TheScientist
OCTOBER 2017 | WWW.THE-SCIENTIST.COM
EXPLORING LIFE, INSPIRING INNOVATION

A NATURAL ARCHIVE

THE PRACTICAL CHALLENGES OF STORING DATA IN DNA

THE EVOLUTION OF MORE-VIRULENT PATHOGENS

THE MULTITASKING MACROPHAGE

HOW TO DRUG INTRINSICALLY DISORDERED PROTEINS

PLUS

CRISPR PATENTS: A PRIMER

Compact and lightweight with a modern interface, it fits any benchtop – and budget

Building on our family of benchtop multimode plate readers, the VICTOR Nivo™ system packs all popular detection modes into the industry's smallest multimode reader footprint. The perfect system for everyday biochemical and cell-based assays, it measures at any wavelength, with top and bottom reading on all technologies. And its software interface is easy to learn and use, and lets you control the instrument from almost any device, anywhere – perfect for multiuser environments. Combine with our assays and reagents to get you started right away. The VICTOR Nivo system: The next big thing in plate readers is now the smallest.

Learn more at www.perkinelmer.com/nivo

VICTOR Nivo™ Multimode Plate Reader

PerkinElmer®
For the Better

NEW ENGLAND BioLabs® Inc.

be INSPIRED
drive DISCOVERY
stay GENUINE

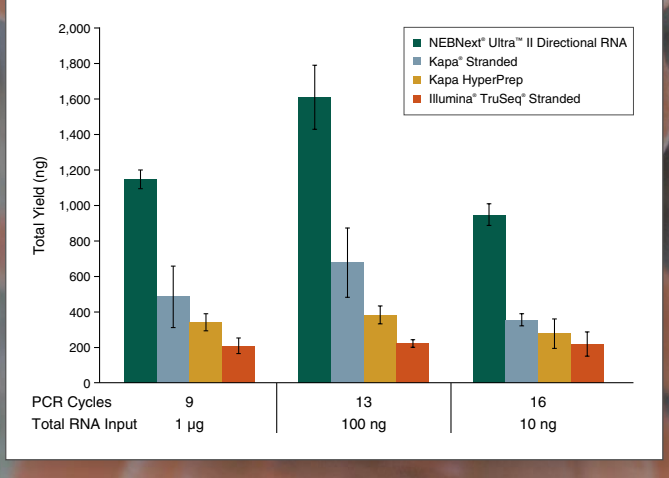
Even more *from* less for RNA.

NEBNext® Ultra™ II RNA Library Prep Kits for NGS

Do you need increased sensitivity and specificity from your RNA-seq experiments? Do you have ever-decreasing amounts of input RNA? Our new NEBNext Ultra II RNA kits are optimized for low ng to µg inputs, have streamlined, automatable workflows and are available for directional (strand-specific, using the "dUTP method") and non-directional library prep. Compatible with poly(A) mRNA enrichment or rRNA depletion, the kits are available with the option of SPRISelect® beads for size-selection and clean-up steps.

Visit **UltraIIRNA.com** to learn more and request a sample.

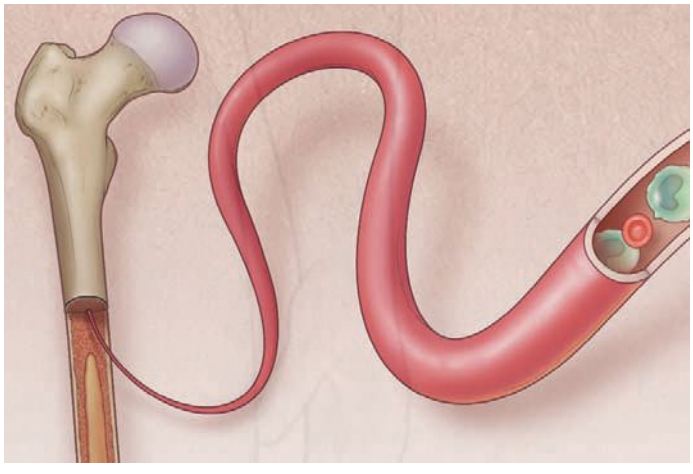
NEBNext Ultra II Directional RNA produces the highest yields, from a range of input amounts



Poly(A)-containing mRNA was isolated from 10 ng, 100 ng and 1 µg of Universal Human Reference RNA (Agilent® #740000) and libraries were made using the NEBNext Ultra II Directional RNA Kit (plus the NEBNext poly(A) mRNA Magnetic Isolation Kit), Kapa Stranded mRNA-Seq Kit, Kapa mRNA HyperPrep Kit and Illumina TruSeq Stranded mRNA Kit. The input RNA amount and number of PCR cycles are indicated.

Contents

THE SCIENTIST | THE-SCIENTIST.COM | VOLUME 31 NUMBER 10



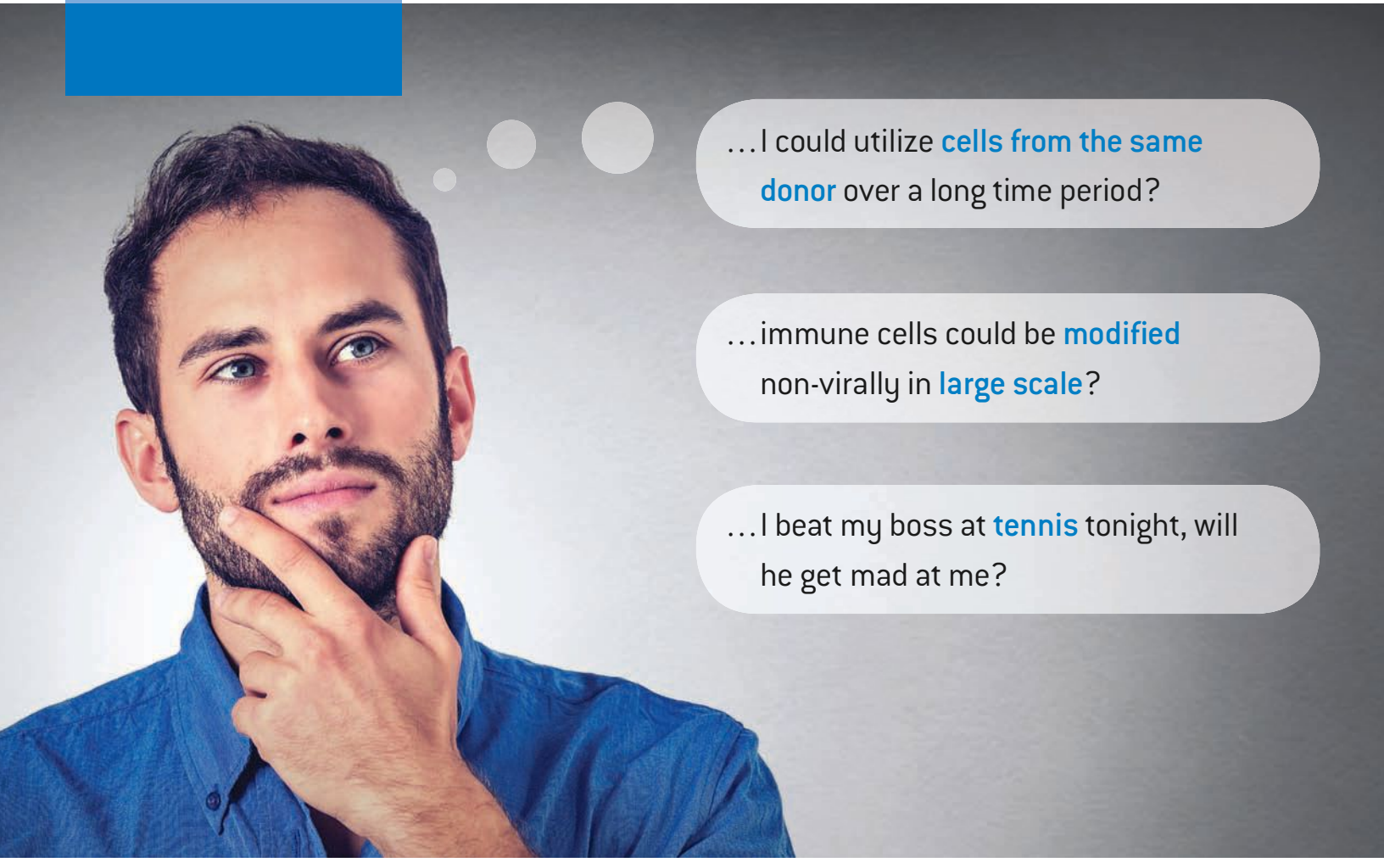
Features

ON THE COVER: ILLUSTRATION BY SHANNON MAY

BioResearch

Lonza

What if...



Solving Today's Challenges in Immunotherapy

Lonza can't help you with your temperamental boss, but we can provide high quality primary cells, transfection technology and services to support your transition from research into therapy.

- Large inventory of primary blood cells from individual donors
- NEW! Large-scale transfection of 10^9 primary immune and stem cells
- Clinical development and commercial manufacturing services for cell therapies

Visit our website for immunotherapy-related technical resources.

4D-Nucleofector™ LV Unit
for large-scale transfection

www.lonza.com/immuno

Department Contents

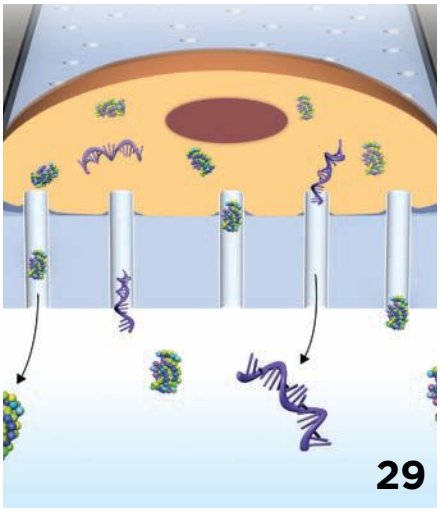
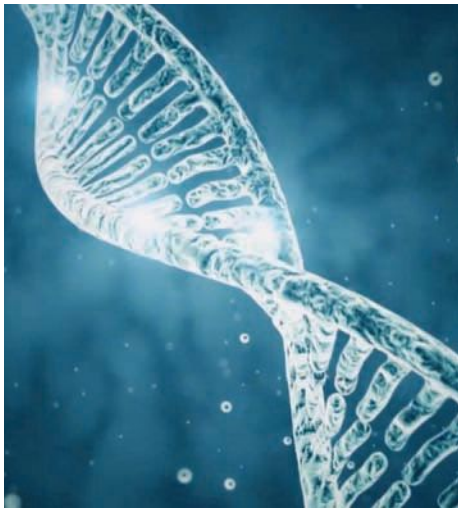


IMAGE COURTESY OF KRAIG BIOCRAFT LABORATORIES; © GEORGE RETSECK; © STEFAN FUERTBAUER

PUZZLE ON PAGE 14

L	I	F	E	L	I	S	T	■	S	N	I	P
E	■	A	■	A	■	H	■	■	■	A	■	A
A	C	K	E	R	M	A	N	■	M	U	I	R
F	■	E	■	G	■	R	■	P	■	T	■	A
■	■	■	B	E	E	K	E	E	P	I	N	G
P	■	I	■	M	■	S	■	R	■	L	■	U
R	A	N	D	O	M	■	F	I	B	U	L	A
E	■	V	■	U	■	E	■	W	■	S	■	Y
G	I	A	N	T	S	Q	U	I	D	■	■	■
N	■	S	■	H	■	U	■	N	■	H	■	C
A	C	I	D	■	N	I	C	K	N	A	M	E
N	■	V	■	■	■	N	■	L	■	L	■	L
T	O	E	S	■	G	E	N	E	P	O	O	L

Online Contents



THIS MONTH AT THE-SCIENTIST.COM:

VIDEO
Introducing Batman
Daniel Kish, who is blind, uses vocal clicks to navigate the world by echolocation.

VIDEO
Meet the Damage Patroller
Profilee Steve Elledge received a 2017 Breakthrough Prize in Life Sciences for his work on how DNA damage is controlled in eukaryotic cells.

VIDEO
Spider Silk
Kraig Biocraft Laboratories has genetically engineered a silkworm to spin spider silk, which might be used for futuristic products.

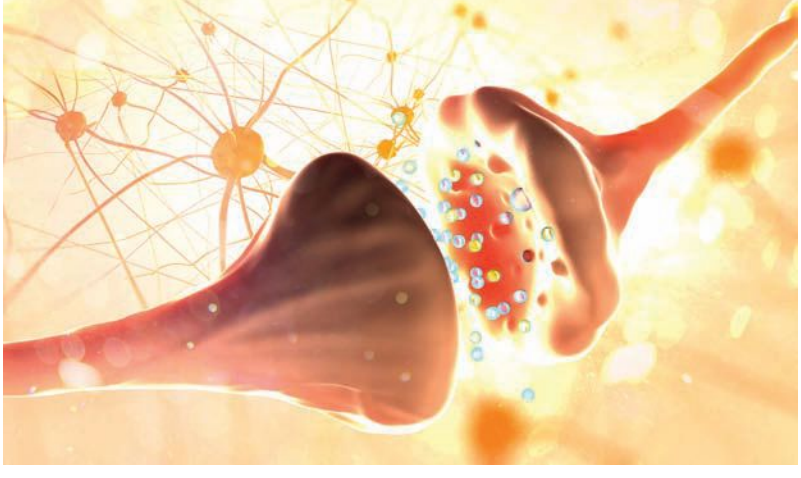
AS ALWAYS, FIND BREAKING NEWS EVERY DAY, AND LEAVE YOUR COMMENTS ON INDIVIDUAL STORIES ON OUR WEBSITE.

Coming in November

HERE'S WHAT YOU'LL FIND IN NEXT MONTH'S ISSUE:

- Documenting mosaicism in brain neurons
- Moving drugs across the blood-brain barrier
- Magnetic recording of nerve signals
- Funding research with private dollars
- Plus: Annual Salary Survey

AND MUCH MORE



KISH: WIKIMEDIA COMMONS; SPIDER: © ISTOCK.COM/BRUCEBLOCK; SYNAPSE: © ANDRII VODOLAZHSKYI/SHUTTERSTOCK.COM

invitrogen

Streamline analysis, expand possibilities

E-Gel Power Snap Electrophoresis System

DNA separation

Simplify DNA electrophoresis with the only integrated gel running and imaging platform

The new Invitrogen™ E-Gel™ Power Snap Electrophoresis System combines the convenience of rapid, real-time nucleic acid analysis with high-resolution image capture. The integrated design helps reduce workflow time and accelerate discovery.

Find out more at **thermofisher.com/powersnap**

ThermoFisher SCIENTIFIC

The Scientist
EXPLORING LIFE, INSPIRING INNOVATION

415 Madison Avenue,
Suite 1508,
New York, NY
10017
E-mail: info@the-scientist.com

POSTMASTER: Send address changes to *The Scientist,* PO Box 2015, Skokie, Illinois 60076. Canada Publications Agreement #40641071 *The Scientist* is indexed in Current Contents, Science Citation Index, BasicBIOS IS, and other databases. Articles published in *The Scientist* reflect the views of their authors and are not the official views of the publication, its editorial staff, or its ownership. *The Scientist* is a registered trademark of LabX Media Group Inc. *The Scientist*® (ISSN 0890-3670) is published monthly.

Advertising Office: *The Scientist,* 415 Madison Avenue, Suite 1508, New York, NY 10017. Periodical Postage Paid at New York, NY, and at additional mailing offices.

SUBSCRIPTION RATES & SERVICES
In the United States & Canada individual subscriptions: $39.95. Rest of the world: air cargo add $25.

For assistance with a new or existing subscription please contact us at:

Phone: 847.513.6029
Fax: 847.763.9674
E-mail: thescientist@halldata.com
Mail: The Scientist, PO Box 2015, Skokie, Illinois 60076

For institutional subscription rates and services visit www.the-scientist.com/info/subscribe or e-mail institutions@the-scientist.com

LIST RENTALS
Contact Statlistics, Jennifer Felling at 203-778-8700 or j.felling@statlistics.com

REPRINTS
Contact Jennifer Barron at jbarron@the-scientist.com

PERMISSIONS
For photocopy and reprint permissions, contact Copyright Clearance Center at www.copyright.com

eppendorf

REGISTER FOR A YO-YO

More Capacity

Centrifuge 5920 R

The new Centrifuge 5920 R delivers extraordinary high capacity in a very compact and ergonomic product design. Visit the website below for a chance to receive a special edition yo-yo modeled after the rotors for the 5920 R.

- > Max. capacity: 4 x 1000 mL or 52 x 50 mL conical
- > Very low noise level for a pleasant work environment
- > Unique two-stop lid for easy access to samples
- > Advanced universal bucket design for tubes and plates

www.eppendorf.com/5920RSCI1 • 800-645-3050

Watch video!

Contributors

Andrew Read's interest in natural history and evolution began when he was a child growing up in New Zealand. The islands are home to many "strange and weird birds and animals," he says; their uniqueness and diversity prompted him to think about how they came to be. After obtaining a bachelor's degree from the University of Otago, Read moved to the University of Oxford to pursue graduate studies in bird evolution. There, his interests began to diverge from birds to something much smaller and faster-evolving: pathogens. The pace of infections enthralled him, and he recognized that by studying infectious disease, he could observe evolution in real time. "I've been working on them ever since," he says. How pathogens evolve is fundamentally important to human health, he says, and such research could positively affect society. After a series of faculty positions, first at the University of Tromsø in Norway and then the University of Edinburgh, Read moved to the U.S. in 2007 to set up a lab at Penn State's Center for Infectious Disease Dynamics. Throughout his career, he's pursued a variety of questions from a population-biology perspective, including why certain pathogens make us sicker than others, how pathogens compete within the same host, and what factors lead to drug resistance.

In his early years as a practicing veterinarian, **Peter Kerr** had no intention of pursuing a career in research. "I thought I wanted to be a country veterinarian," he says. "I loved working with animals." But after working as a consultant to help control disease on pig farms, Kerr began to wonder how the same pathogen could be completely benign on one farm and cause rampant disease on another. To understand the answers to this question, Kerr knew he needed to arm himself with a solid background in molecular virology. This became the focus of his PhD studies at the Australian National University, and ultimately, "led me into this area of viral pathogenesis, of trying to understand how viruses cause disease," he explains.

After completing his PhD, Kerr was recruited by the Commonwealth Scientific and Industrial Research Organisation (CSIRO) in 1990. There he spent 24 years pursuing his interest in viral virulence. He then moved to the University of Sydney, from which he officially retired at the end of last year. Unofficially, however, "I'm keeping busy." Kerr is an honorary fellow at the Marie Bashir Institute at the University of Sydney, and since 2008, he's been working with colleagues Andrew Read and Edward Holmes on questions surrounding the evolution of viral virulence.

In this issue, Read and Kerr delve into the evolution of microbial pathogenicity on page 40.

To peer into the brains of dogs, Emory University professor of psychology **Gregory Berns** and his team have figured out how to train them to sit still inside MRI scanners. Berns uses these neuroimaging techniques to study social behavior, emotions, and reward processing in dogs with the goal of understanding "why dogs are what they are," he says, and "what they're thinking and feeling." According to Berns, after thousands of years co-evolving and forming social bonds with humans, "[dogs] themselves are special." Berns didn't begin his career by wanting to delve into the canine psyche, however. As a medical student, he became interested the brain's reward circuits while conducting research in Terry Sejnowski's lab at the Salk Institute. After obtaining his MD from the University of California, San Diego, in 1994, he did a residency in psychiatry and subsequently began to study reward circuits in humans, choosing neuroscience research over clinical medicine. In 2011, he began what he calls "a crazy side project" in canine neuroimaging that, over time, became his primary research focus. Since then, he's branched out into imaging the brains of other mammals, including those that are endangered, in an "attempt to map the 3-D structure of a wide range of species before they're gone." Even an animal's extinct status hasn't stopped Berns from wanting to learn about its brain. Learn more about his research on both extinct and living animals in his essay based on his new book, *What It's Like to Be a Dog: And Other Adventures in Animal Neuroscience*, on page 72.

BRAD ZIEGLER; PETER KERR; HELEN BERNS

www.labwrench.com

Ask Questions

Post Answers

Lab Equipment Troubleshooting, Recommendations, Tips and Tricks

LabWrench

Lab Equipment Information and Discussions.

DeNovix

DS-11 FX Series

The World's Most Sensitive Microvolume Spectrophotometer

Patent US9442009

Microvolume UV-Vis

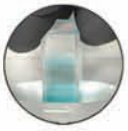
0.75 - 37,500 ng/µL dsDNA

Calibration-Free SmartPath® Technology

Integrated Fluorescence

0.0005 - 4,000 ng/µL dsDNA

UV, Blue, Green, Red channels



Cuvette UV-Vis

0.04 - 75 ng/µL dsDNA

OD600, Kinetics, Colorimetrics

Fully Connected

Wi-Fi, Ethernet, USB

E-mail, LIMS, Printers, Networks

The DeNovix DS-11 FX Series offers your choice of combined UV-Vis and Fluorometer modes in one space-saving unit. Pre-installed EasyApps® and an intuitive Android operating system make it easy to rapidly quantify samples. DeNovix instruments are calibrated for life and include an industry leading three-year factory warranty.

NanoDrop™ ND-1000 Retirement Offer

Manufacturer support for the ND-1000 spectrophotometer will end in December 2017. For details of promotional packages available to NanoDrop™ users wishing to upgrade to the DS-11 series, visit www.denovix.com/special-offers.

SCIENTISTS' CHOICE AWARDS 17 WINNER

Reviewers' Choice Life Science Product of the Year

"This is a wonderful product, compact and easy to use."

Rating: 5.0 ★★★★★

"I love it, because of the accuracy and convenience."

Rating: 5.0 ★★★★★

Recommended by Scientists Worldwide • Free Trial Program

SelectScience 2017 GOLD Seal of Quality

Biocompare The Buyer's Guide for Life Scientists ★★★★★

SelectScience® Independent Product Reviews ★★★★★

TheScientist TOP 10 INNOVATIONS

Puzzle Me This

Looking at words on many different levels

BY MARY BETH ABERLIN

A secret I would like to share: I am addicted. My particular compulsion is the need to try my hand at any word puzzle I encounter: crosswords, double-crostics, whirlpools, switchbacks, scrambles, you name it. Midair, thumbing through in-flight magazines; in doctors' waiting rooms; perusing the tabloids; wherever, I'm licking my pencil. I have withdrawal pangs if I can't get my hands on the two puzzle pages published every Sunday in *The New York Times Magazine*. I have a crush on Will Shortz.

One of my favorite types of word teasers (sadly, not found in the *Times*) is the cryptogram, which requires deciphering a quote or sentence encoded using the 26 letters of the English alphabet. I love cryptograms because they allow me to fantasize about what it might have been like to be at Bletchley during WWII (obviously, a most elementary-level fantasy).

