry oncogenic *KRAS* mutations, yet the gene for epidermal growth factor receptor, a cell-surface receptor linked with numerous cancers, including colorectal cancer, and its molecular partners are hit in only the DLD1 line.

In a separate, as-yet-unpublished line of work, Hart's group conducted a follow-on screen in HPAF-II pancreatic cancer cells. The genetic screen detects numerous secondary processes involved in cell proliferation—for example, post-translational modifications and receptor endocytosis. "In many senses, this is an embarrassment of riches you get from one of these screens," Hart says.

PANELISTS

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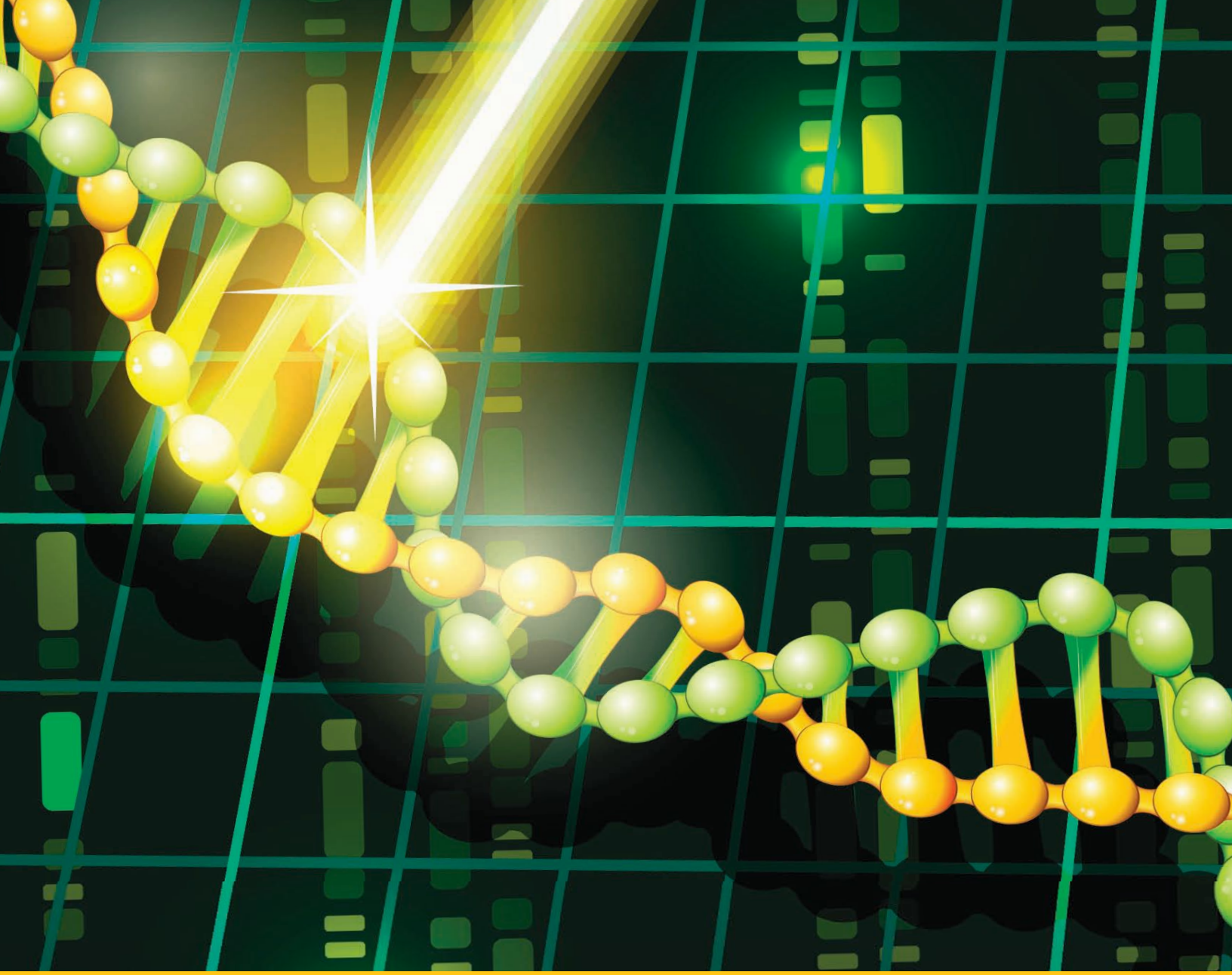
In addition, the screens fingered specific members of receptor families—1 of 10 in the frizzled receptor family and 1 of 17 Wnt ligands—as essential. "Not only do we get the breadth of the pathways and processes that are required to support wild-type growth in this cell line, but we also get very specific, potentially actionable targets," he says.