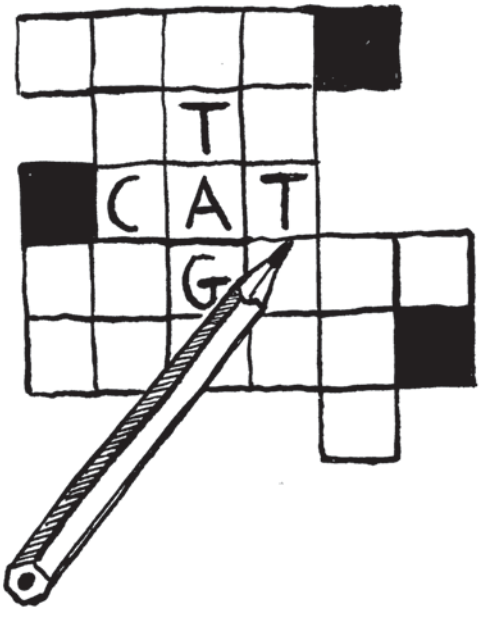
Of course, the work of Alan Turing and all those dedicated to cracking the Enigma code contributed to the invention of modern-day computers and their storage of information using binary units of 0 and 1. It makes me wonder how astonished those codebreakers would be if they could read this month's cover story about encoding messages in DNA to solve the problem of how to store the mind-boggling amounts of data generated in this day and age. In "DNA Hard Drives" (page 32), our new assistant editor Catherine Offord reports on how researchers, using admittedly gimmicky proofs of concept (a poster, a book, a movie, and a computer operating system, among others), are capitalizing on the incredible capacity of the double helix—derived from its four-base sequence—to archive "well into the millions of gigabytes per gram." Sounds great, but there are practical challenges that range from the actual machines used to construct the DNA fragments, to data-storage and reading errors, to picking and choosing exactly the particular data one wishes to retrieve. It's a fascinating story.

The writing and reading of DNA code figure centrally in several other stories in this issue. A profile of Harvard Medical School's Stephen Elledge details his career spent determining how eukaryotic cells have an internal sensing and signaling system to respond to DNA damage (page 58). Elledge describes the molecule's initial lure thus: "The fact that you could take [DNA] apart and put it back together and test ideas on genes—that totally blew my mind." In "Designer DNA" (page 65), Rachel Berkowitz describes some of the computational tools available to synthetic biologists for reducing sequence errors, predicting protein structure, gleaning function from sequence data, and improving the design of gene circuits that work together. For a red-hot tinkering technique, check out the CRISPR patent primer on page 68.

Coding data into DNA and decoding it from the resulting sequence really does have an allure akin to solving word puzzles.

Coding data into DNA and decoding it from the resulting sequence really does have an allure akin to solving word puzzles. When, out of the blue, puzzlemakers extraordinaire Henry Cox and Emily Rathvon contacted *The Scientist* offering to supply us with science-related word puzzles, they tapped right into my addiction. No way was I going to refuse this offer from two more of my puzzle-making heroes. So you will note some changes to our quotes page, "Speaking of Science" (page 14). This month features the duo's first offering: a crossword puzzle.

At *TS*, words are important to us. Why? A recent paper in *eLife* finds that the lingo in scientific journals has made research articles unintelligible to all but the most highly educated, leaving lay readers more often than not at a loss to understand the relevance of published findings. Decoding scientific literature is what we love to do, and we constantly strive to bring you well-written and timely articles that are as jargon-free as we can make them. ■

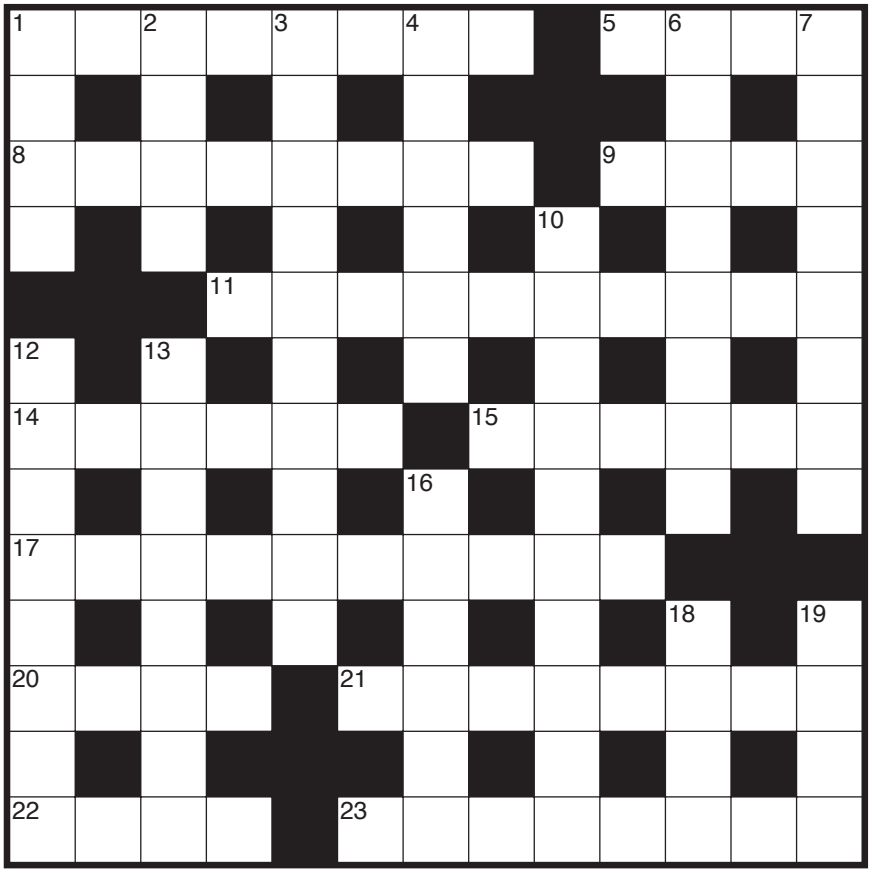


MBA

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

ANDRZEJ KRAUZE

Speaking of Science



BY EMILY COX AND HENRY RATHVON

ACROSS

1. What a serious birder maintains (2 wds)
5. Cut, as DNA with CRISPR
8. Author of *A Natural History of the Senses*
9. Founder of the Sierra Club
11. Pursuit involving drones
14. Happening by chance, like some mutations
15. Bone between knee and ankle
17. Cephalopod in *20,000 Leagues Under the Sea* and *Dr. No* (2 wds)
20. Substance with a pH lower than 7
21. "Lucy" or "Ardi," in anthropology
22. What digitigrades go on
23. Collective DNA of a population (2 wds)

DOWN

1. Photosynthesis factory
2. Like jackalopes or "Piltdown man"
3. Bass with a distinctive jaw
4. Apex predators of the sea
6. Source of a spiral in nature
7. Landlocked country with jaguars, tapirs, and harpy eagles
10. Small, edible whelk; violet groundcover
12. What a male seahorse can become
13. Like kudzu or zebra mussels
16. Like quaggas and hinnies
18. What a coronavirus's ringlike fringe suggests
19. Amoeba or diatom, essentially

Answer key on page 5

Science denial, as a behavior rather than a label, is a consequential and not-to-be ignored part of society. . . . When people ignore important messages from science, the consequences can be dire.

—George Mason University cognitive scientist **John Cook**, in an essay on the dangers of discounting scientific fact in policymaking and public opinion (*National Review*, May 2017)

I think it is pretty dumb not to ask some hard questions about why more rain is now falling, and has fallen in the Houston area, as I understand it, than any time that people can have measured.

—Senator **Bernie Sanders** (D-VT), on the possible contribution of climate change to Hurricane Harvey (*CNN*, August 31)

"Gentlemen, it's time we gave some serious thought to the effects of global warming."

Buy a UV5Nano, get a FREE RAININ pipette and tip box

UV5Nano

One Drop UV/VIS Spectroscopy

- Measure wide DNA and RNA concentration ranges precisely
- Double application power wrapped in ergonomic design
- Fast and reliable results within two seconds
- Save precious samples, only 1 µL of sample is required
- Ready-to-use direct DNA, RNA and protein measurements

reddot award 2016
winner

For more information:

www.mt.com/microvolume-uvvis

METTLER TOLEDO

Notebook

OCTOBER 2017

IMAGE COURTESY OF KRAIG BIOCRAFT LABORATORIES

Spinmeisters

In Jon Rice's office is a small incubator full of tiny insect eggs—one of many such incubators kept at Kraig Biocraft Laboratories (KBL), the Michigan-based polymer development company where Rice is chief operations officer. From these eggs will hatch tiny silkworms, caterpillars of the domesticated silk moth *Bombyx mori*, which will then set to chomping down on mulberry leaves and preparing themselves for the demanding task of spinning silk cocoons to pupate in just a few weeks later.

But these are no ordinary silkworms, a fact you might notice "if you know what you're looking for," Rice says. For a start, "the eyes and the feet of our silkworms glow, if you look at them under the right UV filter," he explains. And the cocoons the silkworms later produce "have a slight greenish hue."

The glow comes courtesy of a fluorescent protein used as a marker to confirm that several genes for spider-silk proteins have been successfully edited into the silkworm genome. Unlike regular domestic silkworms, which are reared in teeming millions around the world to spin fibers for use in clothing and furnishings, KBL's stock is raised to produce what the company hopes may be one of the toughest fibers on earth.

Combining strength comparable to that of steel with an elasticity per weight that rivals rubber's, spider silks have some of the highest measures of toughness—a reflection of the energy needed to break the material—of any fiber out there, beating the sturdiest man-made fibers such as nylon or Kevlar several times over. "Spider silk is unique," Rice says. "We can't recreate that synthetically." Researchers have long touted uses for such a material in varied applications, including parachute cords, high-performance sportswear, and, thanks to spider silk's unusual biocompatibility, suture thread.

However, obtaining silk from spiders presents a considerable challenge. For a start, spiders' webs consist of multiple types of silk—not all of which have desirable mechanical properties. Worse,

SILK MERCHANTS: With the help of inserted spider genes, these newly hatched transgenic silkworms can spin silk that is closer to that spun by arachnids.

GMMMMMMMM: Transgenic silkworm adults at Kraig Biocraft Laboratories munch on mulberry leaves in preparation for spinning their spidery silken cocoons.

IMAGE COURTESY OF KRAIG BIOCRAFT LABORATORIES

"spiders have two personality defects," explains Randy Lewis, director of the Synthetic Bioproducts Center at Utah State University, and one of the inventors of the first spider silk–spinning transgenic silkworm. "They're both cannibalistic and territorial. Unlike silkworms, where you just throw a bunch of them in, and they eat and keep perfectly happy, spiders want to have a certain amount of room and will kill to keep that room."

Consequently, the last few decades have seen multiple attempts to take live spiders out of the equation. At KBL, "we think the silkworm approach is the best way forward," Rice says. "Silkworms make silk. That aspect is fully understood. The only challenge we have is changing the recipe." The company has created 20 transgenic lines of silkworms that spin cocoons containing spider silk proteins. Dragon Silk, one of the latest products made from the fibers of these cocoons, is stronger than steel and tougher, lighter, and more flexible than Kevlar (though it has slightly lower tensile strength than this synthetic fiber). The company now holds a million-dollar contract with the US Army, which is exploring possible uses in defensive clothing and other gear.

But some researchers point out that the transgenic silkworm approach has its own pitfalls. "Silkworms and spiders do make silk in different ways," says Karolinska Institute silk researcher Janne Johansson. Silkworms spin thicker fibers than arachnids do, and add their own proteins in addition to the fibers themselves. For example, their silk contains a protein called sericin, a gluey substance that sticks fibers together in the cocoon, Johansson says. "You need to treat silk from *Bombyx* before you use it for anything."

The internal spinning machinery differs between the two organisms too. In a spider's silk gland, unspun silk, or "dope," passes through a pH gradient, gradually being exposed to more-acidic conditions that help silk proteins aggregate. Silkworms also have a pH gradient, "but it's less pronounced," Johansson says. "To the extent that spider silk proteins are optimized for experiencing this more extensive pH gradient in the spider silk gland, it will not work [the same way] in the silkworm."

Several research groups have turned to other organisms to produce spider silk proteins. Lewis's group made headlines in the 2000s with the creation of transgenic goats whose milk contained large quantities of silk proteins usually made by the arachnids. Other approaches include the creation of transgenic tobacco plants, potato, alfalfa, yeast, and biology's go-to bacterium, *E. coli*, which is "simple, easy, and cheap to scale up," says Thomas Scheibel, a biomaterials researcher at the University of Bayreuth in Germany. "It's a nice system."

Of course, without a spinning host, researchers have to spin the silk themselves. After tweaking the proportion of silk proteins expressed in *E. coli*, Scheibel's group recently used wet-spinning—drawing fibers from silk proteins that have been allowed to self-assemble in a bath of water and alcohol—to make fibers with a toughness comparable to that of natural spider silk, albeit with lower tensile strength (*Adv Mater*, 27:2189-94, 2015).

And earlier this year, Johansson, along with Karolinska Institute collaborator Anna Rising, published an approach that mimics pH changes in a spider through what Johansson calls "an almost embarrassingly simple setup." After keeping a highly concentrated solution of *E. coli*–produced recombinant silk proteins at pH 7.5, "we pump it through a narrow capillary and out into a beaker filled with buffer solution at pH 5," he says. "That turns it almost instantaneously into a silk fiber that we can reel up." The result is the toughest artificial spider silk fiber so far (*Nat Chem Biol*, 13:262-64, 2017).

At this rate, scaled-up, cheap production of reliably tough, recombinant silk fibers may be only a few years away. "We feel pretty good about it," Lewis says. "Do we have the same properties as spider silk? No. Are we close? Yes." The key now, as many researchers see it, will be finding the right applications for these fibers once they're made. "You have to look for, 'Where does silk give us the boost?'" says Scheibel, whose spinout company AMSilk also works on non-fiber spider silk applications, such as biocompatible coatings for silicone breast implants and 3-D–printed scaffolds for biofabrication. "Not just, 'I make a product because I can do it.'"

Lewis agrees. "The unique property of spider silk is a combination of elasticity *and* strength," he says, adding that many purported applications are likely to add little value by incorporating the fibers. "I guarantee you I can make a bulletproof

vest—it's just going to stop the bullet on the wrong side of your chest. If you just want strength, use Kevlar."

—Catherine Offord

Cage Sweet Cage

"What human trial would propose studying the effect of a drug only in 43-year-old males who are all twin brothers living in one small town in California, with identical studio apartments, identical educations, identical monotonous jobs, identical furniture, identical monotonous diets, identical locked thermostats set to uncomfortably cold temperatures, where the house is cleaned by a grizzly bear that erases all of their social media every two weeks?"

This challenge to fellow biomedical researchers, issued in the pages of the journal *Lab Animal* in April of this year (doi:10.1038/laban.1224), was accompanied by a proposal that was no less bold: solve science's reproducibility crisis and problems with translating laboratory biology to humans by overhauling how animal research is conceived of and conducted. The five authors of the review argued that part of that overhaul should be a greater focus on research animals' needs.

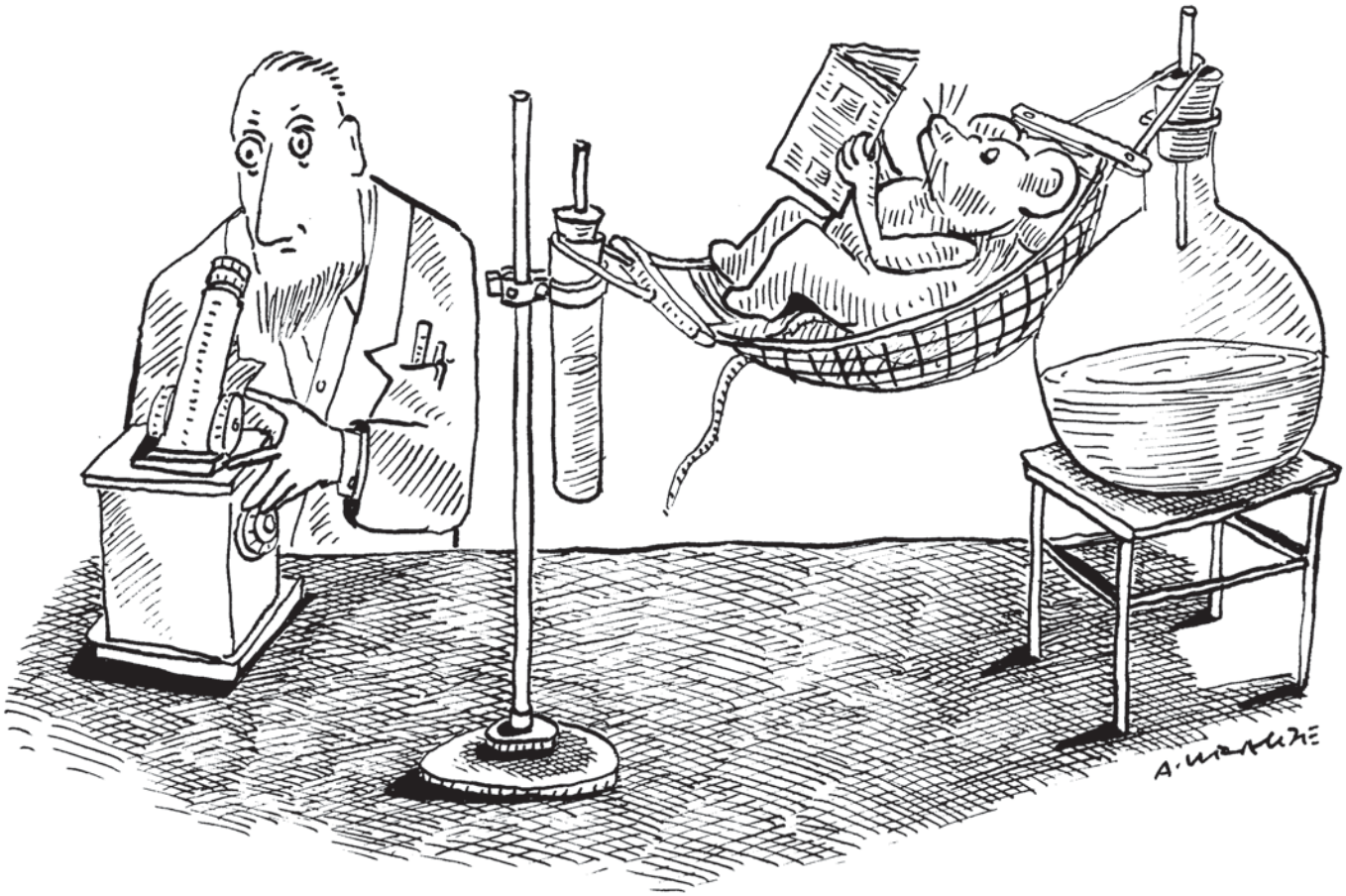
A few months later, neuroscientist Garet Lahvis of the Oregon Health & Science University took to *eLife* (6:e27438) to suggest that conventional laboratory cages should be done away with altogether in favor of large, naturalistic enclosures, or allowing research animals to roam freely in the wild, tracked and monitored with GPS and other technologies.

For many, the relationship between animals' well-being and the validity of research results may not seem immediately obvious, but to Lahvis, a clear pattern emerges from decades of experimentation in disparate fields. "I think biomedical researchers are making a big mistake. . . . The mistake is that we keep studying rodents and primates in cages to understand human beings that are not in cages," he says. Using caged animals as models of some aspect of human experience is, he says, "just a step I can no longer take. I don't think that's valid."

Animals shouldn't be furry test tubes; they should be practice patients.

—Kathleen Pritchett-Corning, Harvard University

Kathleen Pritchett-Corning, a lab animal veterinarian at Harvard University and one of the authors of the *Lab Animal* review, reached a similar conclusion. "Animals shouldn't be furry test tubes; they should be practice patients . . . because a lot of this research goes on to be translated into humans," she says. "The more you can respect the



ANDRZEJ KRAUZE

biology, the behavior, and what a mouse needs, the better you're going to find your results in terms of getting honest answers about the translatability of your work to the human platform." For example, she says, mice are stressed by puffs of air in today's individually ventilated cages; commonly used corncob bedding contains phytoestrogens that have been shown to adversely affect mouse health; and transparent cage walls are another source of stress. "The animals, I think, would prefer opaque caging, where these apex predators walking around every day taking care of them can't see them," she says.

Even a practice that would seem to protect animals—avoiding any exposure to pathogens—may render results less translatable to people and uncaged animals, who exist in a soup of germs. Lahvis cites a finding that wild mice have a type of T cell found in humans, but not in laboratory mice (*Nature*, 532:512-26, 2016). "There's probably no human being on earth that's immunologically naive, or very few of them," he says.

Pritchett-Corning says that funding is virtually nonexistent for studies on how housing conditions affect results; many published findings have been accidental outcomes of experiments gone awry. There are exceptions, however. In the 1980s, researchers at the University of Illinois Urbana-Champaign compared the brains of mice reared in a "complex" environment, with toys and daily access to a larger space, to those kept in smaller, toyless cages either by themselves or with another mouse. The differences in the mice given toys included more synapses and mitochondria per neuron, and more capillary volume. More recently, separate studies have shown dramatic effects of social isolation on the ability of mice and rats to survive breast cancer.

When it comes to remedies, though, Lahvis and the review authors part ways. Lahvis acknowledges that studying animals in the wild or in large, naturalistic enclosures would not be an easy change to implement. "There are probably obstacles to the vision that I see now, but there are a lot of brilliant scientists out there that can figure this out," he says. His own lab has studied the social lives of squirrels in captivity and in the wild, and found similar results in each. "I'm not saying biomedical research is wrong," Lahvis says. "I'm saying, we've got to pay a hell of a lot of attention to this, and really move in this direction, because . . . at the level of biological systems, we don't know when we're right or when we're wrong."

Review coauthor, Brianna Gaskill, an applied pathologist at Purdue University, counters that even if those changes could be implemented, they would bring problems of their own. "On one hand, maybe behaviorally it might be ideal, but on the other hand you're probably going to have a lot more health issues," she says. As for much larger enclosures, "it's probably not something that, at least, we're going to see on a wide-scale basis, especially for mice,

just because of the sheer volume of animals that we use and the volume of space you would need in order to accommodate that new housing."

In lieu of such a radical shift, small adjustments to lab animals' environment, such as cages that offer places to hide, or provision of sufficient bedding, could make a big difference, suggests Pritchett-Corning. (See "Mouse Traps," *The Scientist*, November 2014.) Even these tweaks face the obstacles of rewriting current protocols, potentially wasting money sunk into existing infrastructure, and navigating the limited offerings of laboratory cage manufacturers. But, she says, they have a greater chance of being implemented than Lahvis's suggested overhaul.

The review authors also argue that to make their results more robust, animal researchers may need to take a page from those who conduct studies on humans that allow a bit more variability into their experiments, not less. "Variability is not really our enemy," says Gaskill.

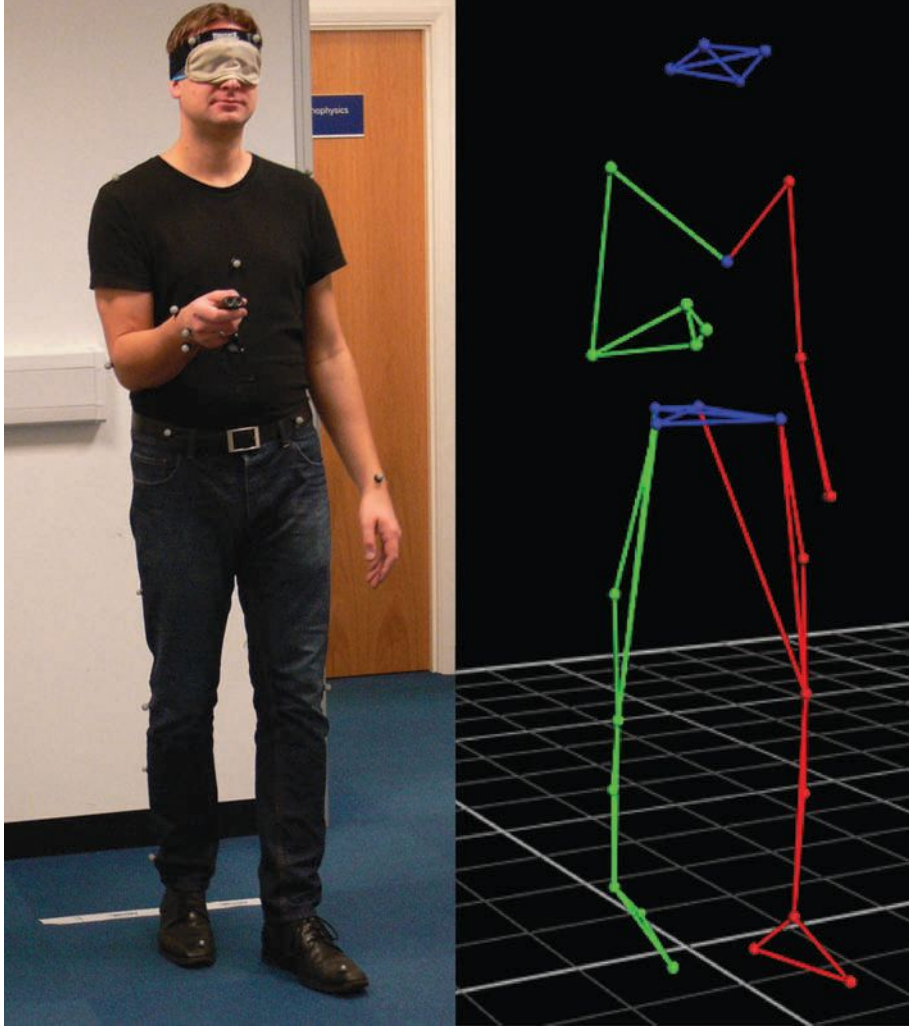
For Lahvis, though, adjustments to the current cages would not be enough. "A standard cage is about 280,000 times smaller than [a mouse's] natural home range. For a rhesus macaque it's 7 million times smaller," he says. Increasing the size of the cage by a bit and adding toys or furniture for the animals is "not enrichment; it's just less harsh impoverishment," he says.

—Shawna Williams

Click Bait

Daniel Kish, an expert echolocator, uses sound to see the world. After losing his eyesight to retinoblastoma at the age of one, he learned to navigate using the noise from his tongue clicks bouncing off nearby surfaces. Dubbed "Batman" for his abilities, Kish is able to independently bike down streets and hike through the wilderness with ease.

While some may perceive echolocation as an almost superhuman sense, it's a surprisingly ubiquitous ability. Although the vast majority of people are unable to navigate using echolocation alone, even those without training can use this skill to sense their environments—for example, by hearing the difference between standing in a cathedral and a soundproof room. "We hear echoes all the time," says Daniel Rowan, an audiologist at the University of Southampton in the U.K. "What blind people [like Daniel Kish] are doing is . . . putting together a range of different skills that we already have, more or less, and taking them to a level of expertise that you and I wouldn't have."



BATTING PRACTICE: Participants in Daniel Rowan's experiment tried to navigate a virtual environment using only their sense of hearing.

AMY SCARFE

For people who lack sight, echolocation can be a valuable skill. Since this technique is particularly useful for detecting objects at eye level, it is typically used as an addition to—rather than a replacement for—canes and guide dogs, which are helpful for identifying things on the ground.

As people age, however, their hearing often worsens, which can impede their ability to use echoes to identify their surroundings. Rowan wants to tackle this issue by first trying to piece together what types of acoustic information people with normal hearing can use for echolocation.

To address this question, Rowan and colleagues recently conducted a series of experiments on both blind and sighted participants who were inexperienced in echolocation. The trial subjects wore headphones through which played long sequences of clicks that Rowan calls "virtual objects." His team created these in a soundproof room by placing items around an acoustic mannequin (a model of the human head and ears). The researchers played a sound near the mannequin, which would hit the object and bounce back to the model head where it was recorded, simulating the perception of reflected sound that an echolocator might hear.

"The advantage of doing what they did is that you have good control over the information that people are getting—that's important because we know relatively little about the acoustic cues that people may use," says Lore Thaler, a psychologist at Durham University in the U.K. who was not involved in the work. "[However,] I think that an important step would be to

DISCOVER
THE NEXT WAVE
OF IMMUNE CELL ANALYSIS

Detect Immune Cell Activation in Real Time with Agilent Seahorse XF Technology

Don't wait hours for immune cell activation results. Agilent Seahorse XF technology detects activation within minutes of stimulation by measuring discrete changes in cellular bioenergetics in live cells, in real time. Rapid detection opens a window to the earliest events of immune cell activation, enabling researchers to identify changes in activation kinetics and the magnitude of activation.

Accelerate immune cell activation analysis from hours to minutes

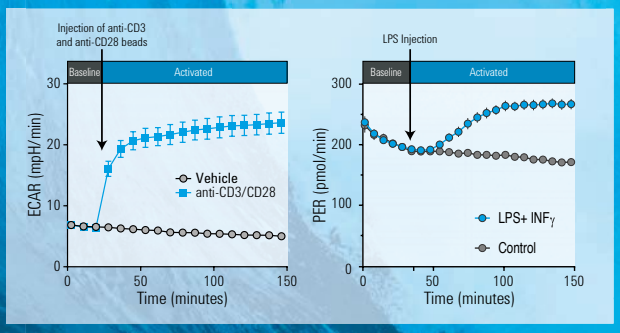
Modulate activation with integrated compound injection ports

Investigate immune cell metabolism through standardized assays and software

Learn more about Agilent Seahorse XF products and immune cell analysis at **www.agilent.com/chem/immunology**

T cell Activation Assay using naïve T cells

Macrophage Activation Assay using polarized M1 macrophages



Agilent Technologies

While some may perceive echolocation as an almost superhuman sense, it's a surprisingly ubiquitous ability.

try to get from this paradigm to a paradigm where one can use a similar [method] but participants make their own mouth clicks."

Rowan's team discovered that inexperienced, sighted listeners could detect objects up to four meters away. However, these individuals performed poorly on tasks where high frequencies were removed from the acoustic stimuli. The researchers found similar results in their small sample of five blind participants (*Hearing Research*, 350:205-16, 2017).

According to Thaler, her lab and others have found that people who use higher-frequency clicks tend to perform better on echolocation tasks (*PLOS ONE*, 11:e0154868, 2016; *J Neuro*, 37:1614-27, 2017). "This is a correlation, but it fits with what they have found here, [which is] that there's something within the higher frequency range that's informative," she says. "When we teach these mouth clicks, knowing that people who make brighter clicks tend to perform better, we emphasize that people make a click that's relatively brief and bright."

"[This study] is quite nice because it [highlights] the importance of high-frequency listening," says Andrew Kolarik, a research associate studying echolocation at the University of Cambridge who was not involved in the work. "Unfortunately, this seems to be the frequency that's the first to go as people get older and they start to lose their hearing."

Kolarik adds that the finding that individuals can use echoes to identify objects up to four meters away "makes us think that [this] is the possible level to aspire to, and that maybe we can train people or change the environment to try to make it easier to use echolocation." He points out that previous experiments, including his own, have found that effective echolocation is only possible at much shorter distances—around two meters at most (*PLOS ONE*, 12: e0175750, 2017).

More studies are necessary to tease apart what types of useful information high frequencies provide. However, researchers are now starting to think about ways to help blind individuals with compromised hearing.

According to Rowan, one potential approach will be to develop technologies to convert those high frequencies to lower frequencies. He adds that most conventional hearing aids are unlikely to fulfill this need because they don't usually receive frequencies beyond the 3,000 Hz typically required for echolocation. Even those hearing aids with extended bandwidth, between 8,000 to 10,000 Hz, may be limited, as recent evidence (*PLOS ONE,* 13:e1005670, 2017) suggests there is useful information to echolocators beyond those frequencies as well.

"We need an improvement in technology in order to give blind people access to the information that's above the current reach of hearing aids," Rowan says. "We all lose our high-frequency hearing as we get older. So that's a problem that health-care services need to tackle for blind people."

—Diana Kwon

The Bitter Taste of Preterm Labor

Over the past 15 years, researchers have begun to discover that the taste receptors that sense sweet, bitter, salty, sour, and umami flavors are found in tissues far removed from our mouths. For example, taste receptors expressed in the gut appear to play a role in digestion, while receptors in the airway may play a role in respiration. (See "What Sensory Receptors Do Outside of Sense Organs," *The Scientist*, September 2016.)

When Ronghua ZhuGe, a physiologist at the University of Massachusetts Medical School, came across a 2010 study that had identified bitter taste receptors on human airway smooth muscle cells (*Nat Med*, 16:1299-304), he was intrigued. The paper's authors had found that activation of these receptors caused the cells to relax, dilating the airway. The research-

TARGETING TASTE: Will doctors one day be able to drug taste receptors in the uterus to prevent preterm labor?

ers hypothesized that calcium-activated potassium channels underlay the taste-mediated relaxation, but they didn't measure this directly. Having studied ion channels for many years, ZhuGe wanted to investigate this mechanism further.

Directly measuring the channel function with a patch clamp, ZhuGe and his colleagues instead identified a novel mechanism involving calcium channels (*Nat Med*, 18:648-50, 2012; *PLOS Biol*, 11:e1001501, 2013). Around the same time, other researchers were reporting the presence of bitter taste receptors on cells in the smooth muscle tissue that lines blood vessels. ZhuGe wondered if the mechanism his team had identified might also be at play there, as well as in a third type of smooth muscle—the myometrium, which forms the middle layer of the uterine wall. If bitter taste receptors had a similar relaxing effect in those tissues, he thought, perhaps targeting them could prevent or interrupt preterm labor, a condition which medicine has made little progress in treating.

ZhuGe had already collaborated with researchers in China who study reproductive biology, so he quickly got them on board to explore the presence of bitter taste receptors in the uterus. Sure enough, examining both mouse and human tissue, the group found certain subtypes of bitter taste receptors expressed in myometrial muscle cells. The next step was to see what happened to the tissue when those receptors were activated.

The researchers isolated strips of uterine tissue and placed them in a device that measures the contractile force that muscles generate. They then triggered the muscle to clench by exposing it to oxytocin, a hormone known to initiate contractions during labor. Next, they added a bitter compound called chloroquine and found that it caused the muscle to relax. "It completely [reversed the contractions] in the myometrium," ZhuGe says. "That's striking." The team also tested four medications currently used to treat human preterm labor, all of which induced only partial muscle relaxation.

Steve Liggett, a molecular biologist now at the University of South Florida's Morsani College of Medicine who coauthored the 2010 publication on the relaxation effect of bitter taste receptors in the airway, says he wasn't surprised by the finding. "The lineage of airway smooth muscles, vascular smooth muscle, and myometrial muscle is all the same, and I had a feeling that these receptors were going to be present in all of them. And that has turned out to be the case."

To see if the same thing would happen in vivo, ZhuGe and his colleagues tested the effect of chloroquine in mice. The researchers injected the bacterial endotoxin LPS, which initiates preterm labor, into the uteruses of pregnant ani-

mals. Three hours later, they injected chloroquine into the uteruses of some of the mice. While chloroquine did not prevent preterm labor in 100 percent of these mice, it delayed birth by two days in about half of them, and all of the delayed litters survived. By comparison, mice treated with one currently used human medication (magnesium sulfate) all delivered their litters the day of labor induction, and none of the pups survived. A second current medication (albuterol) provided protection more in line with chloroquine, delaying birth in half the animals, and most, but not all, of the pups survived (*FASEB J*, doi:10.1096/fj.201601323RR, 2017).

However, Wolfgang Meyerhof, a molecular biologist at the German Institute of Human Nutrition, warns that it's too early to make any claims about a potential treatment for preterm labor that targets bitter taste receptors in the uterus. More information is needed about the abundance of the receptors in the uterus, he says, as well as the types of compounds that target them. He also noted that bitter compounds likely have off-target effects.

But Liggett says he is "very encouraged by the data." He notes that off-target effects may be lessened by the fact that bitter compounds could conceivably be administered directly into the uterus, and adds that researchers have plenty of candidates to start testing. "There are probably 10,000 known compounds that activate these receptors." There are risks, however. For example, evidence is accumulating that the activation of bitter taste receptors in the vasculature has a similar relaxation effect. "I would not be surprised that the blood vessels that go to the uterus are also vasodilated when you're treating," says Liggett. "That would mean that . . . the bleeding risk would be higher."

More research is needed to assess the practicality of targeting bitter taste receptors in the uterus to treat preterm labor. But as more studies surface about the roles of these receptors around the body, Liggett is excited to learn more about their functions outside of taste. Earlier this year in *Cellular Signalling*, he and Steven An of Johns Hopkins Bloomberg School of Public Health proposed that bitter taste receptors and other odorant receptors comprise an entire chemosensory system expressed all over the body. "By the time we're done, it's going to turn out to be one of those situations where the original assignment of the receptor location is relatively minor," he says. "It just happens to be what they came across first."

But Meyerhof disagrees. "I think they are, in the first instance, bitter taste receptors." For one thing, research points to their expression being much higher in tongue tissue than in other tissues, he says, and "if these receptors would carry out predominant functions outside the taste systems, we would have discovered these receptors much earlier. The fact that we detected these receptors late . . . tells me at least their main action is in taste."

"I'm not saying there is no action," he adds, "but I'm saying I think we have a number of premature reports, and we have to do much more work to be clear about these extra-oral taste functions." —Jef Akst

INSIGHTS AMPLIFIED

Shape the future of neuroscience

By enabling the spread of new information and ideas, we make it easier for neuroscientists to extend the reach, and influence, of their work. The challenges ahead are big, and so is our network.

Join us for our 5th Annual Satellite Symposium Event at NEUROSCIENCE 2017

NEUROINFLAMMATION:
Determining Molecular and Functional Heterogeneity

Learn more or Register at
SigmaAldrich.com/sfn

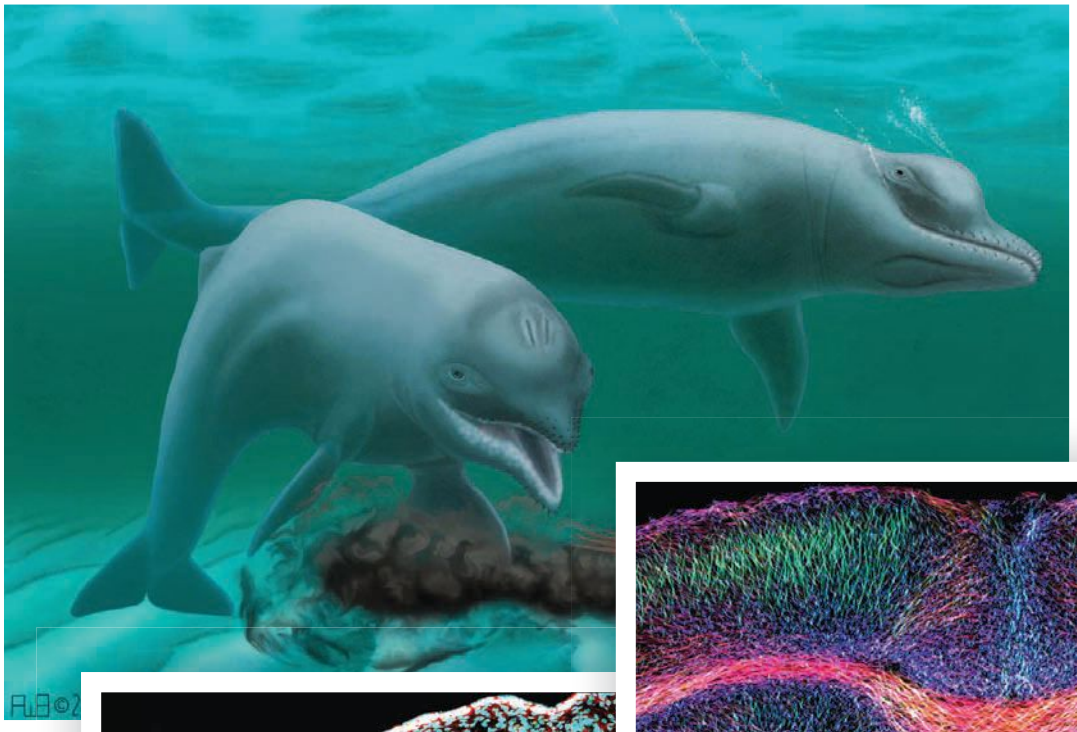
The life science business of Merck KGaA, Darmstadt, Germany operates as MilliporeSigma in the U.S. and Canada.

MilliporeSigma

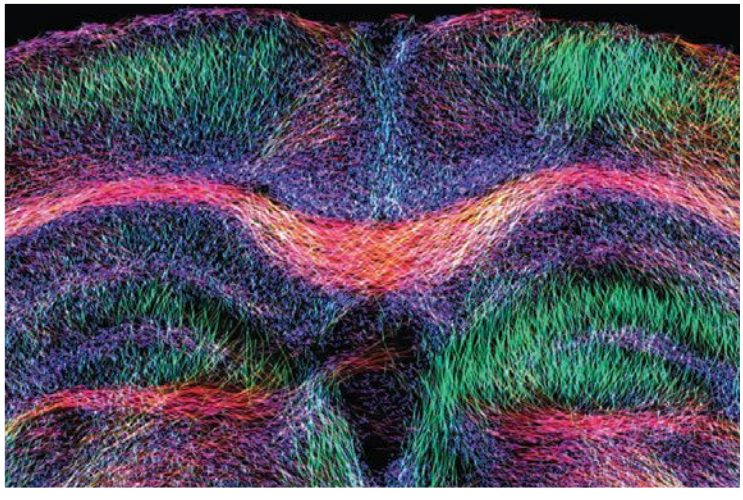
2017-01161

Caught on Camera

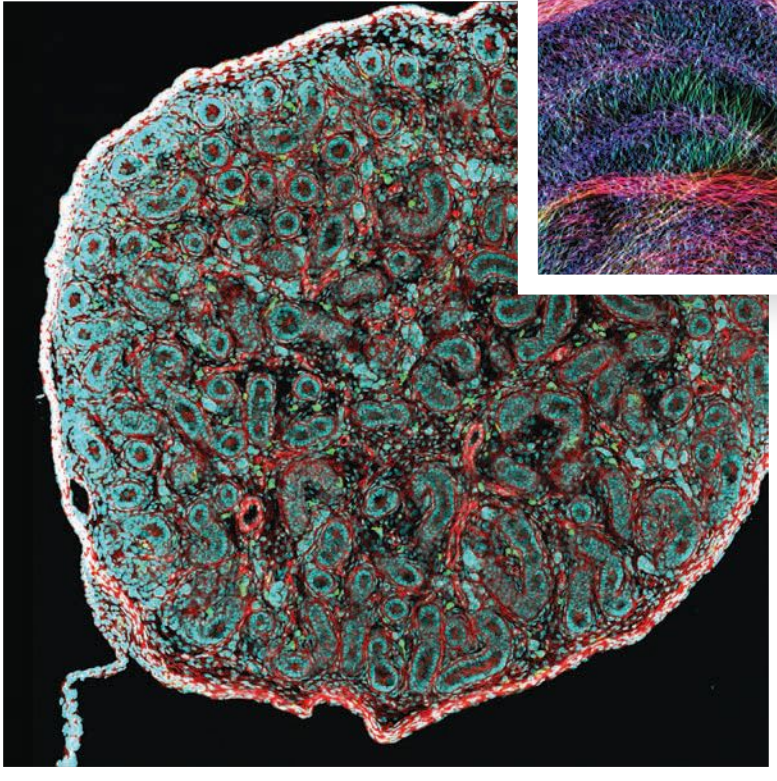
Selected Images of the Day from the-scientist.com



« NO TABLE MANNERS
Ancient bones of the newly described toothless, stout-nosed dolphin (*Inermorostrum xenops*), depicted here in an artist's rendering, suggest that the animal slurped its food.
Posted: *August 24, 2017*



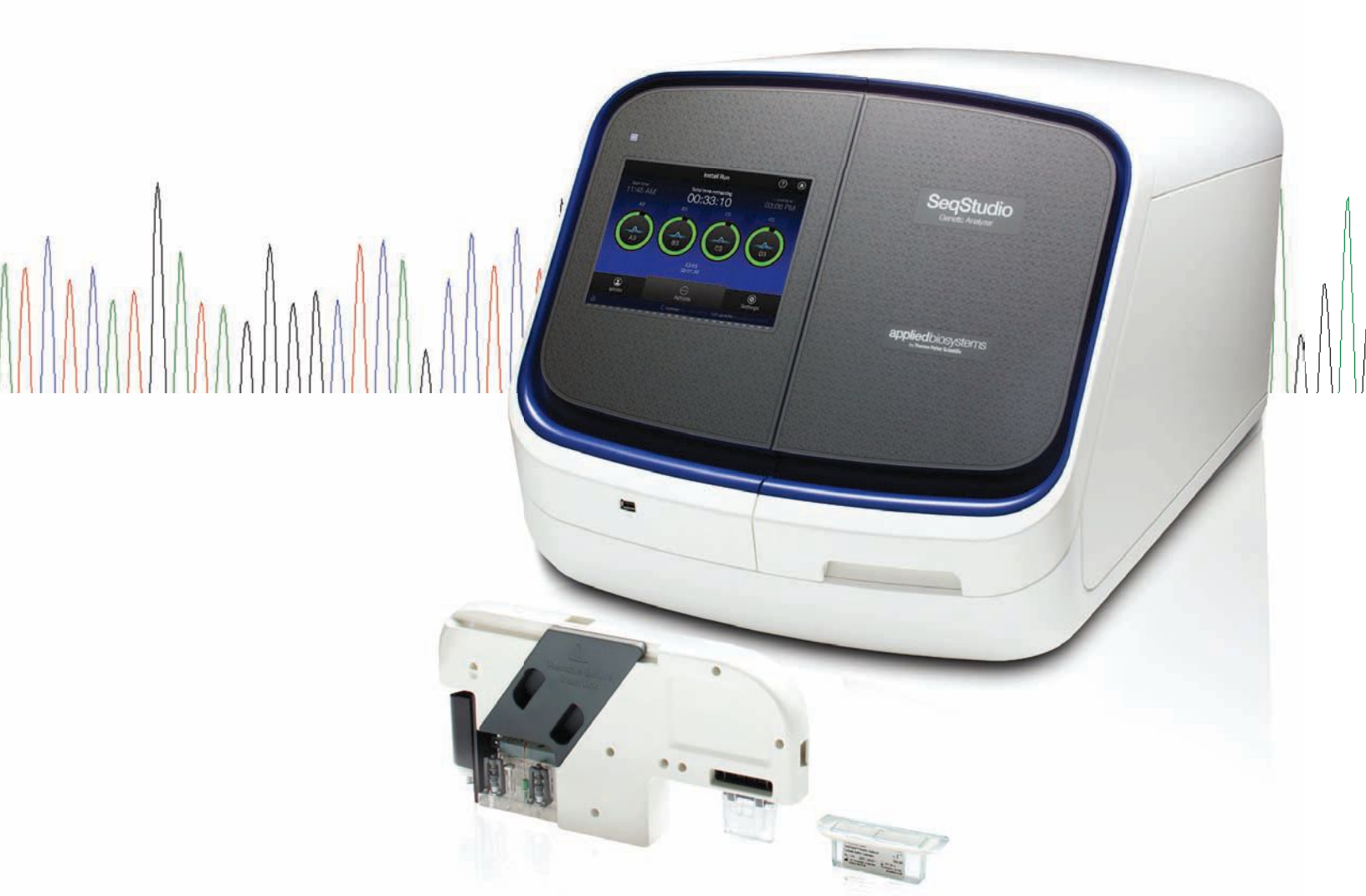
RAINBOW MATTER »
Using diffusion-weighted tractography, scientists have produced a detailed image of the minute neural fibers within a mouse brain to relate microstructural alterations to neurological diseases.
Posted: *August 14, 2017*



« SPERM PROTECTORS
Among their many functions, macrophages (green) in mouse testes guard sperm against attacks by other immune cells.
Posted: *August 18, 2017*

CLOCKWISE FROM TOP: © ROBERT BOESSENECKER; NIELS SCHWADERLAPP, DEPARTMENT OF RADIOLOGY, MEDICAL PHYSICS, UNIVERSITY OF FREIBURG, GERMANY; © NOUSHINE MOSSADEGH-KELLER AND SÉBASTIEN MAILFERT/CIML

appliedbiosystems



Nothing has changed, **except everything**

Sanger sequencing and fragment analysis like you have never experienced before. Same workflow, same trusted technology, now with an innovative all-in-one cartridge that takes setup time from hours to minutes. Introducing the **Applied Biosystems™ SeqStudio™ Genetic Analyzer**.