Core essential genes in cancer cell lines can also be explored as therapeutic targets. In 2012, Dana-Farber Cancer Institute researchers showed that when mutating cancers delete regions coding for tumor suppressor genes they also remove various "passenger" genes that do not directly play a role in cancer but that may leave tumor cells vulnerable to additional specific insults (*Cell*, 150:842-54, 2012). This concept has since been dubbed "collateral lethality" and is believed by Hart and others to be an opportunity for therapy (*Trends Cancer*, 1:161-73, 2015).

Deleting core essential genes in the vicinity of a tumor suppressor gene presents a therapeutic window, Hart says. For example, *POLR2A* is a core essential gene that encodes an RNA polymerase subunit, and a copy of the gene is almost always co-deleted with the well-known tumor suppressor gene *TP53* in ovarian cancer. Last year, Xiongbin Lu's team at MD Anderson showed that *TP53* loss sensitized colorectal cancer cells to polymerase inhibition (*Nature*, 520:697-701, 2015).

The inventory of core essential genes will continue to grow, and sharpen, as more researchers conduct CRISPR screens, Hart says. "They are scattered all over the genome, and as idiosyncratic copy losses occur in tumors, any one of these might then become a therapeutic target," he says. "This is a very exciting frontier for cancer research—not just the context sensitivity but also the core essentials are potential therapeutic targets." ■

A video link to the webinar can be found at
http://bit.ly/GeneticScreensWebinar



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Finders Keepers

A petition recently filed with the Supreme Court triggers renewed debate about the role of patents in stimulating innovation in the diagnostics sector.

BY CATHERINE OFFORD

Seventeen years ago, Arupa Ganguly received a disturbing legal letter asking her to stop her work. Recently appointed to the faculty at the University of Pennsylvania's Perelman School of Medicine, Ganguly, along with her colleagues, was offering screens for *BRCA1* and *BRCA2*—two genes involved in DNA repair that, when mutated, increase a woman's risk of developing breast and ovarian cancers. But in the late 1990s, molecular diagnostics company Myriad Genetics had acquired patents covering the *BRCA* genes, as well as dozens of *BRCA* mutations and methods to isolate and detect them, establishing a monopoly over the use of the genes in diagnostic testing. In the cease-and-desist letter received by Ganguly, the company asserted that the right to perform *BRCA* screens and return results to patients belonged solely to Myriad.

"I was very angry, to say the least. I was disappointed. I was sad," says Ganguly, now the director of the Genetic Diagnostic Laboratory at Perelman. "But I had to go with it. No one was going to fight Myriad because, they thought, 'A law is a law.'"

Following receipt of that letter, Ganguly avoided working on genes that were not in the public domain. Then, a decade later, the Association for Molecular Pathology organized a lawsuit to challenge Myriad's *BRCA* patent claims, and Ganguly testified to a US District Court in New York that Myriad's action had compelled her to halt *BRCA* research and screening. Over the next few years, the case made its way through the US Court of Appeals for the Federal Circuit to the Supreme Court, where, following a well-publicized hearing in April 2013, the justices unanimously ruled that the *BRCA* genes—and, indeed, any "naturally occurring" DNA sequences—"lie beyond the domain of patent protection" as products of nature and not of human engineering.

"When I came out of that chamber, I could feel the excitement," Ganguly said. "It had been clear from the way the questions were being asked that the ruling would very likely go in our favor, but when it did, it was a very exciting moment. I was very happy that day."

Ganguly's relief was shared by many in the scientific community. Francis Collins, director of the National Institutes of Health (NIH), issued a statement calling the decision "a victory for all those eagerly awaiting more individualized, gene-based approaches to medical care." On Twitter, Collins expressed himself more simply: "Woo Hoo!!!" But for others, the ruling—and the justifications it invoked—reflected part of a gradual erosion of patent eligibility that threatens to limit research and development in the diagnostics sector.

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Now, a patent dispute between biotech companies Sequenom and Ariosa Diagnostics on a related theme—the analysis of naturally occurring fetal DNA for use in noninvasive prenatal diagnostic tests—is making headlines. And as the arguments for each side make their way toward the Supreme Court, the case is reinvigorating an unresolved debate about how to balance freedom in research with the demands of commercialization. Ganguly—along with many others who have a stake, either financially or as a matter of principle—is waiting with bated breath for a decision that could influence development in the diagnostics sector and basic research for years to come.