Find out more at **thermofisher.com/seqstudio**

ThermoFisher SCIENTIFIC

Live-Cell Extractions

Nanostraws that collect specimens from cells without killing them allow for repeated sampling.

BY RUTH WILLIAMS

Analyzing cells en masse provides a general idea of the happenings within a given cell type, but misses the subtle yet significant variations between individual cells—variations that may result in different responses to developmental signals, drugs, and other factors.

To better explore the inherent heterogeneity of cell populations, "many people are trying to do single-cell analyses," says Orane Guillaume-Gentil of the Swiss Federal Institute of Technology (ETH). But, she adds, the approaches are limited. "You have to kill the cells, so you cannot see anything dynamic, and you also lose the [spatial] context of the cells."

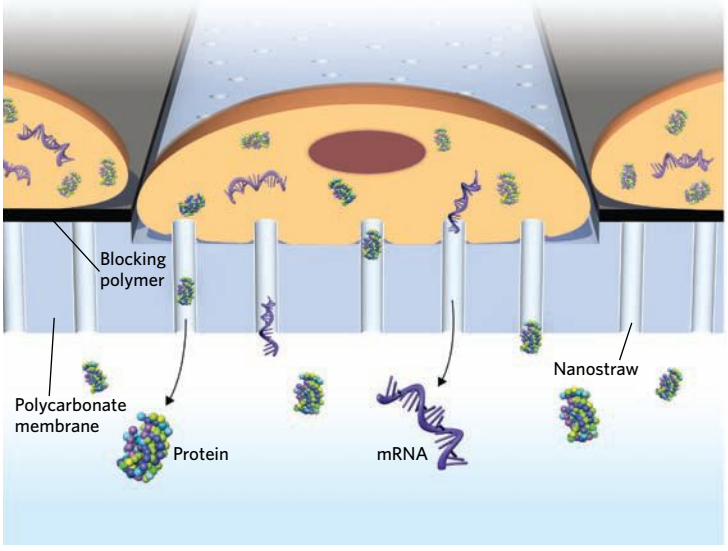
The problem, agrees Nicholas Melosh of Stanford University, is that "you want to know what a cell is, but [current single-cell approaches] tell you what it was."

Researchers are therefore developing nondisruptive techniques to enable the sampling of live cells without having to isolate or destroy them. Melosh recently devised a nanostraw extraction (NEX) technique that is the latest addition to this toolkit.

While previous techniques, from Guillaume-Gentil and others, used nanopipettes, nanotubes, or similar sampling devices that penetrate cells from above, in Melosh's approach, cells are grown on a polycarbonate membrane containing alumina nanostraws, which protrude through the membrane from below in a defined location. The nanostraws do not pierce the cells under normal conditions, but an electric current passed through the straws briefly opens pores in the cell membrane, allowing contents to diffuse into the straws for collection.

By having a nanostraw-dotted surface rather than a single sampling device, Melosh's approach has the potential to become high-throughput, says Guillaume-Gentil, who was not involved in the study.

Melosh's team used NEX to analyze mRNAs and fluorescent proteins expressed in single cells or small groups of cells for a period of several days.



EXTRACTION WITHOUT KILLING: Cells are cultured atop a polycarbonate membrane perforated in spots by vertical aluminum oxide nanostraws. At defined locations, where the membrane has been etched away lithographically, the nanostraws protrude from the membrane and contact the cells. A brief electric voltage is passed across the nanostraws, causing temporary perforations in the cell membrane. This allows small volumes of cytoplasm to diffuse into the nanostraws for collection in the reservoir of extraction buffer below the polycarbonate membrane.

While "other approaches have shown good cell viability and the potential to do such [dynamic studies]," says Guillaume-Gentil, Melosh and colleagues "are the first ones that really showed that they can [do it]. It was an important proof of principle." (*PNAS*, 114:E1866-74, 2017) ■

AT A GLANCE

APPROACH	HOW IT WORKS	DNA MUTATION ANALYSIS	TRANSCRIPTOMICS	PROTEIN ANALYSIS
Current single-cell analyses	Single cells are isolated and lysed and their contents analyzed by sequencing or proteomics methods.	Commercially available kits for whole or partial genome sequencing	Commercially available kits for RNA sequencing	A number of techniques exist, some commercially available, that analyze proteins via antibody binding, mass spectrometry, or other means.
NEX	Live cells in culture have a small portion of their cytoplasmic contents removed for analysis. The cell is minimally disturbed, survives, and can be repeatedly sampled.	Not yet tested	Messenger RNAs from small groups of cells can be analyzed by sequencing, but single-cell analysis is not yet possible.	Specific fluorescent proteins can be monitored over several days in single cells or small groups of cells.

TS WEBINARS

COMING**SOON** | Spheroid Cell Culture: New Dimensions in 3-D Assays

High attrition in clinical trials and the need to replace animal models in a variety of applications has driven researchers to develop in vitro assays with greater physiological relevance. Three-dimensional (3-D) methods are deemed superior relative to layering cells in a monolayer on lab plasticware due to increased extracellular matrix (ECM) formation, cell-to-cell and cell-to-matrix interactions, important for differentiation, proliferation, and cellular functions in vivo. Perhaps the most popular and straightforward method of 3-D cell culture is aggregating cells into spheroids. *The Scientist* is bringing together a panel of experts to discuss spheroid culture systems, and to explore the technical benefits of making the switch from 2-D to 3-D culture. Attendees will have the opportunity to interact with the experts, ask questions, and seek advice on topics that are related to their research.

ANTHONY ATALA, MD
Director, Institute for Regenerative Medicine
W.H. Boyce Professor and Chair
Department of Urology
Wake Forest University School of Medicine

ESMAIEL JABBARI, PhD
Professor, Departments of Chemical Engineering and Biomedical Engineering
College of Engineering and Computing
University of South Carolina

TUESDAY, OCTOBER 17, 2017
2:30–4:00 PM EASTERN TIME

REGISTER NOW!
www.the-scientist.com/3dassays
The webinar video will also be available at this link.

TOPICS TO BE COVERED:

- Using 3-D culture to turn individual cells into organoids and organs
- Novel options for 3-D culture scaffolding

WEBINAR SPONSORED BY:

BioTek

COMING**SOON** | Cancer Stem Cells: Getting to the Root of Cancer

The stem cell theory of cancer implies that anticancer therapies must target and destroy all resident cancer stem cells in order to produce a durable response. Therapies that target cancer stem cells are currently being tested to confirm their safety and efficacy, but research into the vulnerabilities of cancer stem cells continues. To explore the knowns and unknowns in the field of cancer stem cell research, *The Scientist* is bringing together a panel of experts to share their results, as well as the lessons they've learned from studying the root cause of cancer. Attendees will have the opportunity to interact with the experts, ask questions, and seek advice on topics related to their research.

IRVING WEISSMAN, MD
Director, Institute for Stem Cell Biology and Regenerative Medicine
Stanford University School of Medicine

ALKA MANSUKHANI, PhD
Associate Professor, Department of Microbiology
New York University School of Medicine

THURSDAY, OCTOBER 19, 2017
2:30–4:00 PM EASTERN TIME

REGISTER NOW!
www.the-scientist.com/rootofcancer
The webinar video will also be available at this link.

TOPICS COVERED:

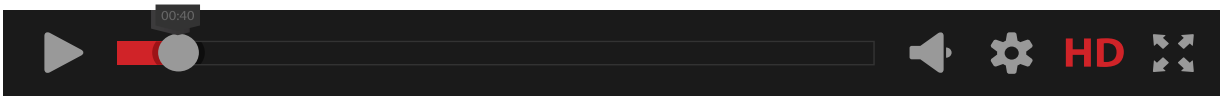
- How stem cells become cancer stem cells
- Methods for constraining the proliferation of cancer stem cells

WEBINAR SPONSORED BY:

METTLER TOLEDO | R&D SYSTEMS a biotechne brand | ROCKLAND antibodies & assays

ONDEMAND | Is Immunotherapy Living up to its Promise?

Immunotherapy has been hailed as a breakthrough for treating the untreatable, but it has not yet lived up to its promise to eradicate cancer and infectious disease. While there have been a number of exciting advances, there are still several real-world hurdles for immunotherapy to surmount before it becomes a standard option for most patients. *The Scientist* brings together a panel of experts to weigh in on the progress that immunotherapy has made, particularly in the search for effective anticancer treatments, and to discuss the steps still needed before immunotherapy becomes standard treatment in the clinic.



WATCH NOW! www.the-scientist.com/promiseofimmunotherapy

JILL O'DONNELL-TORMEY, PhD
CEO and Director of Scientific Affairs
Cancer Research Institute

ALEX Y. HUANG, MD, PhD
Professor, Departments of Pediatrics,
Pathology, BME, and General Medical Sciences
Case Western Reserve University School of Medicine
UH Rainbow Babies & Children's Hospital

TOPICS COVERED:

- Where immunotherapies have succeeded and where they have failed
- Current progress in immunotherapy research, development, and deployment

WEBINAR SPONSORED BY:

Beckman Coulter Life Sciences

ONDEMAND | Mining the Tumor Microenvironment: Advanced Tools and Protocols for Tumor-Cell Signaling

The tumor microenvironment forms a complex, privileged zone where conditions are permissive for unchecked tumor progression. Therein, both cancer and stromal cells exhibit aberrant growth and survival signaling, making pathway analyses ever-more difficult. Advanced tools have enabled deeper, more thorough investigation into how the tumor microenvironment has adapted to evade the immune system, and how we might counteract those adaptations. *The Scientist* brings together a panel of experts to discuss the interplay of cells within the tumor microenvironment and to share their latest methods and findings.

WATCH NOW! www.the-scientist.com/miningthemicroenvironment

SHIJIE SHENG, PhD
Professor, Departments of Pathology and Oncology
Wayne State University School of Medicine
Leader, Tumor Biology
and Microenvironment Program
Karamos Cancer Institute

GAGAN DEEP, PhD
Associate Professor
Department of Cancer Biology
Wake Forest University School of Medicine

TOPICS COVERED:

- Studying pro-cancer signaling within the tumor niche
- New assays for analyzing cell behavior and signaling within the tumor microenvironment

WEBINAR SPONSORED BY:

biotechne

DNA Hard Drives

A few kilograms of DNA could theoretically store all of humanity's data, but there are practical challenges to overcome before nucleic acid storage units become a reality.

BY CATHERINE OFFORD

In the late 1970s, a bizarre theory began making its way around the scientific community. DNA sequencing pioneer Frederick Sanger of the Medical Research Council's Laboratory of Molecular Biology and his colleagues had just published their landmark paper on the genome of virus Phi X174 (or ϕX174), a well-studied bacteriophage found in *E. coli*.[1] That genome, some said in the excitement that followed, contained a message from aliens.

In what they termed a "preliminary effort . . . to investigate whether or not phage ϕX174 DNA carries a message from an advanced society," Japanese researchers Hiromitsu Yokoo and Tairo Oshima explored some of the reasons extraterrestrials might choose to communicate with humans via a DNA code.[2] DNA is durable, the authors noted in their 1979 article, and can be easily replicated. What's more, it is ubiquitous on Earth, and unlikely to become obsolete as long as life continues—convenient for aliens waiting for humans to develop the sequencing technologies necessary to decode their messages.

The thesis wasn't taken terribly seriously, and the researchers themselves admitted there was no obvious pattern to Phi X174's genome. But for biologist George Church, then a Harvard University graduate student learning how to sequence DNA under Walter Gilbert, the speculation in the paper was intriguing. "I didn't believe it," he says of the alien theory, "but it planted the idea that one could encode messages into biological DNA."

At the time, of course, there was a glaring obstacle: cost. Back then, "we synthesized 10 nucleotides for $6,000, and that was considered a pretty good deal," says Church, now a professor of genetics at Harvard. "Obviously, you can't encode much information in 10 nucleotides."

A few decades later, however, things began to change. Oligonucleotide synthesis was becoming more routine, and researchers could write small amounts of arbitrary information into nucleic acids for under a dollar per base. In 2001, for example, a team at Mount Sinai School of Medicine wrote out two Dickens quotes totaling 70 bytes in DNA sequences—encoding each letter of the alphabet as combinations of the bases A, C, and T (e.g., AAA = A, AAC = B, etc.). Eight years later, researchers in Toronto created a plasmid library containing more than 200 bytes of coded text, music, and an image from the nursery rhyme "Mary Had a Little Lamb." In 2010, Craig Venter's group demonstrated progress in oligonucleotide synthesis by artificially synthesizing the entire genome of the bacterium *Mycoplasma mycoides*—about 1.1 million base pairs.

Around this time, Church decided to get involved. He and two Harvard colleagues translated an HTML draft of a 50,000-word book on synthetic biology,

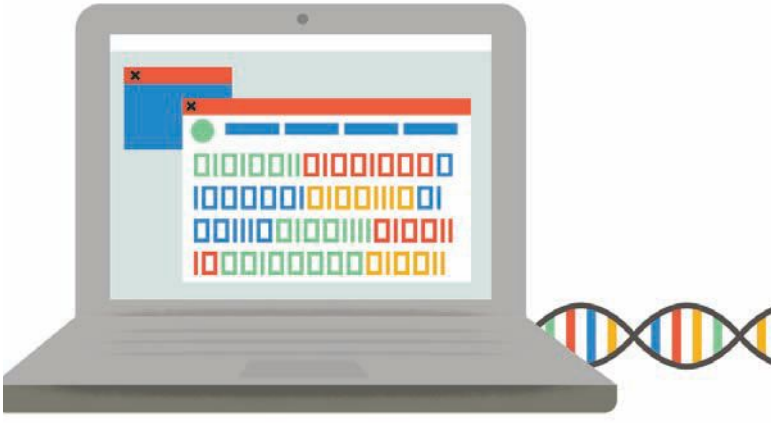
coauthored by Church, into binary code, converted it to a DNA sequence—coding 0s as A or C and 1s as G or T—and "wrote" this sequence with an ink-jet DNA printer onto a microchip as a series of DNA fragments. In total, the team made 54,898 oligonucleotides, each including 96 bases of data along with a 22-base sequence at each end to allow the fragments to be copied in parallel using the polymerase chain reaction (PCR), and a unique, 19-base "address" sequence marking the segment's position in the original document.

The resulting blobs of DNA—which the team later copied with PCR and ran through an Illumina sequencer to retrieve the text—held around 650 kB of data in such a compact form that the team predicted a storage potential for their method of more than 700 terabytes per cubic millimeter.[3] Not only did this result represent far and away the largest volume of data ever artificially encoded in DNA, it showcased a data density for DNA that was several orders of magnitude greater than that of state-of-the-art storage media, never mind the average computer hard drive. (For comparison, an 8-terabyte disk drive has the dimensions of a small book.)

The study's publication in late 2012 was met with excitement, and not only among biologists. In the years since Yokoo and Oshima's discussion on extraterrestrial communiqués, the world of computing had started to acknowledge an impending crisis: humans are running out of space to store their data. "We are approaching limits with silicon-based technology," explains Luis Ceze, a computer architect at the University of Washington in Seattle. Church's paper, along with a similar study published a few months later by Nick Goldman's group at the European Bioinformatics Institute, part of the European Molecular Biology Laboratory (EMBL) in Germany,[4] brought the idea of using DNA for data storage squarely into the spotlight. For Ceze and his colleagues, "the closer we looked, the more it made sense that molecular storage is something that probably has a place in future computer systems."

The idea of a nucleic acid–based archive of humanity's burgeoning volume of information has drawn serious support in recent years, both from researchers across academic disciplines and from heavyweights in the tech industry. Last April, Microsoft made a deal with synthetic biology startup Twist Bioscience for 10 million long oligonucleotides for DNA data storage. "We see DNA being very useful for long-term archival applications," Karin Strauss, a researcher at Microsoft and colleague of Ceze at Washington's Molecular Information Systems Lab, tells *The Scientist* in an email. "Hospitals need to store all health information forever, research institutions have massive amounts of data from research projects, manufacturers want to store the data collected from millions of sensors in their products."

With continued improvements in the volume of information that can be packed into DNA's tiny structure—data can be stored at densities well into millions of gigabytes per gram—such a future doesn't look so fanciful. As the costs of oligonucleotide synthesis and sequencing continue to fall, the challenge for researchers and companies will be to demonstrate that using DNA for storage, and maybe even other tasks currently carried out by electronic devices, is practical.



Minimizing error

In theory, storing information in DNA is straightforward. Researchers synthesize their data into a series of oligonucleotide fragments by translating electronic data—typically written in binary digits, or bits, of zeros and ones—into DNA. As DNA has four bases, the molecule can potentially hold up to two bits per nucleotide, for example, by coding the sequences 00, 01, 10, 11 as A, T, C, G. The resulting fragments, which are usually labeled with an individual address sequence to aid reassembly, can be printed onto a microchip or kept in a test tube and stored somewhere cool, dark, and dry, such as a refrigerator. Recovering the information involves rehydrating the sample, amplifying the fragments using PCR, and then sequencing and reassembling the full nucleotide code. Provided the user knows the strategy employed to generate the DNA, she can then decode the original message.

In reality, though, DNA storage presents several practical challenges that are the focus of current research efforts. The greatest challenge remains the cost of read-

ing and especially writing DNA. Although some companies, such as Twist, offer synthesis for less than 10 cents a base, writing significant volumes of data is still prohibitively expensive, notes EMBL's Goldman. To take DNA data storage beyond proof-of-concept research, "I think we need five orders of magnitude improvement in the price of writing DNA," he says. "It sounds overwhelming, but it's not, if you're used to working in genomics." Church is similarly optimistic. "Things are moving pretty quickly," he says. "I think it's totally feasible."

DNA CAN STORE DATA AT A DENSITY THAT IS SEVERAL ORDERS OF MAGNITUDE GREATER THAN THAT OF STATE-OF-THE-ART STORAGE MEDIA, NEVER MIND THE AVERAGE COMPUTER HARD DRIVE.

A more technical challenge involves minimizing error—a problem familiar to anyone working with electronic equipment such as cell phones or computers. "In all of those [devices], there will be some errors at a certain rate, and you've got to do something to mitigate the errors," Goldman explains. "There's a trade-off between the error rates, how much correction you need, [and] processing costs. . . . Using DNA will be the same."

Some of the errors in DNA data storage are similar to those in electronic media—data can go missing or be corrupted, for example. When reading DNA, "sometimes you simply miss a letter," Goldman says. "You read ACT when it actually was ACGT." One solution is to build in redundancy by writing and reading multiple copies of each oligonucleotide. But this approach inflates the price researchers are so desperate to bring down.

DNA also risks other types of errors that aren't an issue with traditional data storage technology, such as those that arise due to the biochemical properties of the nucleic acid and the molecular machinery used to read and write it. (See "Designer DNA" on page 65.) Sequences containing lots of G nucleotides are difficult to write, for example, because they often produce secondary structures that interfere with synthesis. And polymerase enzymes used in next-generation sequencing are known to "slip" along homopolymers—long sequences of the same nucleotide—resulting in inaccurate readouts. Encoding methods like Church's that write just one bit per nucleotide can avoid problematic sequences—for example, by writing four zeros as an alternating sequence such as ACAC—but greatly reduce the maximum possible information density.

Researchers have explored multiple ways to circumvent these errors while still packing in as much information per nucleotide as possible. In his 2013 paper, published shortly after Church's group encoded the synthetic-biology book into DNA, Goldman and his colleagues used a method called Huffman coding, which has also been adopted by several other labs, to convert their data into a trinary code, using the digits 0, 1, and 2, instead of just 0 and 1. To ensure that no base was used twice in a row, the digit that each DNA base encoded depended on the nucleotide that immediately preceded it. For example, A, C, and G were assigned to represent 0, 1, and 2 at the nucleotide immediately after a T, but following an A, the digits 0, 1, and 2 were encoded as C, G, and T. This strategy avoided the creation of any homopolymers while still making use of DNA's four-base potential. Then, Goldman's team synthesized oligonucleotides carrying 100 bases of data, with an overlap of 75 bases between adjacent fragments, so that each base was represented in four oligonucleotides. Even so, the researchers lost two 25-base stretches during sequencing, which had to be manually corrected before decoding.

More recently, labs have taken advantage of error-correction codes—techniques that add redundancy at specific points in a message to aid reconstruction later. In 2015, a group in Switzerland reported perfect retrieval of 83 kB of data encoded using a Reed-Solomon code, an error-correcting code used in CDs, DVDs, and some television broadcasting technologies.[5] And earlier this year, Columbia University researchers Yaniv Erlich and Dina Zielenski published a method based on a fountain code, an error-correcting code used in video streaming. As part of their method, the pair used the code "to generate many possible oligos on the computer, and then [we screened] them in vitro for desired properties," Erlich tells *The Scientist* in an email. Focusing only on sequences free of homopolymers and high G content, the researchers encoded and read out, error-free, more than 2 MB of compressed data—stored in 72,000 oligonucleotides—including a computer operating system, a movie, and an Amazon gift card.[6]

Along with the more recent development of specialized algorithms to handle the challenges of coding information in DNA specifically, these advances toward error-free DNA data storage and retrieval have helped broaden the appeal of the strategy. At the IEEE International Symposium on Information Theory this year, for example, "there was a whole session on coding for DNA storage," notes University of Illinois computer scientist Olgica Milenkovic, who got involved in the field after reading Church's 2012 paper and seeing the technology's potential. "Coding theorists are getting very excited about it."

Cherry-picking data

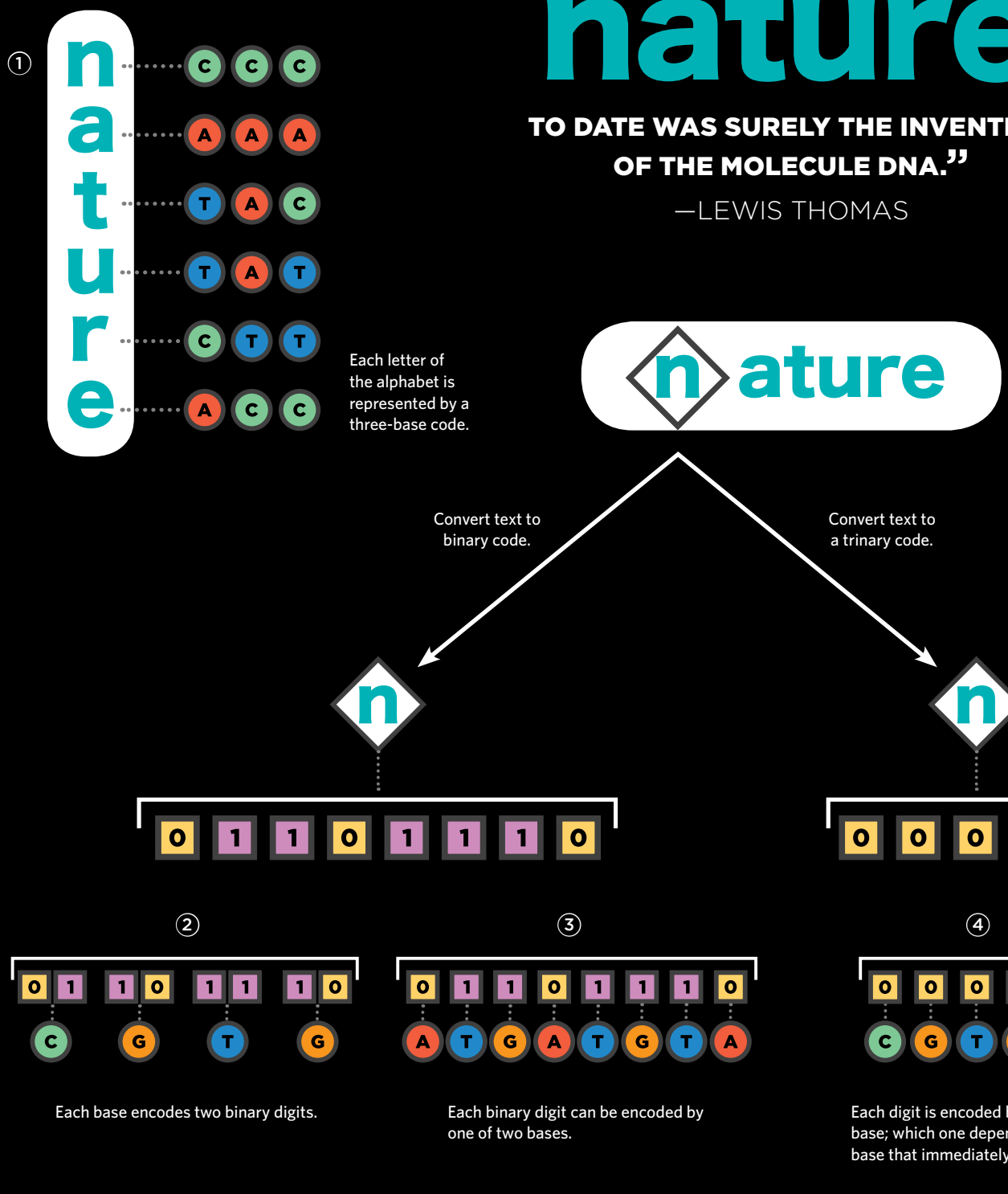
Error isn't the only challenge facing those looking to store data in nucleic acids. Another problem is figuring out how to retrieve just part of the information stored in a system, what's known in computing as "random access." In electronics, Milen-

"THE GREATEST SINGLE ACHIEVEMENT OF

nature

TO DATE WAS SURELY THE INVENTION OF THE MOLECULE DNA."

—LEWIS THOMAS



THE SCIENTIST STAFF

WRITING WITH DNA

There are many possible ways to encode information into DNA. If just encoding text, one way is to convert each letter of the alphabet into a three-letter code. Using three bases, such as A, C, and T, gives 27 combinations—enough for the English alphabet plus a space—with a code such as AAA = A, AAC = B, and so on (1). However, researchers often want to encode more than just text, so most current methods instead first translate data into binary code—the language of 1s and 0s used in electronic media. Using binary, the four bases of DNA could theoretically store up to two bits of information per nucleotide, with a code such as A = 00, C = 01, and so on (2).

In reality, though, biochemical features of nucleic acids make some combinations of bases more desirable than others. Particularly problematic are homopolymers—long strands of the same nucleotide—which are difficult to write and read using current methods. One way to avoid homopolymers is by allocating two bases to each binary digit; long runs of the same digit can then be encoded by alternating base pairs (3). A more efficient method is to convert text or other data into a code that employs three digits rather than two, and then write bases so that no base is used twice in a row—for example by encoding 0, 1, and 2 as C, G, and T after an A, but as G, T, and A after a C (4). Newer methods include more complex codes, as well as error-correcting techniques, to pack as much information as possible into DNA while maximizing the accuracy of information retrieval.

Sources for methods depicted: 1. Bancroft et al., 2001; 3. Church et al., 2012; 4. Goldman et al., 2013.

kovic explains, "every storage system has random access. If you're on a CD, you have to be able to retrieve a certain song. You don't want to go all the way through the disk until the song starts playing." Many published DNA storage methods, though, require sequencing all the data at once—a costly and time-consuming approach for large archives.

A couple of years ago, Milenkovic's lab came up with a solution: instead of using a single unique address sequence to tag each synthesized oligonucleotide, plus separate flanking sequences for PCR that were common across all the oligos in a sample—which meant all of them had to be amplified together—the team proposed just adding two unique sequences to every oligonucleotide, one at each end. By designing primers that were complementary to these unique sequences, the researchers could target PCR amplification to just one oligo of interest simply by adding the unique primers matching each of its flanking sequences.[7] "It's a way to selectively amplify only the sequences that you want," explains Ceze, whose group independently developed a similar primer-based approach.[8] The amplified oligonucleotide will "have high concentration compared to everything else, so when you take a random sample, you only get what you want, and then you sequence that."

To demonstrate the technique's potential, Milenkovic's team encoded 17 kB of text into 32 1,000-base oligonucleotides, each carrying two unique 20-base addresses and 960 base pairs of data. The researchers then successfully amplified and sequenced three specific sequences from that pool. Ceze notes there's potential to massively scale up the approach, provided primers are chosen carefully to avoid accidental amplification of off-target oligonucleotides. His group's most recent work, a joint project with Microsoft's Strauss, selectively amplified specific sequences from a DNA sample of more than 10 million oligonucleotides, a subset of 13 million oligos that stored 200 MB—more data than had ever been stored in DNA before.[9]

In addition to making information access faster and cheaper for future potential data-storage systems, these projects have brought computer scientists and biologists into closer collaboration to solve the biological and computational barriers to making DNA storage possible, Ceze says. "There is a little bit of language adjustment, and even different ways of thinking," he acknowledges, but "the field is so exciting that I think it's going to happen more and more."

THE RESEARCHERS ENCODED AND READ OUT, ERROR-FREE, MORE THAN 2 MB OF COMPRESSED DATA—STORED IN 72,000 OLIGONUCLEOTIDES—INCLUDING A COMPUTER OPERATING SYSTEM, A MOVIE, AND AN AMAZON GIFT CARD.

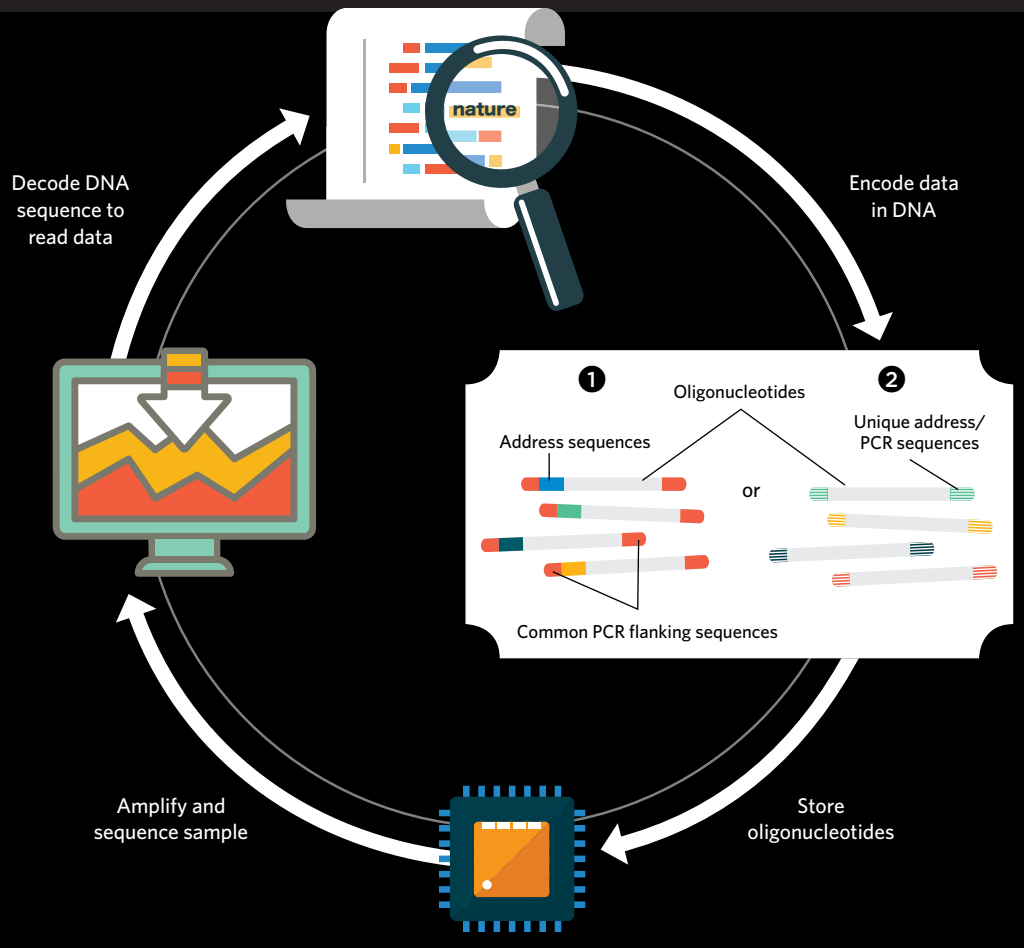
Thinking outside the box

Until recently, most research on ensuring the accuracy and accessibility of information written into nucleic acids has been framed under the assumption that data-storing DNA will be confined to one or a few central storage units—rather like the temperature-controlled Global Seed Vault—where information is only intended to be accessed infrequently. But there's a push in the research community to consider a wider spectrum of possibilities. "People coming into this from the industry

STORAGE CYCLE

After an encoding method is chosen, researchers write the DNA message into a series of long oligonucleotides. In earlier methods, these fragments were each tagged with a unique address sequence to aid reassembly, as well as common flanking sequences that allow amplification by PCR ①. Newer methods incorporate selective retrieval of specific sections of stored data, known as random access, by combining the address and PCR sequences into unique codes on either side of every oligonucleotide. Appropriate primers allow researchers to select and amplify only a sequence of interest ②.

These oligonucleotides are synthesized into tiny test tubes or printed onto DNA microchips, which are stored in a cold, dry, dark place. When the message needs to be read, researchers rehydrate the sample and add primers corresponding to the addresses of the sequences of interest. The amplified product is then sequenced and decoded in order to retrieve the original message.



side are looking long-term," says Goldman. "They're definitely wanting to encourage discussion about making devices that are not just for rarely accessed, archival, backup copies of data."

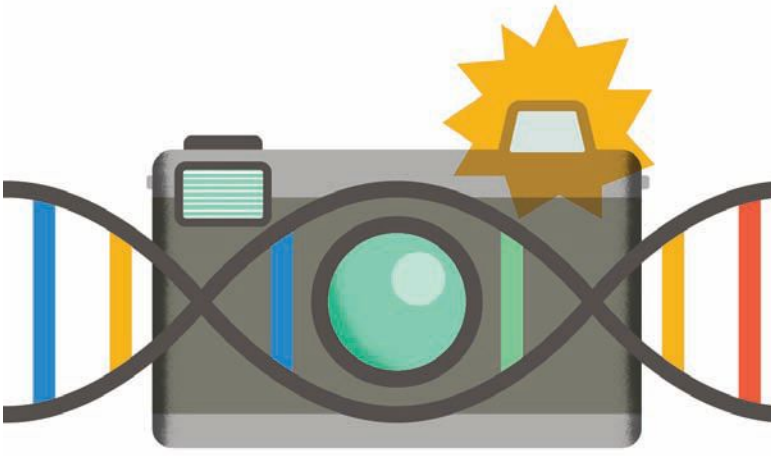
With more-frequent data access in mind, Milenkovic's group has been working on making at least part of DNA storage systems portable. For data reading, "every solution so far has exclusively focused on Illumina sequencing," she says. But "Illumina machines are exceptionally expensive," not to mention bulky. "You wouldn't want to carry one on your back when you want to read your data." Instead, Milenkovic and her colleagues are using a newer technology, MinION—Oxford Nanopore's handheld sequencer. Though error-prone, nanopore sequencing is fast and cheap, and Milenkovic's group has devised error-correcting algorithms specifically for the kinds of mistakes the MinION makes. The result is an error-free read-out, demonstrated earlier this year when the team stored and sequenced around 3.6 kB of binary data coding for two compressed images (a *Citizen Kane* poster and a smiley face emoji).[10]

Other groups are working on combining DNA storage with different molecular technologies. Church's lab, for example, envisages incorporating information capture into the DNA storage system itself. "I'm interested in making biological cameras that don't have any electronic or mechanical components," says Church. Instead, the information "goes straight into DNA." The lab has been laying the groundwork for such a system with research using CRISPR genome-targeting technology in living bacterial cells, paired with Cas1 and Cas2 enzymes that add oligonucleotides into the genome in an ordered way, such that new integrations are upstream of older ones. This summer, the group reported recording a 2.6 kB GIF of a running horse in bacterial DNA by supplying the cells with an ordered sequence of synthetic oligonucleotide sets, one set coding for each of the five frames.[11] "We turn the time axis into a DNA axis,"

THE SCIENTIST STAFF

Church explains. The movie can then be "read" by lysing the bacteria, and sequencing and decoding the oligonucleotides.

Combined with photosensitive elements that capture information in the first place—much like the tissues that allow animals to see or plants to photosynthesize—DNA recorders could archive audiovisual information all by themselves, Church suggests. "You could paint it up on walls, and if anything interesting happens, just scrape a little bit off and read it—it's not that far off." Plus, he adds, the information wouldn't have to be of the audiovisual variety. "You could record biological events in your body," Church says, noting that the team is currently working on developing an in vivo DNA recorder for neural activity. "We'd like to record essentially from all the neurons in the brain simultaneously—something that would be very difficult to do with electrodes."



Meanwhile, researchers at the US Defense Advanced Research Projects Agency (DARPA) have announced a project to develop DNA storage in conjunction with molecular computing—a related area of research that performs operations through interactions between fragments of DNA and other biochemical molecules. DNA computers hold appeal because, to a greater extent than silicon-based computers, they could carry out many parallel computations as billions of molecules interact with each other simultaneously. In a statement this March, Anne Fischer, DARPA's molecular informatics program manager, explained: "Fundamentally, we want to discover what it means to do 'computing' with a molecule in a way that takes all the bounds off of what we know, and lets us do something completely different."

Right now, combining DNA storage and computing sounds a little ambitious to some in the field. "It's going to be pretty hard," says Milenkovic. "We're still not there with simple storage, never mind trying to couple it with computing." Columbia's Erlich also expressed skepticism. "In storage, we leverage DNA properties that have been developed over three billion years of evolution, such as durability and miniaturization," Erlich says. "However, DNA is not known for its great computation speed." But Ceze, whose group is currently researching applications of DNA computing and storage, notes that one solution might be a hybrid electronic-molecular design. "Some things we can do with electronics can't be beaten with molecules," he says. "But you can do some things in molecular form much better than in electronics. We want to perform part of the computation directly in chemical form, and part in electronics."

Whatever the future of DNA in these more complex technologies, such projects are a testament to the perceived potential of molecular data storage—and an indicator of just how much the field has progressed in a very short period of time. Just five years ago, Church recalls feeling "a little skeptical" about how his team's first DNA storage study would be received by the scientific community. "We were just trying to show what was possible," he says. "I wasn't sure people were going to take it seriously." Now, with his lab just one of many research groups aiming to make DNA part of the future of data storage, it appears that his concerns were unfounded. ■

References

1. F. Sanger et al., "Nucleotide sequence of bacteriophage ϕX174 DNA," *Nature*, 265:687-95, 1977.
2. H. Yokoo, T. Oshima, "Is bacteriophage phi X174 DNA a message from an extraterrestrial intelligence?" *Icarus*, 38:148-53, 1979.
3. G.M. Church et al., "Next-generation digital information storage in DNA," *Science*, 337:1628-29, 2012.
4. N. Goldman et al., "Towards practical, high-capacity, low-maintenance information storage in synthesized DNA," *Nature*, 494:77-80, 2013.
5. R.N. Grass et al., "Robust chemical preservation of digital information on DNA in silica with error-correcting codes," *Angew Chem Int Ed*, 54:2552-55, 2015.
6. Y. Erlich, D. Zielinski, "DNA Fountain enables a robust and efficient storage architecture," *Science*, 355:950-54, 2017.
7. S.M.H.T. Yazdi et al., "A rewritable, random-access DNA-based storage system," *Sci Rep*, 5:14138, 2015.
8. J. Bornholt et al., "A DNA-based archival storage system," *ASPLOS '16*, 637-49, 2016.
9. L. Organick et al., "Scaling up DNA data storage and random access retrieval," *bioRxiv*, doi:10.1101/114553, 2017.
10. S.M.H.T. Yazdi et al., "Portable and error-free DNA-based storage," *Sci Rep*, 7:5011, 2017.
11. S.L. Shipman et al., "CRISPR-Cas encoding of a digital movie into the genomes of a population of living bacteria," *Nature*, doi:10.1038/nature23017, 2017.

WILDLIFE DISEASE: Left, a European rabbit (*Oryctolagus cuniculus*) suffering from myxomatosis. Below, a house finch (*Haemorhous mexicanus*) infected with the bacterium *Mycoplasma gallisepticum*.

PROTECTION AT A PRICE

Emerging infections in birds and mammals suggest that increased host resistance—such as that provided by vaccination—could lead to the evolution of more-virulent pathogens.

BY ANDREW F. READ AND PETER J. KERR

One of the most remarkable events in the history of infectious diseases began at the end of 1950. A smallpox-like virus that was being trialed as a biological control agent for the invasive rabbit populations in Australia escaped from test sites and caused an outbreak of unprecedented scale, speed, and carnage. Within just six months, it had spread up the river systems in four states and was decimating rabbit populations across a million square miles. "In places it was possible to drive for a day or more through country that had previously been swarming with rabbits and see only isolated survivors," one research team reported.[1] Tens, perhaps hundreds, of millions of rabbits were eliminated in that initial wave. For farmers whose livelihoods were being devoured by hordes of rabbits, it was something of a miracle.

To everyone's delight, the carnage continued, helped by subsequent deliberate releases in other parts of Australia. Over the ensuing decade, rabbit populations in wide swaths of the country were reduced to a tenth of what they had been.[2] Since that time, rabbit populations have rebounded somewhat, but are nowhere near what they once were. The culpable agent, myxoma virus (MYXV), has generated billions of dollars of savings for Australian agricultural industries to date,[3] surely one of the most cost-effective interventions in the history of agriculture.

The episode also presented a unique opportunity to study the evolutionary arms race between a pathogen and its host animal. Australian microbiologist Frank Fenner took advantage, setting up just the right experiments at just the right time—and he and colleagues kept them going for more than 35 years.[4] The body of work he produced and inspired in others has generated a detailed view of the evolution that ensues when a virus emerges in a new host population.[2] In the process, it also offers important insights into how pathogens might react when interventions such as vaccination and genetic engineering make hosts more resistant to their infections—an important ambition in agricultural and human medicine.

RABBIT: © JOE BLOSSOM/ALAMY STOCK PHOTO; FINCH: ANDY DAVIS, UNIVERSITY OF GEORGIA

Viral virulence

In fully susceptible rabbits, the strain of MYXV that started it all causes classical myxomatosis, a nasty disease in which the eyes, ears, and genitals swell and then, as the eyes seal shut with discharge and the head begins to puff up, mucoid lesions develop on the skin. Almost every infected rabbit dies within two weeks. The question Fenner asked was: What happens when such a virulent virus spreads through a very susceptible host species on a continental scale? He focused on two possibilities. First, the highly lethal virus might evolve to become less lethal. Second, the highly susceptible rabbits might evolve resistance. Thanks to Fenner, we now know both happened.

Let's start with the virus. It's impossible to tell if a pathogen is getting more or less nasty by simply looking for changes in death rates: lots of things can contribute to a change in apparent virulence. Most obviously, hosts can acquire immunity or develop resistance, and so reduce disease severity without any genetic change in the pathogen. The only way to know for sure if a pathogen is evolving to be more or less nasty is to make comparisons in what is called a common garden, a standard setting that does not change. Fenner realized this immediately, and he soon began comparing the lethality of viruses isolated from the field in laboratory rabbits of the same species.[4] (See illustration on page 44.)

The work showed that the almost invariably lethal progenitor virus strain was replaced within a few years by strains with case fatality rates of 70 percent to 95 percent. Some field isolates killed fewer than half the lab rabbits. Over the next few decades, things settled down, and strains at both ends of the lethality spectrum become increasingly difficult to find. Fenner showed why. The highly lethal progenitor virus killed rabbits so fast that its infectious period was shorter than that of the less lethal viral mutants. That meant that the less lethal strains were able to infect more new victims and spread throughout the population.

Natural selection thus favored reductions in virulence. But it did not favor substantial reductions. Benign strains, it turned out, were also less infectious, this time because host immunity was able to control and clear them more rapidly. This work—the time series of isolates tested in a common garden and the experimental dissection of the relationship between virulence and transmission—made MYXV the poster child of virulence evolution: a highly lethal pathogen became less lethal over time. But it was still pretty nasty. It had not become benign.

> We know of no cases where controlled experiments have shown declines in pathogen virulence in the face of rising host resistance.

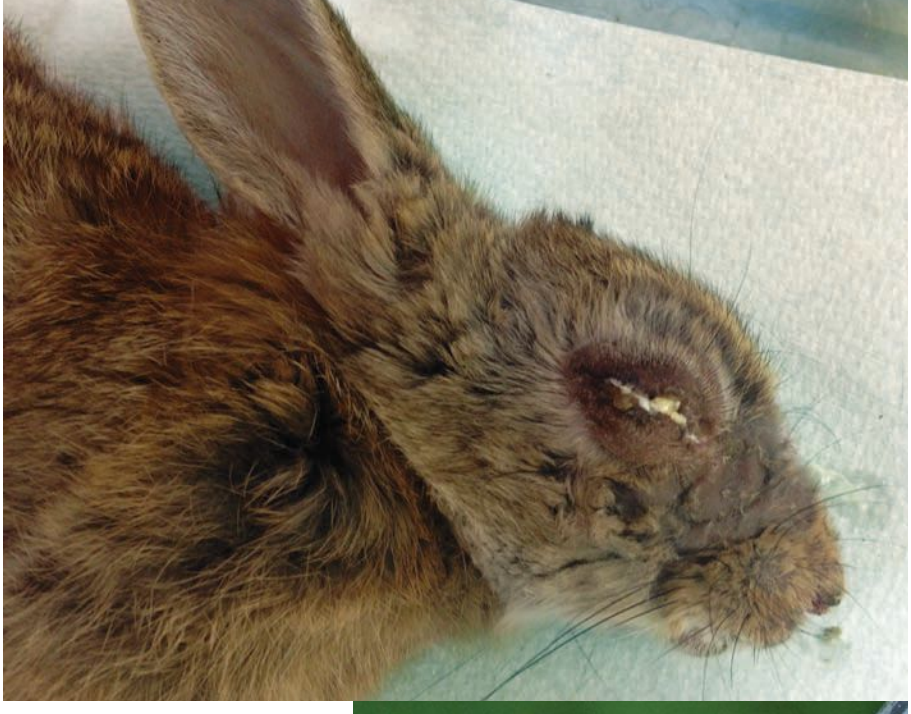
Escalation

In most textbooks, the story stops there. But the virus continued to evolve. From the late 1970s, reports began to accumulate that MYXV was becoming more lethal again. The picture was not simple, partly because the sampling was not as extensive as it had been during Fenner's studies, and partly because there was substantial regional variation. Fascinated by the possibility that the textbook evolutionary trajectory of virulence had reversed, we, together with Eddie Holmes of the University of Sydney and Penn State University's Isabella Cattadori, have been using Fenner's common garden protocols to find out what happened.