A natural minefield

When San Diego, California–based Sequenom began acquiring patent licenses in 2005 for methods to analyze cell-free fetal DNA (cffDNA) in maternal blood, the concept was certainly a new one. Company researchers later exploited the discovery of cffDNA to develop a noninvasive diagnostic test, called MaterniT21, that allowed them to estimate the relative abundance of each chromosome. "The theory is that if a pregnant woman has a fetus with, for example, trisomy 21 [Down syndrome], then the relative amount of chromosome 21 will be elevated," Sequenom executive vice president of research Dirk van den Boom told *The Scientist* shortly after the test's release in 2011.

A few months later, when San Jose, California–based competitor Ariosa Diagnostics released a test that also detected trisomies in fetal DNA, Sequenom sued for patent infringement—a procedure employed routinely by diagnostics companies as a means to protect the temporary rights to intellectual property afforded by patents. In retaliation, Ariosa filed a lawsuit claiming that

Sequenom's patent claims were never valid in the first place, because they depended on naturally occurring DNA and obvious (i.e., not inventive and therefore not patent-eligible) analytical procedures.

In June 2013, after reviewing the dispute, a US District Court in California sided with Ariosa, and was backed up by a Federal Circuit panel in 2015 on the basis of language from both the *Myriad* ruling and another recent decision concerning the patent eligibility of measuring levels of a drug metabolite (*Mayo v. Prometheus*). Because naturally occurring DNA is a "product of nature," and methods to amplify and sequence this DNA applied techniques that have become "routine, conventional, and well-understood," Sequenom's patent claims for its fetal DNA test were invalid, the courts ruled.

Following a subsequent denial of appeal by the Federal Circuit last December, Sequenom filed a petition with the Supreme Court on March 21; the nation's top court will decide whether to review the case later this year. Of course, there's more riding on the outcome than just determining which company has the right to market its noninvasive prenatal tests. As one Federal Circuit Court judge put it, the current state of patent eligibility in the diagnostics sector is a potential harbinger of a "crisis of patent law and medical innovation."

The problem, as many are quick to point out, is that "products of nature" and "conventional" techniques describe a significant portion of modern diagnostic tests, meaning that large swathes of previously patent-eligible methodology could suddenly become impossible to protect. "A lot of medical inventions—and, to a certain extent, Sequenom's invention—are informational," says Jacob Sherkow, a professor at New York Law School. "It's about thinking about doing something a different way, and developing current technology to do it like that. It's tricky to say whether that's a piece of engineering, or whether that's a piece of nature."

Open to all

One of the principal concerns surrounding the issuing of patents in diagnostics research is that allowing ownership of the discovery and detection of naturally occurring molecules could hinder scientific progress. In 2000, a survey of more than 1,200 US geneticists in industry, government, and academia found that approximately three-quarters of respondents disapproved of patenting DNA altogether, while half noted that patents related to genetic testing had at some point limited their research. And these numbers may well increase among university scientists as they learn more about existing patents relevant to their work, says Lisa Campo-Engelstein, a professor with joint appointments in bioethics and obstetrics and gynecology at Albany Medical College in New York.

"Academic institutions are becoming more aware of the fact that researchers are not checking or don't know about patents," she says. "They're becoming more concerned about liability, and they're notifying their researchers." This, in turn, can pressure scientists to adjust their research questions to avoid any sort of legal confrontation with industry, she adds. "I'm concerned about the effects it will have on scientific research."

Having experienced some of those effects directly, Ganguly is well aware of the potential dangers of allowing a single company to essentially own an entire research area, and argues that the same logic applied in the *Myriad* ruling ought to apply more broadly. "These are all natural products," she says of genetic markers. "They're something that nature is doing, not us. You're just finding out [the sequence or mutation] and improving your methods so you can one day detect that in one cell out of millions."

With the widespread adoption of next-generation sequencing, those methods increasingly consist of "conventional" techniques that many labs can reproduce—a situation that became clear during the *Myriad* proceedings, notes the University of Utah's Sean Tavtigian, a former Myriad researcher and the lead inventor on the company's now largely invalidated US patent for *BRCA2*. "Once massively parallel sequencing came along, it became considerably less expensive for other organizations to enter the testing field," he says.