To our great surprise, the most virulent of the isolates harvested from the field and frozen in the 1990s caused our susceptible laboratory rabbits to develop a highly lethal immune collapse syndrome akin to septic shock. This disease syndrome had never been seen before. Rabbits die at about the same rate as those infected with the ancestral virus, but they do so without developing classical myxomatosis. Instead, death is associated with a form of toxic or septic shock characterized by an almost complete absence of cellular inflammatory responses, allowing normally well-controlled bacteria to run rampant in the test rabbits. Evidently, sometime after Fenner's detailed work, MYXV evolved the ability to very profoundly immunosuppress rabbits. From our phylogenetic and molecular-clock dating studies, our best estimate is that viral genes encoding this phenotype first arose sometime between the mid-1970s and the early 1980s.[5]

Why did the virus evolve in this way? The most likely explanation is that hyper-immunosuppression was MYXV's answer to genetic resistance that evolved in wild Australian rabbits. Using the common garden approach but in reverse, this time experimentally infecting wild-caught rabbits with a control virus, Fenner and colleagues showed that genetic resistance had rapidly evolved in wild rabbit populations in the 1950s—hardly surprising given the devastation MYXV wreaked on rabbit populations and the fact that surviving rabbits can breed like, well, rabbits. The resistance that evolved clears MYXV infections more rapidly, and so reduces virus transmission. Importantly, resistance is not perfect: it does not prevent infection or transmission. The virus can thus evolve in resistant rabbit populations, and so any viral mutants that are better able to overcome enhanced antiviral host defenses will be favored by natural selection. Hyper-immunosuppression is precisely the sort of viral adaptation that could arise in such circumstances.

It is important to recall that we discovered the immune collapse when we tested viral strains in genetically susceptible lab rabbits. In the field, these same viral strains cause a classical myxomatosis presentation. Apparently, the net effect of hyper-immunosuppressive viruses in resistant wild rabbits is a disease syndrome not unlike the original. It is much like ducks staying calmly in place on a fast-flowing river: frantic paddling resulting in little change on the surface. Fenner's common garden protocols make it possible to see what's going on below the surface: the rabbits have become more resistant and, in turn, the viruses have evolved to suppress the host



NASTY PATHOGENS: Infections that circulate among wild animals, such as the myxoma virus in rabbits or the bacterium *Mycoplasma gallisepticum* in house finches, tend to increase in virulence as hosts develop resistance. If the same holds true for farmed animals, there's concern that breeding and vaccination efforts aimed at increasing host resistance could fuel the evolution of more virulent pathogens. Chris Cairns (bottom, left) and Andrew Read of Penn State University are studying this phenomenon, pictured here sampling broiler chickens for Marek's disease virus in central Pennsylvania.

RABBIT: PETER KERR; FINCH: BOB VUXINIC; CHICKENS: ASHLEY CHAN

immune system on a large scale, allowing MYXV to continue to manifest the classical disease.

A common theme

Common garden experiments have shown that escalating viral virulence in response to increases in host resistance is not unique to MYXV in Australia. In a remarkable case of parallel evolution, the same thing happened in Europe after a different strain of MYXV was released for rabbit control following the Australian successes. Wild rabbits became more resistant over time, and field isolates of the virus ramped up in virulence. Viral strains isolated in the U.K. around 2010 even caused hyper-immunosuppression in lab rabbits, just like the viruses that had evolved earlier in Australia.[6]

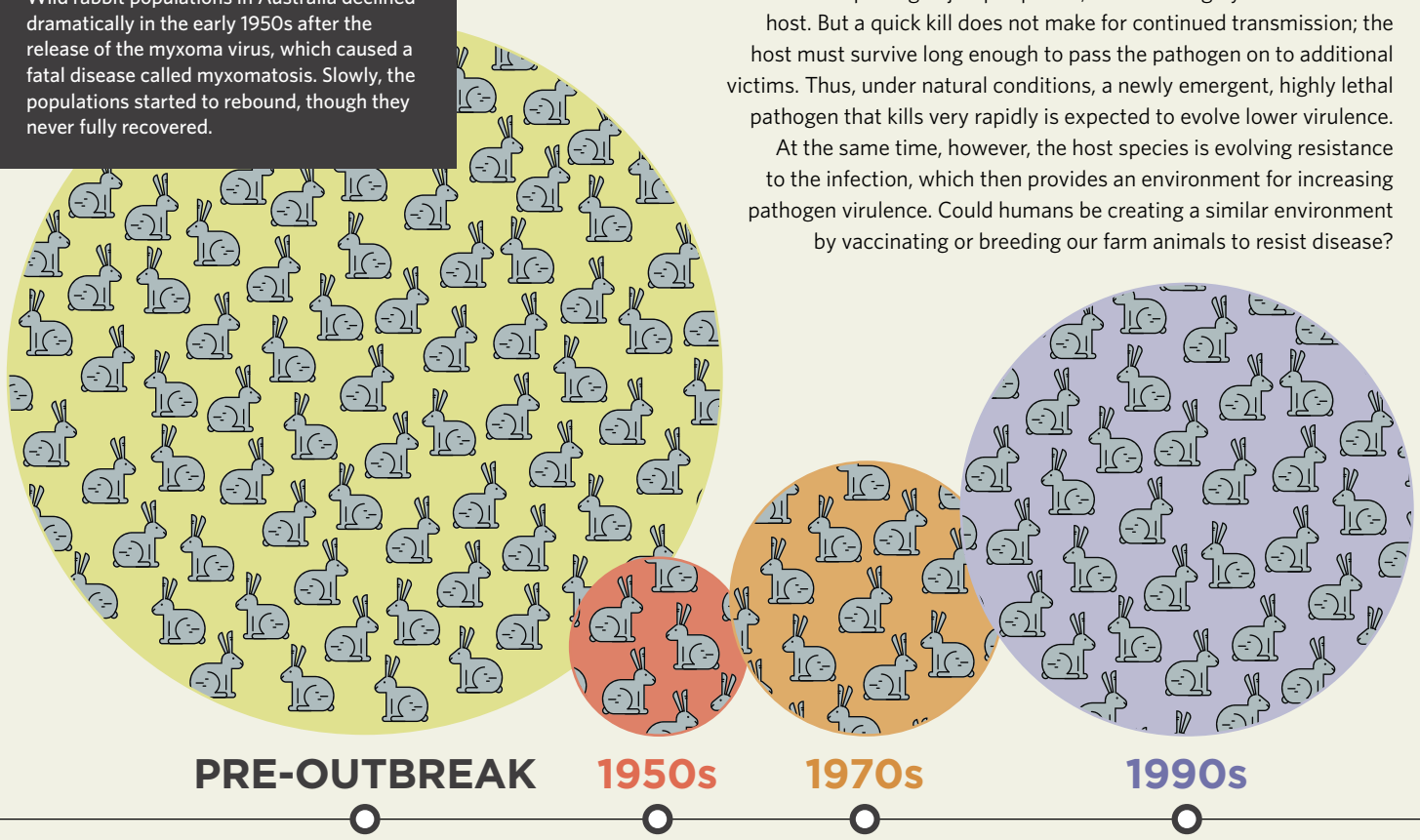
And it's not just MYXV. Few diseases have been subject to the scale of common garden experimentation that Fenner and colleagues lavished on MYXV (these studies are not easy or cheap), but escalating virulence in response to naturally selected host resistance seems to have occurred wherever researchers have looked for it. For instance, in the late 1990s, highly lethal rabbit hemorrhagic disease virus (RHDV) escaped from quarantine while Australian authorities were investigating it as a possible biocontrol agent against rabbits. It, too, decimated wild rabbit populations, which consequently evolved resistance against RHDV. In turn, even more virulent viruses evolved.[7] Similarly, the mosquito-borne West Nile virus (WNV) spread across the U.S. after first appearing in New York in 1999. It infects a wide range of hosts, including humans, but its core reservoir is wild birds. House sparrows have become more resistant through time, and the virus has correspondingly become more virulent.[8] (See "A Race Against Extinction," *The Scientist,* December 2014.)

Virulence also increased after a bacterial pathogen of poultry, *Mycoplasma gallisepticum* (MG), jumped into the Eastern US house finch population sometime in the mid-1990s. In house finches, MG causes severe conjunctival inflammation that affects over-winter survival. When

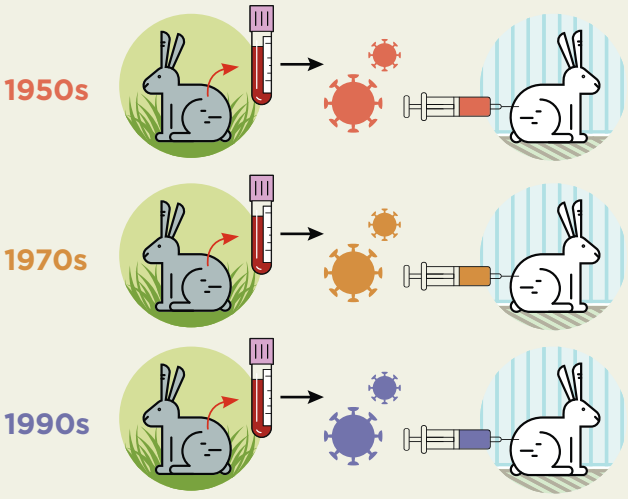
EVOLVING VIRULENCE

When a pathogen jumps species, it is often highly lethal in its new host. But a quick kill does not make for continued transmission; the host must survive long enough to pass the pathogen on to additional victims. Thus, under natural conditions, a newly emergent, highly lethal pathogen that kills very rapidly is expected to evolve lower virulence. At the same time, however, the host species is evolving resistance to the infection, which then provides an environment for increasing pathogen virulence. Could humans be creating a similar environment by vaccinating or breeding our farm animals to resist disease?

Wild rabbit populations in Australia declined dramatically in the early 1950s after the release of the myxoma virus, which caused a fatal disease called myxomatosis. Slowly, the populations started to rebound, though they never fully recovered.

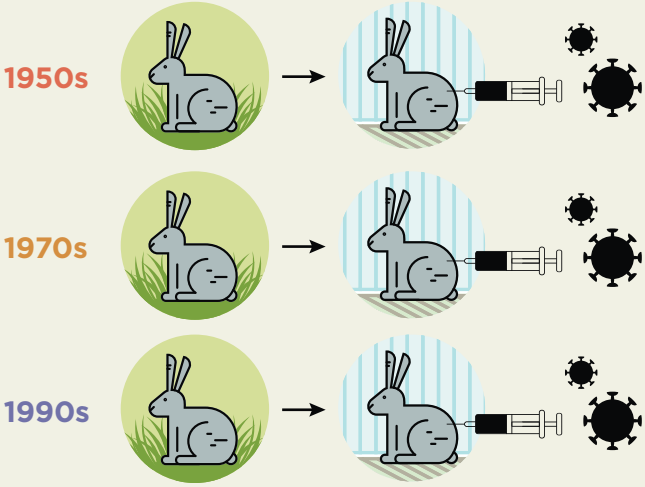


TRACKING PATHOGEN VIRULENCE



Viruses isolated from the field tested in laboratory rabbits

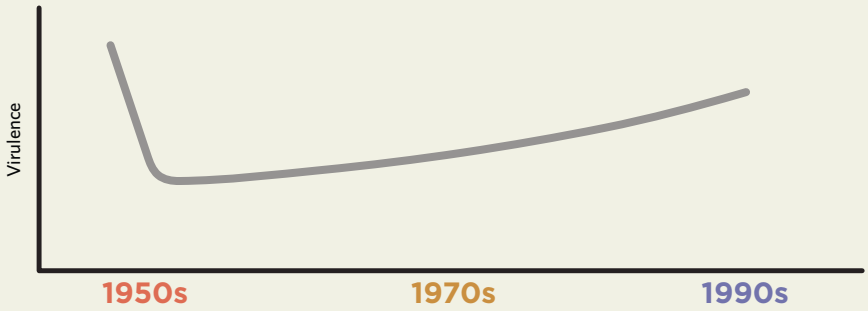
TRACKING HOST RESISTANCE



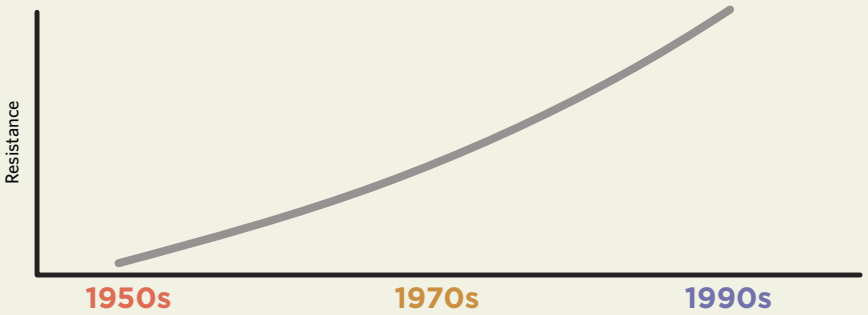
Test virus administered to wild-caught rabbits

To track the myxoma virus (MYXV) as it devastated the invasive rabbit populations of Australia, researchers conducted what are known as common garden experiments, testing the effects of the evolving viral strains on laboratory rabbits, as well as the effects of a standard virus on different samples of rabbits in the wild over time.

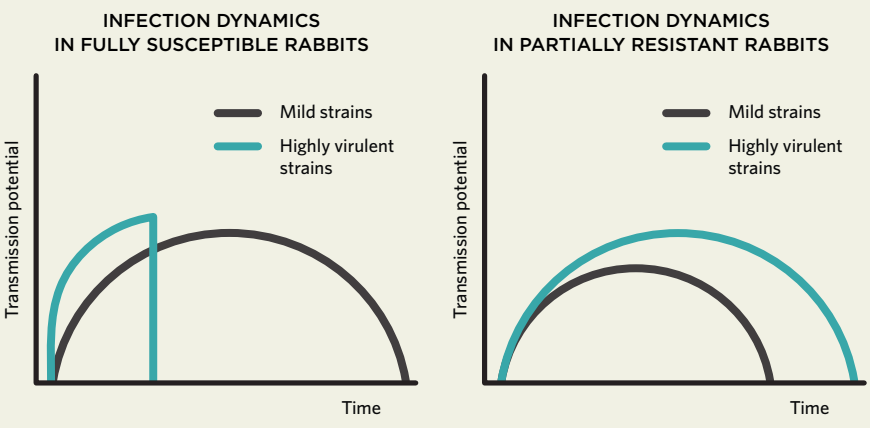
EVOLUTION OF VIRAL VIRULENCE AND HOST RESISTANCE



When MYXV first infected the Australian rabbit population in 1950, it caused a severe disease known as myxomatosis that killed more than 99 percent of its victims. Natural selection favored strains with reduced lethality and therefore longer infectious periods. Within a few years, circulating viruses had fatality rates between 95 percent and less than 50 percent.



Meanwhile, the rabbits were evolving resistance to the viral infection, though the protection was not complete, allowing the virus to continue evolving.



Host resistance likely decreased the virus's transmission rate, thus setting the stage for the selection of more virulent strains. Sometime between the mid-1970s and the early 1980s, strains arose that massively suppressed the cellular inflammatory response of laboratory rabbits. In wild rabbits, the combination of host resistance and increased viral virulence resulted in typical myxomatosis presentation, but when naive rabbits were exposed to the new viral strains, bacterial infections bloomed in their immunosuppressed bodies, killing nearly all of the hosts before they developed the classic disease.

it first emerged, house finch populations declined by up to 60 percent. Over the subsequent 15 years, MG virulence increased. In the early 2000's, a relatively low-virulence strain of MG established itself in Pacific house finch populations, and the same thing happened again: virulence increased through time. On both sides of the continent, these increases occurred as partially immune survivors became common in finch populations.[9]

Thus, nasty pathogens of birds and mammals evolved to become even nastier following six separate emergence events on three continents. Importantly, these six cases cover a diversity of pathogens, including a large DNA virus (MYXV), small single-stranded RNA viruses (RHDV and WNV), and a bacterium (MG). For two of these (MYXV and MG), virulence increases occurred on two separate occasions. The quality of the evidence that the changes in pathogen virulence was caused by rising resistance in the hosts varies, but it is hard to imagine in any of these cases that the increasingly virulent strains could have much of an evolutionary future in highly susceptible hosts, which would likely die before the infectious agent could be transmitted. In all likelihood, the hosts had gained sufficient resistance to ensure somewhat prolonged infectious periods for the more virulent strains. We know of no cases where controlled experiments have shown declines in pathogen virulence in the face of rising host resistance.

Implications for agriculture

Enhancing the resistance of farm animals to infectious disease is an aspiration of veterinary medicine and most agricultural industries, not least because intensive farming is only possible if infectious diseases can be controlled. Traditional selective breeding, genetic engineering, and immunization can all be used to make animals more resistant to infections. If pathogens in nature respond to increases in host resistance by evolving greater virulence, however, is it possible that such efforts will unintentionally select for the same response in pathogens infecting farm animals?

Nothing will happen if hosts are made completely resistant: stop onward transmission, and evolution will cease as well. But artificially enhanced resistance is often imperfect. Many vaccines used on farms do not render hosts impervious to infection, and animal breeders have yet to produce animals completely resistant to a number of different infections. In those situations, pathogens will evolve in newly resistant hosts, just as MYXV, RHDV, WNV, and MG did. Given what we now know about pathogen-host arms races, we think we have to take seriously the possibility that by creating resistant hosts, humans might trigger the evolution of more-virulent animal pathogens.

In fact, this may have already happened. Marek's disease virus (MDV) is a highly contagious cancer-causing herpesvirus of poultry. Fenner-style common garden experiments clearly show that MDV has become more virulent over the last 50 years.[10] When the poultry industry began to ramp up in the 1950s, MDV caused mild disease and had little economic impact. Currently, MDV strains that kill all unvaccinated birds in just 10 days are common in the US and Europe. Birds have to be vaccinated or the losses are devastating. Critically, and for reasons not fully understood, MDV vaccines protect against disease but they do not generate so-called sterilizing immunity: vaccinated hosts can become infected and transmit viruses to other chickens.

In a series of experiments with strains of varying virulence, one of us (AR), together with Venu Nair and colleagues at the Pirbright Institute in England, found that the hypervirulent, or "hot," strains of MDV that dominate nowadays can exist only in vaccinated flocks. In unvaccinated birds, they kill before they have a chance to be transmitted. Vaccines keep birds infected with the hot strains alive and so massively increase their transmission potential.[11] We can't know for sure that vaccination caused the evolution of the hot strains in the first place (sadly, no Fenner-equivalent experiments tracked the initial evolution), but we can say that without vaccination, there would be no hot strains: vaccination creates the conditions for hot strains to emerge and persist.

We can't help but wonder if something similar is happening in other poultry diseases. Highly pathogenic strains of several viruses—most notably, those that cause infectious bursal disease, avian influenza, and Newcastle disease—arise from circulating strains that are less virulent. The resulting outbreaks can be economically devastating. In all those cases, vaccines are available and often widely used. But none of the vaccines generate sterilizing immunity. We think it should be a top priority to determine whether, by reducing bird fatalities and hence the death rates of hypervirulent strains, vaccines are actually increasing the risk of outbreaks of highly pathogenic avian influenza in birds.

> The best bird would be one that dropped dead as fast as possible, before it has started transmitting virus to other birds.

In addition to vaccination, breeding companies that raise poultry and other livestock often try to use selective breeding to enhance resistance. For example, particular major histocompatibility complex alleles in poultry reduce the severity of disease caused by Marek's disease virus, and there are concerted efforts to spread those alleles through national flocks. This breeding, as well as the increasing development of genetically engineered resistance,[12] may further encourage the evolution and spread of virulent strains. For instance, transgenic chickens have recently been constructed that suppress the replication and transmission of avian influenza, but don't block it entirely.[13] This is directly analogous to the antiviral effects of MYXV resistance that arose in Australia's rabbits. Were such chickens to go into widespread use, it is easy to imagine that, just like the rabbits in Australia, they would cause the evolution of more-virulent viruses.

Our suggestion is that breeders and engineers try to do the reverse: breed for susceptibility. The best bird would be one that dropped dead as fast as possible, before it has started transmitting virus to other birds. If death can't be arranged, engineer an animal that becomes obvious to a farmer on first infection—perhaps something as dramatic as a change of color, which could be monitored by cameras—so it can be removed from the flock before it starts an outbreak. Convincing the industry to employ such a counterintuitive strategy will undoubtedly be difficult, of course.

Moreover, virulence is defined in a standardized host, often one that is fully susceptible. If industrial animals are made more resistant, it may not matter if pathogens become more virulent in response. The threat only exists for those animals that remain susceptible.

For example, there is absolutely no question that MDV has become substantially more virulent over the last 50 years, but industry losses to Marek's disease are nothing like they were when less virulent strains circulated.[14] One reason is that in vaccinated birds, even today's hypervirulent strains cause less-severe disease than did milder strains in unprotected birds. Current viral strains only cause problems when they get into unvaccinated flocks—for example, some organic operations, small outdoor flocks, or production systems with faulty vaccination practices. And that's the rub.

This issue may be of particular concern when it comes to aquaculture, where not all operations in a particular watershed might have access to vaccines or genetically resistant fish stock, and nearby wild populations might be very vulnerable.[15] Likewise, it is easy to envisage non-GMO poultry operations being threatened by hypervirulent pathogens evolving in flocks engineered for resistance. An ethically challenging possibility is that companies deploying resistance-enhancing technolo-

PUBLIC HEALTH

Could the widespread use of human vaccines lead to the evolution of pathogens that would be more harmful to the unvaccinated? Most of the human vaccines that have been in use for decades generate sterilizing immunity and so would not be expected to promote pathogen evolution. But next-generation vaccines might be less effective. Clearly, we all hope for malaria or HIV vaccines that completely prevent transmission, but in the absence of fundamental breakthroughs, it seems likely that our current list of vaccine-preventable diseases will soon be joined by a list of vaccine-ameliorable diseases, in which symptoms are alleviated but infection and onward transmission continue. In those cases, it will be critical to understand the possible evolutionary trajectories those target pathogens might take once they evolve in populations that can, just like resistant Australian rabbits, control pathogen titers and sickness, but not prevent infection.

Mathematical models and experimental studies point to the possibility that for some diseases and some vaccines, immunized people might create conditions for the evolution of pathogens that cause more-severe disease in the nonimmunized.[1,2] There are controversial suggestions that this might already be so for the nonsterilizing vaccines against pertussis (also known as whooping cough),[3,4,5] and for our money, there is a strong case for examining the evolutionary consequences of vaccines against cervical cancer and typhoid fever. This is not an argument against next-generation vaccines; rather, it is an admonition that, in the future, we may need additional tools to protect those whom vaccines cannot reach.

1. S. Gandon et al., "Imperfect vaccines and the evolution of pathogen virulence," *Nature,* 414:751-56, 2001.
2. V.C. Barclay et al., "The evolutionary consequences of blood-stage vaccination on the rodent malaria *Plasmodium chabaudi," PLOS Biol,* 10:e1001368, 2012.
3. F.R. Mooi et al., "*Bordetella pertussis* strains with increased toxin production associated with pertussis resurgence," *Emerg Infect Diseases,* 15:1206-13, 2009.
4. S. Octavia et al., "Newly emerging clones of *Bordetella pertussis* carrying *prn2* and *ptxP3* alleles implicated in Australian pertussis epidemic in 2008-2010," *J Infect Dis,* 205:1220-24, 2012.
5. M. Clarke et al., "The relationship between *Bordetella pertussis* genotype and clinical severity in Australian children with pertussis," *J Infect,* 72:171-78, 2016.

gies might gain twice: protection for their own animals and the creation of pathogens that could put their competitors out of business.

Planning for the future

Emergent wildlife diseases show that increasingly aggressive pathogens can attempt to overcome novel host resistance mechanisms as they arise. In the case of MYXV, it is unclear what the very long-term outcome of the escalating arms race will be. But so long as there is virus around, there is no going back: less-resistant hosts would, like our experimental animals, be hugely vulnerable to the hypervirulent viruses now circulating. So, what is the lesson in all this for animal breeders, genetic engineers, and vaccine developers? As in politics and war, if you plan to escalate, also plan for escalation by your opponent. ■

Andrew F. Read is an evolutionary microbiologist at Penn State's Center for Infectious Disease Dynamics. Peter J. Kerr is a virologist and honorary fellow at the Marie Bashir Institute for Infectious Diseases and Biosecurity at the University of Sydney.

References

1. F.N. Ratcliffe et al., "Myxomatosis in Australia: A step towards the biological control of the rabbit," *Nature,* 170:7-11, 1952.
2. F. Di Giallonardo, E.C. Holmes, "Viral biocontrol: Grand experiments in disease emergence and evolution," *Trends Microbiol,* 23:83-90, 2015.
3. B. Cooke et al., "The economic benefits of the biological control of rabbits in Australia, 1950–2011," *Aust Econ Hist Rev,* 53:91-107, 2013.
4. F. Fenner, B. Fantini, *Biological Control of Vertebrate Pests* (Wallingford, U.K: CABI Publishing, 1999).
5. P.J. Kerr et al., "Next step in the ongoing arms race between myxoma virus and wild rabbits is a novel disease phenotype," *PNAS,* doi:10.1073/pnas.1710336114, 2017.
6. P.J. Kerr et al., "Genomic and phenotypic characterization of myxoma virus from Great Britain reveals multiple evolutionary pathways distinct from those in Australia," *PLOS Pathog,* 13:e1006252, 2017.
7. P. Elsworth et al., "Increased virulence of rabbit haemorrhagic disease virus associated with genetic resistance in wild Australian rabbits (*Oryctolagus cuniculus*)," *Virology,* 464:415-23, 2014.
8. N.K. Duggal et al., "Evidence for co-evolution of West Nile virus and house sparrows in North America," *PLOS Negl Trop Dis,* 8:e3262, 2014.
9. D.M. Hawley et al., "Parallel patterns of increased virulence in a recently emerged wildlife pathogen," *PLOS Biol,* 11:e1001570, 2013.
10. R.L. Witter, "Increased virulence of Marek's disease virus field isolates," *Avian Dis,* 41:149-63, 1997.
11. A.F. Read et al., "Imperfect vaccination can enhance the transmission of highly virulent pathogens," *PLOS Biol,* 13: e1002198, 2015.
12. L. Tiley, "Transgenic animals resistant to infectious diseases," *Rev Sci Tech,* 35:121-32, 2016.
13. S.J. Byun et al., "Transgenic chickens expressing the 3D8 single chain variable fragment protein suppress avian influenza transmission," *Sci Rep,* 7:5938, 2017.
14. D.A. Kennedy et al., "An observational study of the temporal and spatial patterns of Marek's-disease-associated leukosis condemnation of young chickens in the United States of America," *Prev Vet Med,* 120:328-35, 2015.
15. D.A. Kennedy et al., "Potential drivers of virulence evolution in aquaculture," *Evol Appl,* 9:344-54, 2016.

The Multitasking Macrophage

From guiding branching neurons in the developing brain to maintaining a healthy heartbeat, there seems to be no job that the immune cells can't tackle.

BY CLAIRE ASHER

In the mid-1990s, while researching mice's immune responses to nematode worms, immunoparasitologist Judi Allen of the University of Manchester spotted macrophages accumulating at the site of a multicellular parasite infection.[1] This was unexpected, she told *The Scientist*; at the time, the immune cells were only known for their antimicrobial activity—a different type of immune response from that known to fight large parasitic worms. The mystery continued, as RNA sequencing revealed that the immune cells' gene expression differed greatly from that of macrophages activated by a microbial infection.[2] "It was so shockingly different that we were thrown," says Allen. "It didn't tell us at all what these macrophages were doing, because the list of genes that they were [expressing] were completely unknown."

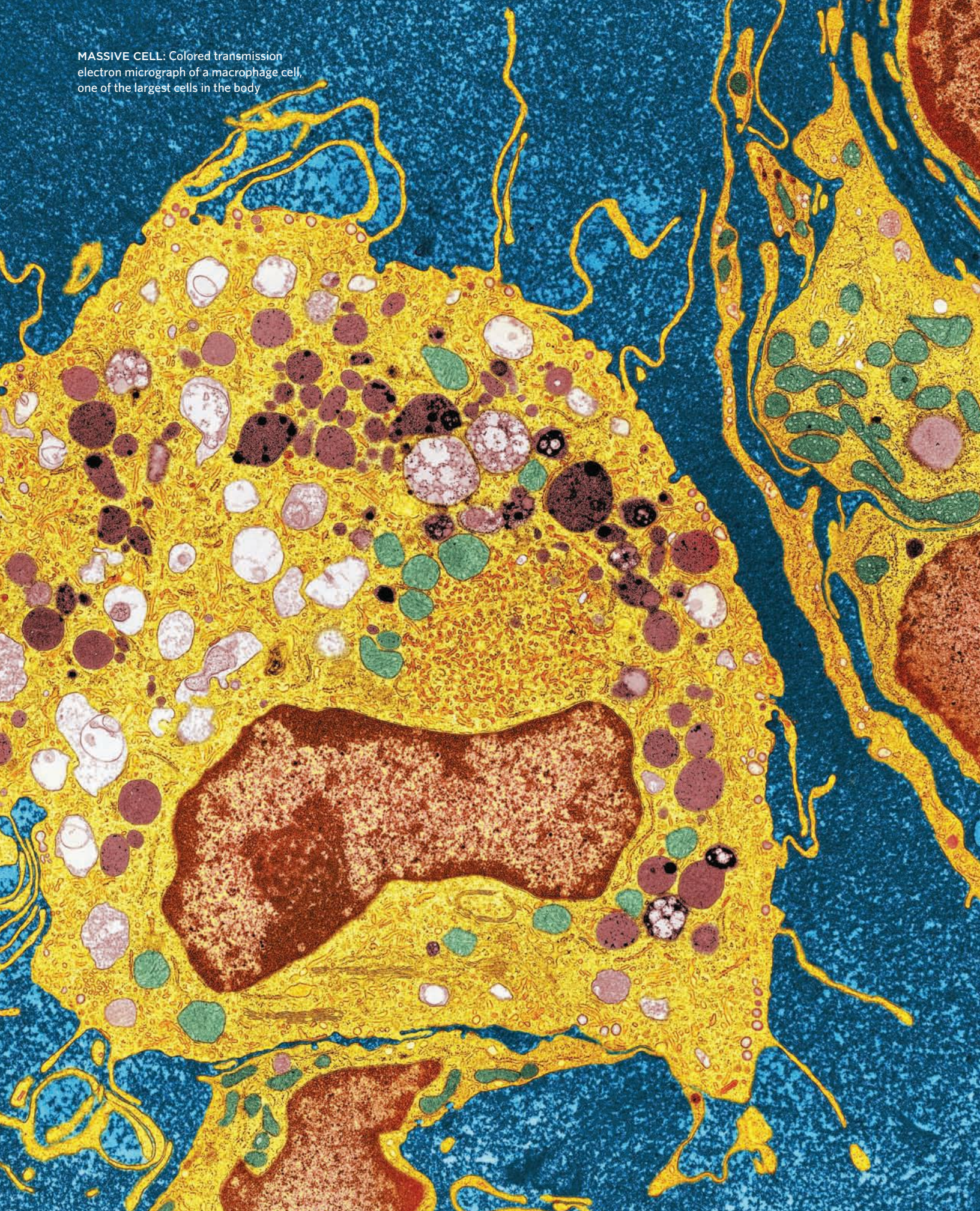
Only years later, when Allen discovered the same profile in macrophages at the site of surgical wounds,[3] did the pieces fall into place—the immune cells appeared to be producing proteins involved in tissue repair, a brand-new function for macrophages.

For more than a century, macrophages—which means "big eaters" in Greek—were considered relatively simple cells whose sole job was to engulf bacteria, other microbes, and cellular debris. Averaging around 20 μm across in humans, they are among the largest cells in the body, which helps them to physically surround and digest their microscopic meals. "You can think of them as the vacuum cleaners of the body," Babak Razani, a physician and researcher at Washington University School of Medicine in St. Louis, writes in an email.

But over the last 10 years, research has demonstrated that this is only part of the picture. In terms of immune defense, investigators now understand that macrophages mount a three-stage response to infection. "Their initial role when our body is invaded with a pathogen is a more inflammatory-and-defense function," says Sourav Ghosh, a biologist at Yale University School of Medicine. Macrophages stimulate inflammation and release a cocktail of molecules to kill microbes. After engulfing a pathogen, macrophages keep hold of pieces of the invader's proteins, known as antigens, and present these to T cells.

In the final stage of macrophages' response to infection, "they will take on a resolving phenotype, where they produce a variety of anti-inflammatory factors that help quiet the inflammation," says Tom Wynn, an immunologist at the National Institute of Allergy and Infectious Diseases. It is during this stage that macrophages stimulate repair of the damage to surrounding tissues, by releasing growth factors and signaling fibroblasts to spring into action and seal the wound.[4] "Macrophages appear to orchestrate the repair as well as to initiate it," says Nadia Rosenthal, a regeneration biologist at the Jackson Laboratory in Maine. "The anti-inflammatory phase is accompanied by a gene expression shift into a more proregenerative profile."

As researchers have begun to take a closer look at these underestimated cells, they have also recognized roles for macrophages outside of immunity. For example, the cells are involved in directing tissue development, transporting chemical messages, even regulating body weight and conducting electrical impulses. "With the advent of single-cell genomics, we're in a moment when we can actually look at individual subtypes of macrophages, and we're



MASSIVE CELL: Colored transmission electron micrograph of a macrophage cell, one of the largest cells in the body

finding that there is an enormous heterogeneity amongst them," explains Rosenthal.

"I think we're learning [that] more and more cells in the body are much more flexible . . . than we originally thought," says Wynn. "Macrophages are probably exceptional in that ability."

Tissue repair

The complexity of the wound-healing process and macrophages' role in it is only just starting to emerge. In a study published in *Science* this May, Ghosh, immunologist Carla Rothlin of Yale University School of Medicine, and their colleagues showed that macrophages integrate multiple signals from their local environment to produce a tailored response to infection and injury.[5] Cytokines recruit more macrophages to the site of inflammation, but these signaling molecules alone are not sufficient to initiate the wound-healing process. "Macrophages can sense cells that die in the tissue, and they can integrate this sense to induce a tissue-repair response," says Rothlin. In the same issue of *Science*, Allen and her colleagues announced the discovery of tissue-specific signals needed to activate macrophages in the lung and liver to repair damaged tissue.[6] (See "Newly Discovered Emergency Responders to Liver Damage," *The Scientist*, August 2016.)

Tissue repair doesn't always go smoothly, however. In chronic injuries, conflicting signals can confuse macrophages into activating all three stages of their response at once. "You can have macrophages that are at the pro-inflammatory stage of activation, macrophages that are pro-wound healing, and macrophages that exhibit some pro-resolving activities, all at the same time," leading to wounds that simply won't heal, explains Wynn.

Other times, wounds do heal, but the injuries leave a scar. Internal scarring, also known as fibrosis, is a hardening of tissues that occurs when macrophages stimulate fibroblasts, which synthesize the extracellular matrix, to produce too much collagen during the repair process. Scar tissue is at the root of many diseases, including liver cirrhosis, and cardiovascular and kidney diseases. "Fibrosis is one of the biggest challenges in Western medicine—that's what most people end up dying of," says Allen.

Perhaps nowhere in the body is the ability to repair damaged tissue more important than in the heart. Although fetal mammals are typically able to regenerate heart tissue without scarring, most species lose that ability as adults, for reasons scientists still don't fully understand. Studying heart repair in mouse fetuses, researchers have learned that, while macrophages are often responsible for the development of fibrosis in adults, they also play a starring role in scar-free repair. Kory Lavine, a researcher and cardiologist at Washington University School of Medicine, suggests that the difference may be attributed to where the macrophages come from.

With the advent of single-cell genomics, we're in a moment when we can actually look at individual subtypes of macrophages, and we're finding that there is an enormous heterogeneity amongst them.

—Nadia Rosenthal, Jackson Laboratory

In addition to the well-studied circulating macrophages (often called monocytes) that are produced in the bone marrow and

REGROWING LOST LIMBS

Mammals' ability to repair tissue is impressive, but some animals are able to regenerate entire appendages. Cellular regeneration is the ultimate form of tissue repair, and recent research suggests that the response of macrophages and other immune cells immediately after injury may play a critical role.

In 2013, for example, Nadia Rosenthal of the Jackson Laboratory and colleagues reported that macrophages were essential for successful limb regeneration in the axolotl (*Ambystoma mexicanum*).[10] "The first five days of wound healing [in most organisms] involves a stately procession of different components of the immune system, which arrive like clockwork at the site of the injury," Rosenthal says. But not so in a regenerating axolotl limb. "Within 24 hours, every cell type that we can identify in the salamander's immune system is on board at that site," suggesting a very rapid progression of normal wound healing factors and the speedy resolution of the inflammatory phase, she says.

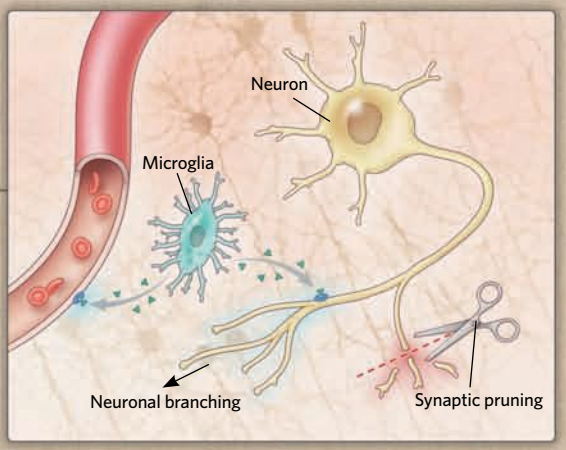
At a localized level, some mammals can also regenerate tissues such as the skin or the tips of their fingers and toes, and macrophages appear to have a hand in such smaller-scale regenerative activities as well. One unique mammal, the African spiny mouse (*Acomys cahirinus*), is capable of regenerating complex tissue in the fleshy part of the ear, including cartilage, hair follicles, and sweat glands.[11] In May, University of Kentucky regeneration biologist Ashley Seifert and his team showed that removing macrophages during injury prevented normal regeneration in the spiny mouse.[12]

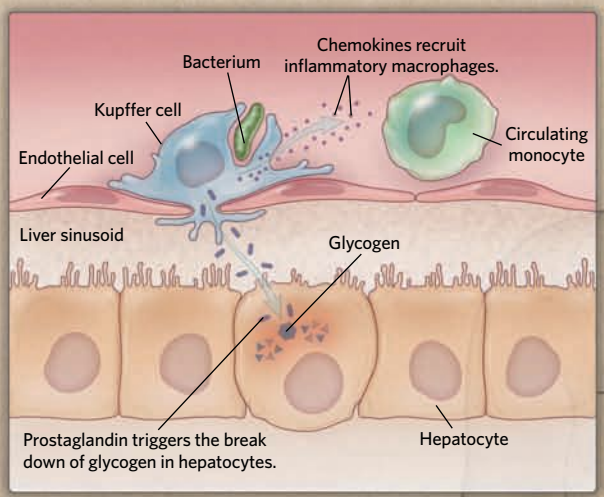
During tissue repair and regeneration, "we think the different immune cell types help facilitate activation of local fibroblasts," Seifert explains. Fibroblasts, under the direction of macrophage-produced signals, can form extracellular structures that act as scaffolding during the repair process.

MACROPHAGES AROUND THE BODY

In addition to circulating in the blood as immune sentinels, macrophages play specialized roles in different organs around the body. Such tissue-resident macrophages, which not only respond to local assaults but also function in normal development and physiology, originate in the yolk sac of the embryo and mature in one particular tissue in the developing fetus, where they acquire tissue-specific roles and change their gene expression profile. By contrast, circulating macrophages are produced throughout life by the bone marrow, then released into the vasculature to respond to infections and injury.

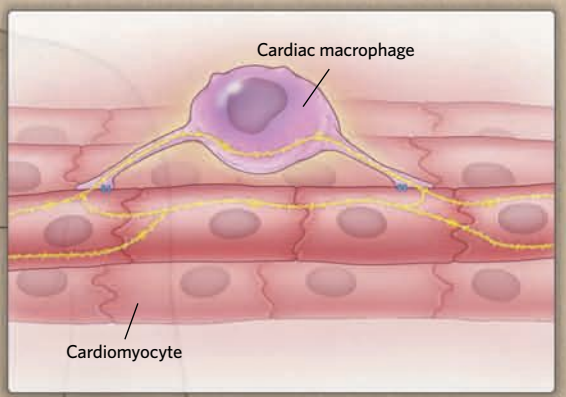
In the developing brain, macrophages called microglia release CD95L (orange triangles) and other signals that bind the CD95 receptor (blue shapes) on blood vessels and neurons, stimulating them to grow and branch, respectively. They also orchestrate a pruning process, so that blood vessels grow and new synapses form according to need.



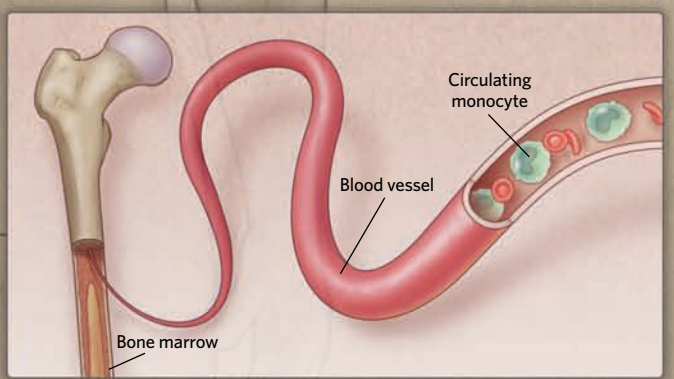


Kupffer cells, the most numerous type of tissue-resident macrophage in the body, digest bacteria and toxins carried to the liver in the blood, break down old red blood cells, and regulate iron and cholesterol levels in the blood. They also help regulate the production and storage of glucose by hepatocytes. In obesity, Kupffer cells can inhibit insulin signaling and activate hepatic glucose production leading to the development of insulin resistance. Injury, infection, and obesity can also cause Kupffer cells to release chemokines that recruit inflammatory macrophages to the liver.

Macrophages in the heart are essential for maintaining a healthy heartbeat, conducting electrical impulses between cardiomyocytes. The cells rhythmically depolarize and repolarize as the electrical impulse passes across them.



Circulating macrophages (called monocytes) primarily patrol the body for infection, but they can also specialize to perform tissue-resident roles, replacing embryonic macrophages that die. Some scientists believe this macrophage replacement may contribute to aging.



are released into the blood to patrol for and respond to infection, in the 1980s researchers began to recognize that some macrophages are produced from embryonic stem cells during early development and remain permanently within one tissue. "The biggest revolution in macrophage biology in the past five years has been this understanding that macrophages that live in the tissues are quite fundamentally different than macrophages that circulate in the blood," explains Allen.

It is these tissue-resident macrophages that contribute to repair in the neonatal heart, Lavine says, explaining that "macrophages [from the blood] really don't enter the heart until the postnatal period." In fact, the resident macrophages appear to actively keep the circulating monocytes out of the developing heart, Lavine adds.[7] Removing the resident macrophages from the heart in neonatal mice results in inflammation and scar-tissue formation.[8]

In adults, on the other hand, the tissue-resident macrophages are depleted after an initial heart injury and circulating monocytes are recruited to replace them, Lavine's group found.[9] These recruited macrophages cause inflammation and tissue damage as they force their way between cells, which may prevent successful tissue regeneration and lead to scarring. In the heart, says Rosenthal, recruited "macrophages do not appear to be able to make that transition to a proregenerative state; [they] continue to be proinflammatory."

Development

Since discovering permanent tissue populations of macrophages, researchers have noted that they appear to be important for the normal development of many tissues. Last year, for example, Lavine and colleagues found that resident macrophages actively shape the development of blood vessels in neonatal mouse hearts. In a growing fetus, blood vessels form a network of capillaries that cover the heart before blood has even begun to flow through them. After blood flow is connected, Lavine's group found, macrophages are attracted to vessels with the highest flow and release molecules that promote their growth. The cells also release signals that direct blood vessels receiving little blood flow to recede, trimming the branching blood vessel network.[13]

A similar process also guides neurons and blood vessels in the developing brain. Resident macrophages in the brain, known as microglia, populate the organ during embryonic development, producing growth factors for neurons and coordinating synaptic pruning—both essential processes for normal brain development. "[Microglia] set up a completely independent population of self-renewing cells," separated from macrophages in the rest of the body by the blood-brain barrier, says Chris Glass, a molecular immunologist at the University of California, San Diego School of Medicine.

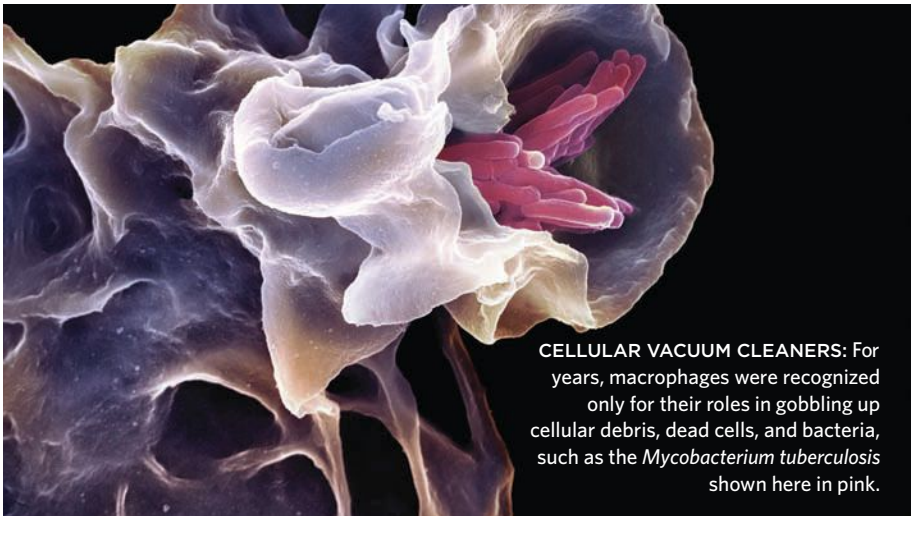
Further evidence for macrophages' role in the development of the brain and its vasculature came earlier this year, when neurobiologist Ana Martin-Villalba at the German Cancer Research Center (DKFZ) and colleagues found that a macrophage-produced protein called CD95L binds to CD95 receptors on the surface of neurons and developing blood vessels in the brains of mouse embryos. The CD95 receptor and its ligand are best known for triggering cell death, or apoptosis, in response to viral infections, cancers, and stressors, such as free radicals and oxygen deprivation. Without CD95L, Martin-Villalba's team showed, neurons branched less frequently, and the resulting adult brain showed less electrical activity.[14] "In the developing brain the macrophages, just using one signal, are shaping the developing neurons and vessels at the same time," she says.

Just as macrophages seem essential for setting up a blood supply for developing organs, they are also complicit in helping tumors develop.

But just as macrophages seem essential for setting up a blood supply for developing organs, they are also complicit in helping tumors develop. "Cancer is nothing [but] a developing organ," explains Martin-Villalba. "Normally, if you find something going on in development, it's pretty much the case that you find this very same issue in cancer."

Developing tumors attract circulating monocytes from the blood and stimulate the immune cells to become tumor-associated macrophages. When the process goes smoothly, these macrophages kill tumor cells and release factors to destroy their blood supply. But when things go wrong, they instead produce growth factors that help the developing tumor grow its network of blood vessels, just as macrophages do for developing organs in a fetus.