If it stands, then, the invalidation of Sequenom's fetal DNA patent claims could improve the landscape for everyone by denying any single company control over conducting, and pricing, related tests, Ganguly concludes. "If Sequenom loses its monopoly, it will open up diagnostic testing to broader competition. It should again become a level playing field."

It's tricky to say whether that's a piece of engineering, or whether that's a piece of nature.

—Jacob Sherkow, New York Law School

A darker side?

The arguments that diagnostics patents inhibit research and prevent healthy commercial competition made a strong appearance in the *Myriad* dispute, and in the intensive media coverage that surrounded it. But support for these arguments, both then and now, is widely contested.

Some studies have suggested that academics are less likely to face obstacles from patents being enforced than from restricted access to patented materials and data—a problem that essentially becomes moot if the techniques being used are "routine, conventional, and well-understood." And Myriad President Peter Meldrum argued in a controversial 2013 letter to *The Washington Post* that, far from limiting scientific progress, the company's test had in fact promoted research, as demonstrated by the 18,000 scientists working on the *BRCA* genes and their more than 10,000 publications.

"In the best cases, patents and open science work in tandem with academics and industries to make things work and make them make a profit," says New York Law School's Sherkow. "The instances of bad behavior"—in which patent holders bully scientists out of doing their research—"have, frankly, been really, really small."

And if patent eligibility requirements become too restrictive, innovation could

suffer as a result of less industry investment, Sherkow adds. "We have a system of very burdensome regulatory approval in the United States that costs lots and lots of money. Companies wouldn't be spending hundreds of millions of dollars doing clinical trials if they weren't going to come out on the other side with some sort of property asset."

Sequenom highlights this very point in its petition, and it's an argument that has seen growing support among other biotech leaders and law professionals over the last few months. While it's conceivable that companies could find ways to work around the decision—by tacking on a therapeutic step post-diagnosis, for example—if the federal courts continue to side with Ariosa, it could change the way the industry approaches innovation, says Kevin Noonan, a biotech patent lawyer at McDonnell Boehnen Hulbert and Berghoff LLP, who filed an amicus brief to the Federal Circuit on Sequenom's behalf in late 2015. "If [the ruling] doesn't get overturned, it is going to make it very, very difficult for companies to protect their intellectual property," he says. Following the 2013 ruling and last December's denial of appeal, Sequenom's shares fell 22 percent and 10 percent, respectively, and earlier this year, the company laid off 20 percent of its workforce and sold its prenatal testing facility in North Carolina.

Researchers at Sequenom aren't the only ones feeling the effects of the shaky state of patent eligibility. Brian Van Ness, a geneticist at the University of Minnesota who heads the gene sequencing and diagnostics startup Target Genomics, is also noticing an apparent loss of investor confidence now that the intellectual property protection offered by patents is harder to guarantee. "Compared to five years ago, the investment community has gotten very, very cautious about what they're investing in," says Van Ness, who also acted as an expert consultant in litigation related to Myriad's patents. "Raising capital has become more difficult."

Far from boosting data sharing and reducing secrecy, as many opposed to such patents hope to achieve, the loss of protection for diagnostic tests and associated decrease in investment could lead to a reduction in research openness, says Noonan. Rather than filing their ideas as public patents, "companies, as much as they can, would try to keep things as trade secrets," he says, adding that it's a pattern he is already beginning to see among clients working with biomarkers and diagnostics. "That would mean, for example, that [companies] would offer a test, but you're not going to know what molecule or gene is being detected. That's just not a good thing."

Of course, it's difficult to measure the impact of legal decisions on research and industry, and even more difficult to predict the downstream effects of these rulings on the lives of the patients that the research is supposed to help. But the *Ariosa v. Sequenom* case may at least offer an opportunity for the Supreme Court to clarify what constitutes protectable innovation in the diagnostics sector.

"Whatever happens, we are going to live with the consequences," says Sherkow. "We'll have to wait and see whether we get the present that we want under the tree at the end of the year, or whether we're getting lumps of coal." ■

DOMINO EFFECT

So far, the US federal court system has sided with Ariosa in its argument that Sequenom's patent on a method to analyze fetal genetic abnormalities from a maternal blood sample is invalid, due to the courts' designation of fetal DNA as a natural product and the techniques employed as routine. If that decision holds, some lawyers have suggested that the fallout could eradicate patent protection for thousands of life science inventions. Below are just three examples of cases that have relied on the *Ariosa* ruling to invalidate diagnostics patents in the last 12 months.

Esoterix Genetic Laboratories LLC v. Qiagen Inc.

In August 2014, Esoterix sued Qiagen for infringing on a patent covering a method to determine the effectiveness of certain cancer drugs on patients' tumors. Qiagen responded in July 2015, citing the Federal Circuit's decision in *Ariosa v. Sequenom*, and argued—successfully—that Esoterix's claims were ineligible due to a reliance on "laws of nature." In September, the Court sided with Qiagen and invalidated Esoterix's patent.

Cleveland Clinic Foundation v. True Health Diagnostics LLC

Until recently, Cleveland Clinic Foundation (CCF) held patents for a test that assesses a patient's risk of developing cardiovascular disease using a combination of enzyme measurements and known risk factors. When sued for infringement by CCF, True Health Diagnostics responded by arguing that CCF's methods pertained to natural phenomena and contained no inventive steps. In February, citing both *Myriad* and *Ariosa v. Sequenom*, a US District Court ruled in True Health's favor, and found three of CCF's patents invalid.

Genetic Technologies Ltd v. Merial LLC

Merial LLC responded to an infringement lawsuit from Genetic Technologies Ltd over a patent covering methods to detect alleles using selective PCR by challenging the patent's validity. In April, a Federal Circuit panel found that the methods in question were "remarkably similar" to the genetic testing involved in *Ariosa v. Sequenom* and upheld an earlier US District Court decision invalidating the patent claims.

False Climbs

Our sense of understanding is rooted in biology, but scientific progress results only when our biology is reliably tethered to reality.

BY J.D. TROUT

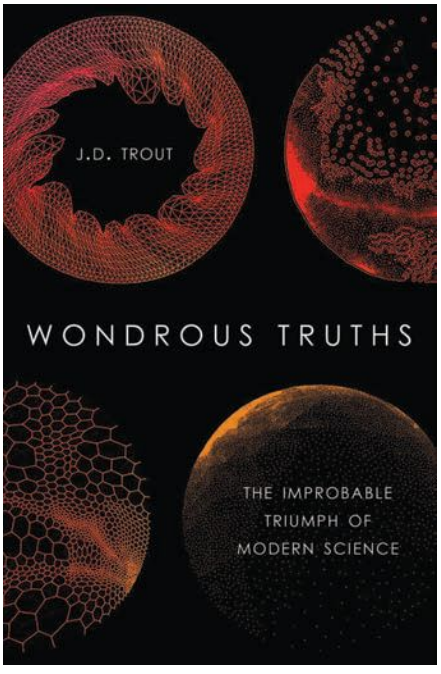
A good explanation—such as the microbial theory of disease, or evolutionary theory—can bring a wondrous sense of understanding. Unfortunately, for a time, so can a bad one—like the medieval miasma (or "foul-cloud") theory of disease, or supernatural accounts of the creation of species. Does our sense of understanding carry any objective signs that we are right? If not, what accounts for the dramatic success of modern science, a success driven by ever-more powerful and accurate explanatory understanding? This is the question I take up in *Wondrous Truths: The Improbable Triumph of Modern Science*.

A sense of understanding exercises a visceral grip on us: visceral because explanation itself has a biological backstory. The simple sea slug *Aplysia* cannot explain, but it can learn. It can learn, through classical conditioning, to retract from a noxious stimulus, and through operant conditioning, to bite more frequently. To learn, an organism need only anticipate: it is all a matter of frequent exposure and coherent expectation. In humans, this learned anticipation of reward or punishment tells us what to expect. We like good feelings, and we want more of them. But that doesn't mean satisfying feelings or actual rewards track consistently with truth.

Objective truth is similarly decoupled from the sensation of insight. Some researchers describe insight as an "aha" moment that can result in new interpretations or solutions. But studies in which subjects are induced to perceive "aha" moments, regardless of whether they are presented with right or wrong answers to puzzles, have shown that people can get a feeling of insight even when they are chronically mistaken. In the real world, scientists seldom know whether the correct solution is contained in their choice set, or even whether their problem *has* a solution. Tracking the truth is that much harder when you have no clue what it looks like.

For us to achieve genuine insight, truth has to cooperate. Without the subtle perceptual and cognitive arrangements that a good theory supplies, we are like pilots flying without instruments on a dark night. Such pilots can fall prey to a phenomenon called "the false climb." Acceleration pushes bones in the middle ear toward the back of our head, causing a sensation of upward trajectory. This happens normally when a plane is *oriented upward*, but it also happens when the plane is accelerating toward the ground. And that is the story of most of the history of science. Steeping oneself in bad theories produces false climbs. Whether apprenticing for years near the furnaces with an adept alchemist or putting in 10,000 hours of study, lab work, and conference preparation as a graduate student and postdoc, you develop a fluency in your thoughts and work that is tightly tied to your cherished theory. There is no certain sign of truth in the sense of understanding.

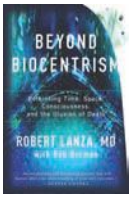
Evolution has left our perceptual and cognitive powers with informal, even ragged, boundaries. So we arrive at true beliefs and good theories by many means, not all of them experimental. The great biologist J.B.S. Haldane once said that he could not conceive of a physical mechanism of heritability and that "no intelligent person who has thoroughly realized its meaning and implications can continue to hold" such a view. He wasn't being coy. In 1914, *no one* had the concept of a physical mechanism that could regulate the development of subtle and complicated biological systems and sustain virtually endless divisions and combinations through successive generations of organisms. But the discovery of DNA was fewer than 40 years away. How did modern biology make this fast transition? Well, the germplasm concept happened to track the actual structure of DNA just closely enough to stimulate accelerating conceptual progress. Once it did, our biological theories firmly oriented our scientific ascent toward the heavens. We have been flying on increasingly accurate instruments ever since. ■



Oxford University Press, May 2016

J.D. Trout is a professor of philosophy and psychology at Loyola University Chicago. Visit the-scientist.com to read an excerpt of Wondrous Truths.

Beyond Biocentrism: Rethinking Time, Space, Consciousness, and the Illusion of Death
Robert Lanza with Bob Berman
BenBella Books, May 2016



In a follow-up to their celebrated 2009 book, *Biocentrism*, stem cell researcher Robert Lanza and astronomer Bob Berman further consider the farthest reaches of science's grip on reality in *Beyond Biocentrism*. As they did in their earlier book, the duo highlight the recent scientific advances that are shaking our understanding of time, space, life, the universe, and our place in it. "[W]elcome back for a deeper and more thorough exploration into [biocentrism], including chapters that solely involve key issues such as death, and important ancillary investigations into topics such as awareness in the botanical world, how we gain information, and whether machines can ever become conscious," the authors write.

Given the pace of research not only in basic biology, but in neuroscience, cosmology, and theoretical physics, it's conceivable that Lanza and Berman will need to revisit the ever-expanding sphere of scientific understanding at regular intervals in the future.

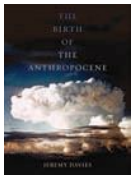
The Sting of the Wild: The Story of the Man Who Got Stung for Science
Justin O. Schmidt
Johns Hopkins University Press, May 2016

Self-experimentation has a storied history in the annals of science. Nobel Laureate Barry Marshall drank a solution of *Helicobacter pylori* to show that the microbe could cause gastritis; Nobel Prize-winning cardiologist Werner Forssmann inserted a catheter into his own heart's atrium; and renowned virologist Jonas Salk administered his polio vaccine not only to himself, but to his wife and children, before beginning double-blind clinical trials. Entomologist Justin Schmidt has taken on the mantle, subjecting himself to dozens of painful stings from the insects he studies. He tells the story of his unorthodox research—and publishes for the first time in a book his "Schmidt Sting Pain Index"—in *The Sting of the Wild*.

In an entertaining narrative of how he stumbled on his calling, Schmidt recalls the encounter with a harvester ant in Georgia that launched him on the path of simultaneously becoming both subject and experimenter. "Wham, an ant stung me. Serendipity had struck," he writes. After surviving the rending, long-lasting pain of that sting, Schmidt and his wife Debbie struck out across the western U.S. seeking to capture and catalog every species of stinging insect they could find. "We had no desire to be intentionally stung, but if we did get stung, we might as well be prepared to record the data. Wasting a good opportunity for a data point seemed crazy."

Schmidt then gives a chapter-by-chapter account of the stings of various insect groups, from fire ants and yellowjackets to tarantula hawks and honeybees.

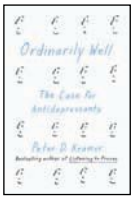
The Birth of the Anthropocene
Jeremy Davies
University of California Press, May 2016



Our species is causing such a drastic shift in Earth's climate that we've brought about a new epoch in the planet's geological evolution. Christened the Anthropocene, the man-made shift is effecting every other living creature with which we share our planet. As University of Leeds English lit lecturer Jeremy Davies describes in *The Birth of the Anthropocene*, two key facts bear out this grim concept: carbon dioxide levels are the highest they've been in three million years, and we are in the midst of the largest mass extinction event since the cataclysm that decimated the dinosaurs.

Even as the concept of the Anthropocene can help humanity frame the crisis into which we've plunged ourselves and the rest of the biosphere, doing so cannot provide a solution to the problem. "It is a way of seeing, not a manifesto," Davies writes. "By providing [environmentally conscious citizens] with a standpoint from which to observe the winding course of earth history, the Anthropocene creates an opportunity to comprehend the environmental calamity in its full dimensions." Addressing the realities of this new epoch, the author suggests, falls squarely on the shoulders of politicians and the activists and scientists whom they rely upon for information.

Ordinarily Well: The Case for Antidepressants
Peter D. Kramer
Farrar, Straus and Giroux, June 2016



Brown Medical School psychiatry professor Peter Kramer has been writing and thinking about depression and antidepressants for a long time. His 1993 book *Listening to Prozac* was met with controversy, as Kramer conducted the thought experiment of envisioning "cosmetic psychopharmacology," in which mood-altering drugs would be used in healthy people to encourage favorable personality traits. With his latest book, *Ordinarily Well*, Kramer defends the use of antidepressants, once-touted "miracle" therapies that have engendered a much more nuanced reaction in recent years, as some studies have linked the drugs to increased risk of suicidal thoughts in adolescent patients.

Kramer reviews current research on antidepressants and defends the careful use of the drugs as treatments for the devastating illnesses he's seen in his own patients. "I will try never to lose sight of our main question, whether antidepressants work," he writes in the book's preface. "We're talking about a major scourge of humankind, depression. We're thinking about many millions of people, those on medication and those not yet offered care. We need to know." —Bob Grant

TS Webinars

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Assistant Professor of Genetics and Genome Sciences
Kavli Fellow (2014-Present)
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ISAAC GARCIA-MURILLAS, PhD
Senior Scientific Officer
Molecular Oncology Team
Institute of Cancer Research, U.K.

WENYING PAN, PhD
Postdoctoral Researcher
Department of Bioengineering
Stanford University

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ONDEMAND | Genetic Screens: A Route to Rapid Progress in Disease Targeting and Drug Development

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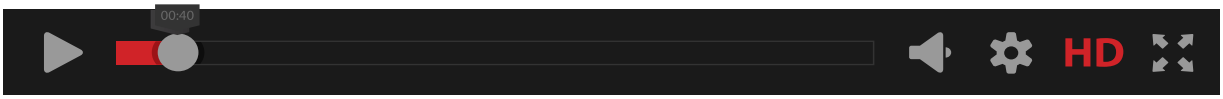

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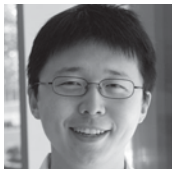

FENG ZHANG, PhD
Core Member
Broad Institute of MIT and Harvard
Keck Career Development Professor
Massachusetts Institute of Technology

TRAVER HART, PhD
Assistant Professor
Department of Bioinformatics and Computational Biology
MD Anderson Cancer Center

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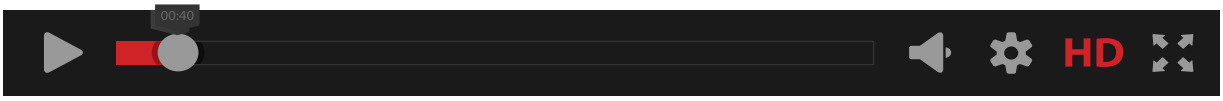

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Assistant Professor
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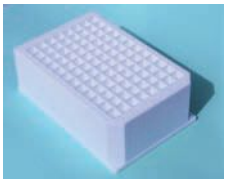

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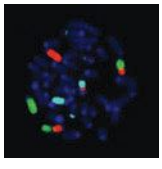

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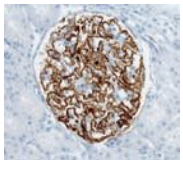

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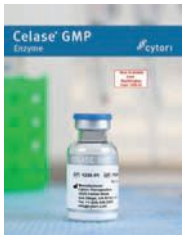

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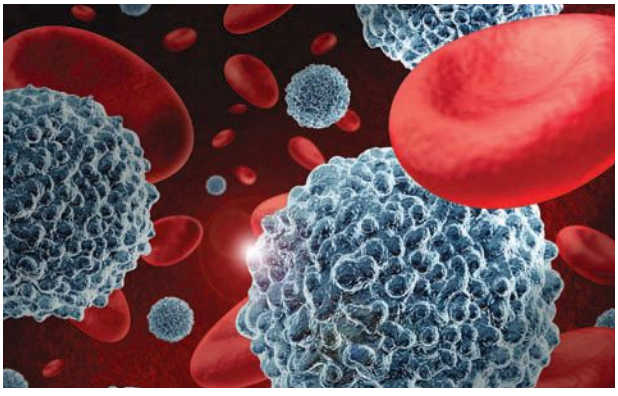

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A Cavalier Experiment, 1885

BY CATHERINE OFFORD

On July 6, 1885, three men in Paris prepared to treat Joseph Meister, a nine-year-old from Alsace who had been bitten multiple times by a rabid dog. Two of the men were medically trained; the third was the therapy's creator, a chemist-turned-microbiologist named Louis Pasteur.

Despite being relatively rare, rabies (or hydrophobia, as it was also known) commanded a fearful fascination in Europe; wildly foaming at the mouth, its victims died painfully and dramatically. But the virus's incubation period also made rabies of interest to Pasteur—already a famous scientist in France—as a candidate for a new type of vaccine.

"The time from the bite to the sickness was pretty long, usually around a month or longer," explains Kendall Smith, an immunologist at Weill Cornell Medical College. "There would be time to intervene with a therapeutic vaccine."

By 1885, five years after starting work on rabies, Pasteur and his colleagues had developed a live viral preparation, which, Pasteur claimed, not only protected dogs from rabies infections, but prevented the disease from becoming symptomatic if administered postexposure.

Still, it was not without reluctance—or concern from his peers—that he agreed to administer a series of viral injections to the asymptomatic young Meister. "This will be another bad night for your father," wrote Pasteur's wife Marie to their children during the treatment. "He cannot come to terms with the idea of applying a measure of last resort to this child."

But it seemed to work—Meister didn't develop rabies. And after starting treatment of another boy that October, Pasteur declared the vaccine a success before the French National Academy of Medicine. The story became international news; even patients from America were soon shipped over to Europe to receive the miracle cure.

Of course, there were critics. "To conclude that a vaccine is successful, you have to compare a trial group against a control group," says Smith. Skeptics argued that as the disease didn't always become symptomatic, the vaccine's efficacy couldn't be confirmed; Pasteur, they said, was taking risks with children's lives.

Pasteur's secretive attitude further fuelled his opponents. "His papers were only three or four pages long," Smith says. "There were no details, and no way you could reproduce any of it."

Nearly a century later, in the 1970s, Pasteur's laboratory notes (in the possession of his heirs until then) were made public. They revealed startling discrepancies between Pasteur's research and his claims: although he had tested a vaccine on dogs, the concoction administered to Meister was made using different methods, essentially untested in animals. Its seeming success? Perhaps the result of an educated guess.

SHROUDED IN SECRECY: Pasteur initially believed that a vaccine had to be live in order to confer immunity. The method he claimed to have used to treat rabies in test subject Joseph Meister was based on his previous research in rabid dogs and rabbits (like the ones Pasteur is shown examining in this panel from *Harper's Weekly*, 1884). This method involved passaging the virus through animals, then desiccating extracted spinal cord tissue for increasing lengths of time in an attempt to reduce its virulence. Meister received a series of injections apparently going from the least to the most virulent spinal cord. But Pasteur's lab notebooks, released in the 1970s, suggest the concoction may instead have been developed with different methods not yet fully tested in dogs.

But appearances mattered more than transparency. In 1888, the Pasteur Institute was opened, and although his vaccine was soon superseded by a chemically inactivated alternative, Pasteur himself is remembered, rightly or wrongly, as a revolutionary scientist and a careful experimentalist. "Let me tell you the secret that has led me to my goal," he is famously quoted as saying. "My strength lies solely in my tenacity." ■

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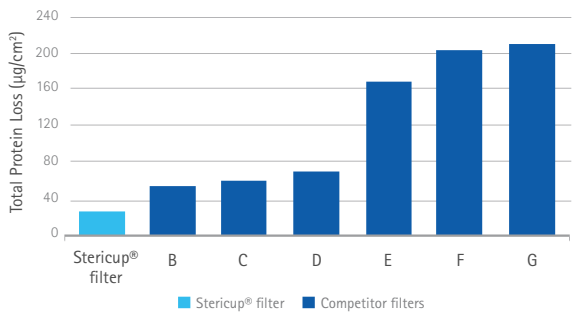

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