The link between macrophages and cancer was first identified in the 1860s, when German physician Rudolf Virchow noted that cancer was often associated with



CELLULAR VACUUM CLEANERS: For years, macrophages were recognized only for their roles in gobbling up cellular debris, dead cells, and bacteria, such as the *Mycobacterium tuberculosis* shown here in pink.

inflammation. But it wasn't until the 20th century that his ideas were taken seriously, as researchers began to find loads of the cells in biopsied tumors. Researchers now know that some 15 percent of cancers are triggered by infection, which can jump-start the inflammatory process. Macrophages are also found at the site of cancers caused by other factors, such as carcinogens or genetic mutations, and cancer initiation and progression has been linked to chronic inflammation.[15] "The most aggressive types of cancer have the highest infiltration of macrophages and other immune cells," says Martin-Villalba.

Researchers have long known that CD95L, also known as the death protein for its role in apoptosis, is expressed by certain immune cells to trigger the process in cancer cells. But tumors can become resistant to CD95L's apoptotic effects. In addition to preventing cancer cell death, this resistance might also help tumors survive and develop by promoting blood-vessel growth, Martin-Villalba suggests. "We postulate that macrophages in a brain tumor can produce signals like CD95L that act on vascularization of the tumor," she says. During Phase 2 clinical trials in 2012, she and her colleagues found increased overall survival in one group of CD95L-expressing brain tumor patients when treated with a protein compound that binds CD95L to prevent it from activating CD95 receptors.

Macrophages may also be involved in a host of neurodegenerative diseases and psychiatric illnesses. Earlier this year, Glass and colleagues analyzed the transcriptomes of microglia from 19 patients undergoing brain surgery, and found that many genes containing risk variants previously associated with diseases such as Alzheimer's, Parkinson's, schizophrenia, and multiple sclerosis were more highly expressed in microglia than in other cells in the brain.[16] For instance, 58 percent of genes known to influence the risk of developing Alzheimer's disease were expressed at levels at least 10 times greater in the microglia than in cortical tissue. This suggests that these genetic variants may increase the risk of disease for patients carrying them by affecting the action of microglia in the brain.

Within hours of being removed from the brain and cultured in the laboratory, however, the microglia had completely changed their expression profile, reducing transcript levels of more than 2,000 genes. Their ability to rapidly change according to environmental conditions might have important implications for scientists studying microglia, making it difficult to generalize from cell-culture studies to macrophages in vivo.

As macrophages' role in development is further elucidated, researchers may uncover additional ways the cells mediate both health and disease. (See "Medical Reprogramming" below.) For example, monocyte-derived cells known as osteoclasts are involved in bone development, and mice that lack these cells develop dense, hardened bones—a rare condition known as osteopetrosis. Macrophages also orchestrate development of the mammary gland and assist in retinal development in the early postnatal period.[17] The next step, says Glass, will be to "carefully evaluate

MEDICINAL REPROGRAMMING

Given macrophages' ever-expanding role in normal development and physiology as well as disease, perhaps it should be no surprise that researchers are now eyeing the cells as potential targets for various therapies. One potential application may be in treating atherosclerosis, plaque build-up in the arteries that can result from inflammatory macrophages engulfing excess lipids. The cells eventually become lipid-engorged, and these "obese macrophages," as Babak Razani of Washington University School of Medicine calls them, tend to stimulate more inflammation and recruit more macrophages, clogging the circulatory system and heart with plaque. But Razani believes he may have found a clever trick to improve macrophages' ability to play a more positive role.

This June, Razani and his colleagues showed that macrophages in the blood vessels can be stimulated with a common sugar molecule to become "super macrophages," with an enhanced ability to degrade plaque.[20] Trehalose, a disaccharide, changes macrophage gene expression, causing the cells to carry more digestive organelles called lysosomes, and resulting in a 30 percent decrease in atherosclerotic plaque in mice injected with trehalose.

"Trehalose is a safe and natural sugar, already being used in many pharmaceuticals and being consumed as a sweetener," Razani says. But he warns a healthy heart is not as simple as just adding more sweeteners to your tea. When consumed in the diet, the enzyme trehalase quickly breaks down trehalose into the simple, nontherapeutic sugar, glucose. The next step towards a medicinal application of this research will be to find ways to deactivate trehalase, so that trehalose can make it into the bloodstream undigested, ready to unleash its macrophage-enhancing powers.

Other potential medical applications for macrophages could include manipulating the cells to promote healthy scar-free wound repair. This might involve devising ways to keep macrophages in the blood from being called in to deal with heart damage, or reprogramming stem cells into wound-healing macrophages that could be injected at the site of a chronic wound. "We don't just want to turn them off in pathological conditions," says biologist Jennifer Simkin, a postdoc at the University of Kentucky. "Instead, we want to try and control what they're secreting and what they're doing."

First, however, researchers must continue to interrogate the cells' basic biology. A better understanding of their roles in health and disease, as well as their therapeutic potential, may then follow, says Razani. "[An] integrative approach to studying [macrophages] will yield long-term dividends in understanding fundamental biological processes, unraveling mechanisms of disease, and eventually modulating their function to treat human disease."

the consequences of removing macrophages from specific tissues during development and asking what the consequences are [for] the development of that tissue."

It is now eminently clear that macrophages are far more than the garbage disposals scientists first viewed them as.

Homeostasis

Beyond their roles in development and wound healing, macrophages are now recognized for their important functions in maintaining the status quo in the adult body. Tissue macrophages are highly sensitive to changing conditions, and respond by releasing cell signaling molecules that trigger a cascade of changes allowing cells to adapt. For instance, macrophages in adipose tissue regulate the production of new fat cells in response to changes in diet or exposure to cold temperatures. Macrophages in the liver, known as Kupffer cells, regulate the breakdown of glucose and lipids in response to dietary changes, and have been linked to obesity and diabetes. "Nowadays they are recognized as major sentinel cells that can sense changes in tissues," says Rothlin. (See "Fat's Immune Sentinels," *The Scientist*, December 2012.)

In the testis, macrophages help create a protective environment for sperm. Sperm cells are at risk of attack by the immune system because they are first produced during puberty, after the immune system has developed. Their specific antigens might be accidentally flagged as foreign, but tissue-resident macrophages in the testis produce immunosuppressant molecules that guard against this.[18]

Macrophages also appear to be critical for the function of the heart. Research has revealed that resident macrophages are present in large numbers, "close to the structures that conduct electricity in the heart," explains Matthias Nahrendorf, a cardiovascular physician at Massachusetts General Hospital's Center for Systems Biology. Curious about the role of these macrophages, Nahrendorf and colleagues at Harvard Medical School engineered mice that lacked them. Unexpectedly, they found that the rodents developed a condition known as an AV block, which inhibits conduction of electricity in the heart, and in humans is usually treated with a pacemaker.[19] "That was a big surprise to me," he says. Without macrophages, "the electrical impulse was not able to travel from the atrium to the ventricle."

Indeed, when they measured the membrane electrical potential of macrophages and cardiomyocytes, the researchers found the cells are directly involved in conducting electricity, polarizing and depolarizing rhythmically to help keep the heart beating regularly. "That was the other big surprise for me," Nahrendorf says—"that there's really electrical connection and cross talk between macrophages and cardiomyocytes." Rosenthal was similarly taken aback. The role of macrophages in electrical conduction "was a complete shock," she says.

It is now eminently clear that the cells are far more than the garbage disposals scientists first viewed them as. "I think the immunologists are staring in disbelief at the armada of nonimmunological laboratories that have suddenly realized that they're going to have to acknowledge the omnipotence of the immune system and how complicated it is," says Rosenthal. "And I'm one of them; I'm a convert." ■

Claire Asher is a freelance science writer living in London, U.K.

References

1. P. Loke et al., "Alternatively activated macrophages induced by nematode infection inhibit proliferation via cell-to-cell contact," *Eur J Immunol*, 30, 2669-78, 2000.
2. P. Loke et al., "IL-4 dependent alternatively-activated macrophages have a distinctive in vivo gene expression phenotype," *BMC Immunol*, 3:7, 2002.
3. P. Loke et al., "Alternative activation is an innate response to injury that requires CD4+ T cells to be sustained during chronic infection," *J Immunol*, 179, 3926-36, 2007.
4. T.A. Wynn, K.M. Vannella, "Macrophages in tissue repair, regeneration, and fibrosis," *Immunity*, 44:450-62, 2016.
5. S. Ghosh et al., "Macrophage function in tissue repair and remodeling requires IL-4 or IL-13 with apoptotic cells," *Science*, 356:1072-76, 2017.
6. C.M. Minutti et al., "Local amplifiers of IL-4R–mediated macrophage activation promote repair in lung and liver," *Science*, 356:1076-80, 2017.
7. S. Epelman et al., "Embryonic and adult-derived resident cardiac macrophages are maintained through distinct mechanisms at steady state and during inflammation," *Immunity*, 40:91-104, 2014.
8. A.B. Aurora et al., "Macrophages are required for neonatal heart regeneration," *J Clin Invest*, 124:1382-92, 2014.
9. K.J. Lavine et al., "Distinct macrophage lineages contribute to disparate patterns of cardiac recovery and remodeling in the neonatal and adult heart," *PNAS*, 111:16029-34, 2014.
10. J.W. Godwin et al., "Macrophages are required for adult salamander limb regeneration," *PNAS*, 110:9415-20, 2013.
11. T.R. Gawriluk et al., "Comparative analysis of ear-hole closure identifies epimorphic regeneration as a discrete trait in mammals," *Nat Commun*, 7:11164, 2016.
12. J. Simkin et al., "Macrophages are necessary for epimorphic regeneration in African spiny mice," *eLife*, 6:e24623, 2017.
13. J. Leid et al., "Primitive embryonic macrophages are required for coronary development and maturation," *Circ Res*, 118:1498-511, 2016.
14. S. Chen et al., "CNS macrophages control neurovascular development via CD95L," *Cell Rep*, 19:1378-93, 2017.
15. A. Mantovani et al., "Cancer-related inflammation," *Nature*, 454:436-44, 2008.
16. D. Gosselin et al., "An environment-dependent transcriptional network specifies human microglia identity," *Science*, 356:eaal3222, 2017.
17. T.A. Wynn et al., "Macrophage biology in development, homeostasis and disease," *Nature*, 496:445-55, 2013.
18. N. Mossadegh-Keller et al., "Developmental origin and maintenance of distinct testicular macrophage populations," *J Exp Med*, 214:10.1084/jem.20170829, 2017.
19. M. Hulsmans et al., "Macrophages facilitate electrical conduction in the heart," *Cell*, 169:510-22.e20, 2017.
20. I. Sergin et al., "Exploiting macrophage autophagy-lysosomal biogenesis as a therapy for atherosclerosis," *Nat Commun*, 8:15750, 2017.

The Scientist wins more kudos for editorial excellence

CREATIVE CONTENT FOR CURIOUS MINDS

AMERICAN SOCIETY OF BUSINESS PUBLICATION EDITORS (AZBEE) 2017 • **March 2016 issue**—Print, Single Topic Coverage by a Team—National Gold and Northeast Regional Gold • **Modus Operandi**—Print, Regular Department—Northeast Regional Bronze • **Magazine of the Year,** More Than $3 Million Revenue—Honorable Mention

FOLIO AWARD S 2016 • **March 2016 issue**—Winner B-to-B Full Issue • **B-to-B News Coverage**—Honorable Mention

TheScientist

The Literature

EDITOR'S CHOICE IN BIOCHEMISTRY

Pressing Pause on Transcription

THE PAPER
W. Shao, J. Zeitlinger, "Paused RNA polymerase II inhibits new transcriptional initiation," *Nat Genet*, 49:1045-51, 2017.

When it comes to regulating gene expression, transcriptional initiation tends to get a lot of attention. But it's become clear in the past decade that RNA polymerase II, the enzyme that transcribes DNA to RNA, frequently pauses after reading just a few dozen base pairs. This break surely affects gene expression rates—though its impact has not been obvious. Julia Zeitlinger and graduate student Wanqing Shao of the Stowers Institute for Medical Research in Kansas City, Missouri, recently found that as long as RNA polymerase remains stalled, very little new transcription is initiated.

Zeitlinger and Shao came to this conclusion by using a drug to freeze RNA polymerase II and transcription factors in place in *Drosophila* cells, then analyzing the positions of polymerases throughout the genome with a technique called ChIP-nexus. "We could clearly see minimal initiation in the presence of paused polymerase," she says. The result was initially surprising, she adds, because it's been shown that promoters with a strong propensity for downstream polymerase-pausing are associated with faster gene expression in response to a developmental signal.

"I think that the idea of having one rate-limiting step is sort of appealing, and a lot of biologists sort of intuitively think that way," she says. "But it's actually not a good way to design a system . . . because it makes it more stochastic, more random." It makes sense that there would be a system in place to ensure transcription isn't simultaneously paused and initiated on the same gene, she says, but the mechanics of how the paused polymerase wards off new initiation remain to be elucidated.

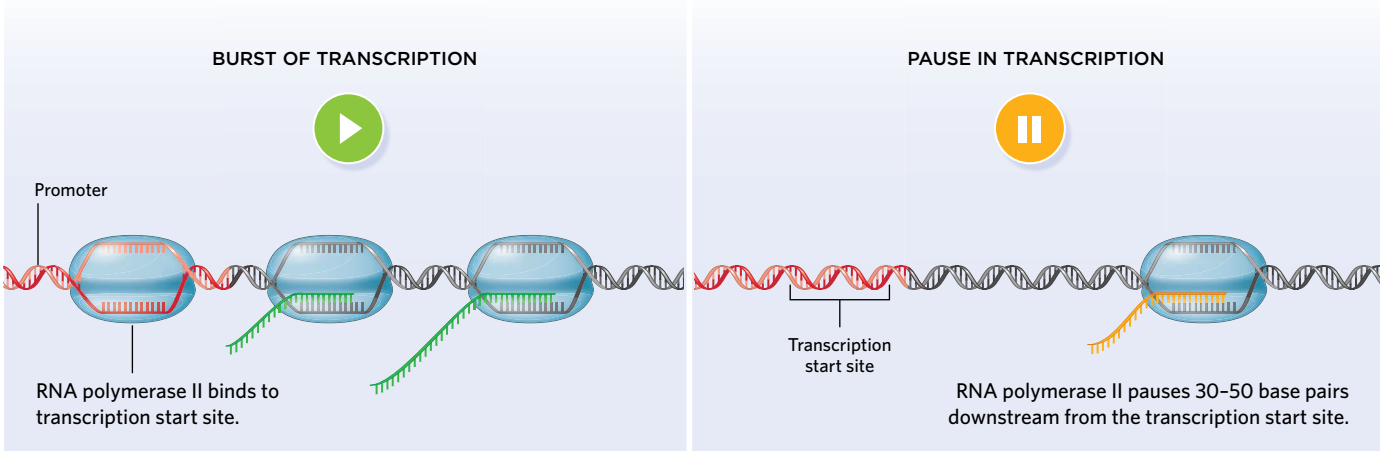
Craig Kaplan, a molecular biologist at Texas A&M University, says this and other recent studies also make it clear that pausing can occur for very different lengths of time depending on the promoter. This helps give cells a "buffet of choices in how expression may happen," he notes. "The regulation doesn't have to be thought of as on or off; it may be how frequently you make a transcript, or whether you're making your transcript in bursts, or whether you're making transcripts evenly."

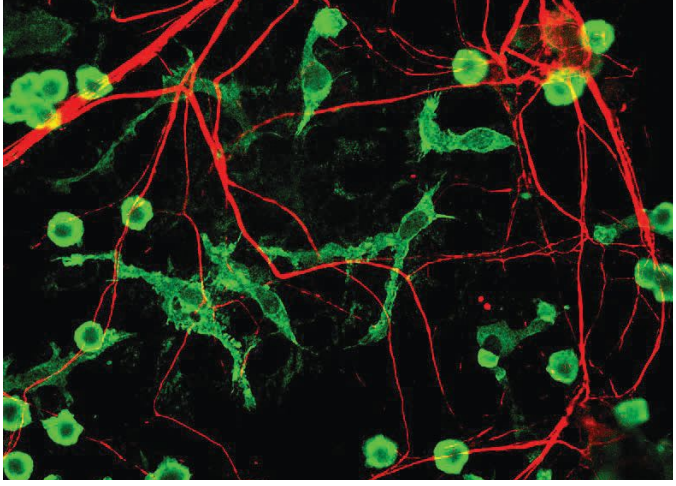
But Patrick Cramer of the Max Planck Institute for Biophysical Chemistry in Göttingen, Germany, wants to see the results confirmed by other studies. "Occupancy changes are certainly an indication for changes in kinetics, but are not a proof, because occupancy can change either because the number of factors bound to DNA changes or because their residency time on DNA changes, or both," he writes in an email to *The Scientist*.

"Transcription is an old field, and I think it's often seen as, 'Oh, it's so well studied,'" says Zeitlinger. "[But] there are a lot of things we don't understand. . . . There are so many open questions in the field that are quite fundamental."

—Shawna Williams

POLYMERASE INTERFERENCE: During gene expression, multiple RNA polymerase II enzymes commonly transcribe a gene simultaneously (left). But if a polymerase pauses on the gene, no new transcription is initiated until it restarts (right).





THE BRAIN'S SENTINELS: Microglia (stained green in this rat brain culture) fight infection in the central nervous system. Neuronal processes stained in red.

NEUROSCIENCE

Changing of the Guard

THE PAPER

P. Réu et al., "The lifespan and turnover of microglia in the human brain," *Cell Rep*, 20:779-84, 2017.

A RENEWABLE RESOURCE?

Evidence has emerged that some of the brain's cells can be renewed in adulthood, but it is difficult to study the turnover of cells in the human brain. When it comes to microglia, immune cells that ward off infection in the central nervous system, it's been unclear how "the maintenance of their numbers is controlled and to what extent they are exchanged," says stem cell researcher Jonas Frisén of the Karolinska Institute in Sweden.

NUCLEAR SIGNATURE

Frisén and colleagues used brain tissue from autopsies, together with the known changes in concentrations of carbon-14 in the atmosphere over time, to estimate how frequently microglia are renewed. They also analyzed microglia from the donated brains of two patients who had received a labeled nucleoside as part of a cancer treatment trial in the 1990s.

SLOW CHURN

Microglia, which populate the brain as blood cell progenitors during fetal development, were replaced at a median rate of 28 percent per year; on average, the cells were 4.2 years old. For Marie-Ève Tremblay, a neuroscientist at the Université Laval in Québec City who was not involved in the study, what stands out is the range of microglia ages found—from brand-new to more than 20 years old. "That's quite striking!" she writes in an email to *The Scientist*.

DIFFERENT STROKES

Tremblay notes that this variation in age meshes with the heterogeneity of microglia types that has begun to emerge in other studies. "With electron microscopy, we find a variety of immune cells in the brain, especially in contexts of disease," she writes.

—Shawna Williams

HOW YOU DOIN'?: A male *L. hesperus* (right) uses its antennae to determine a female's sexual maturity and mating status.

ENTOMOLOGY

Mating Arms Race

THE PAPER

C.S. Brent et al., "An insect anti-antiaphrodisiac," *eLife*, 6:e24063, 2017.

JEALOUS SUITORS

Many male insects deploy "mate-guarding" chemicals that render females unattractive to other males for some time after copulation. The technique gives males' sperm a competitive edge, but can disadvantage females if the effect lasts too long.

LOVE POTION

In a first, Colin Brent of the US Department of Agriculture's Arid Land Agricultural Research Center and colleagues stumbled on a female means of fighting back. In the western tarnished plant bug (*Lygus hesperus*), males' seminal fluid contains antiaphrodisiac pheromones. Over several days or weeks, females convert one of those compounds to geranylgeraniol, which counteracts the antiaphrodisiacs to reveal that she may, in fact, be ready to mate again. "[They're] basically competing signals," Brent says.

GIRL POWER

"This means that the female bugs are not just passive subjects, but they can actively influence the communication and mating system," Sandra Steiger of Ulm University who was not involved in the study tells *The Scientist* in an email. Females continue to produce eggs throughout their lives, so reducing the time between new mates may enable them to have genetically more-diverse offspring, Brent says.

STAY TUNED

Female *Drosophila* had been known to eject an antiaphrodisiac compound from her reproductive tract, but this is the first known instance of an insect countering such a pheromone with a signal of her own. The study's authors predict that more anti-antiaphrodisiacs will be found now that researchers know to look for them. "We need to deepen our understanding of the female part" in post-mating chemical communications, says Steiger.

—Shawna Williams

PROFILE

Damage Patroller

Stephen Elledge has built a career studying how eukaryotic cells maintain genomic integrity.

BY ANNA AZVOLINSKY

When he began a postdoc in Ronald Davis's laboratory at Stanford University in 1984, Stephen Elledge wanted to develop new ways to knock out and mutate specific genes in mammals. His first experimental results contained a serendipitous artifact that laid the foundation for a scientific career studying how eukaryotic cells deal with damage to their DNA.

As a start to designing those gene-targeting tools, Elledge, now a professor of genetics at Harvard Medical School, began by trying to clone the mammalian homolog of *recA,* a bacterial gene required for DNA repair via recombination. Because there was no mammalian *recA* homolog, Elledge attempted to clone the *Saccharomyces cerevisiae* (baker's yeast) homolog using a novel method that included an antibody step. The yeast gene Elledge cloned turned out to be *RNR2,* which encodes the small subunit of ribonucleotide reductase. This enzyme catalyzes the reaction

"DNA was cool itself, but the fact that you could take it apart and put it back together and test ideas on genes—that totally blew my mind. I decided I wanted to do that."

that turns ribonucleotides into the deoxyribonucleotides needed to make new DNA. Elledge had used an anti-RecA antibody that inadvertently cross-reacted with the last four amino acids of Rnr2 in yeast. "It was a depressing day because I did not want to work on nucleotide metabolism—that sounded as boring as you could possibly get for me. So I gave up the project for a while," says Elledge. "But it turned out that this was actually my big break."

Elledge had found that Rnr2 protein levels increased when yeast cells were grown in the presence of agents that damaged DNA. He mentioned this to David Stillman, who was at Stanford to interview for a faculty position, and who studied cell cycle regulation of proteins as a postdoc in Kim Nasmyth's lab at the MRC Laboratory of Molecular Biology in the U.K. Stillman pointed out that ribonucleotide reductase was cell cycle regulated—rather than remaining stable, the RNA and protein levels fluctuate throughout the cell cycle. Elledge decided *RNR2* was worth another look. He found that *RNR2* RNA levels increased dramatically, even more than the protein levels, upon exposure of cells to DNA damage and that mutations in *RNR2* resulted in hypersensitivity to DNA damage.

"I thought, wow, this is gigantic induction. Then I thought, there must be a sensory pathway that recognizes the DNA damage that's going on in the cell," says Elledge. Studying *RNR2*'s regulatory elements, he found those that were necessary to induce the production of higher protein levels in response to DNA damage and identified factors that bind these DNA elements to mediate the response of *RNR2* to DNA damage.

Elledge's idea that eukaryotic cells sense the progress of DNA replication and transform that information into a DNA-damage response was new. While most molecular biologists thought signaling pathways worked by sensing signals extrinsic to the cell and relaying the information to the nucleus, Elledge was proposing an internal signaling pathway that senses cell-intrinsic events. Those results led him to study how cells monitor roadblocks to replication and DNA damage, such as nicks and double-stranded breaks, and how the cell handles that information.

Here, Elledge, talks about how he fell in love with chemistry, how the crux of his graduate thesis was based on a misunderstanding, and why his life partner had to be a scientist.

EMERGING ELLEDGE

It's all matter. Elledge was born in Paris, Illinois, and lived much of his childhood with his paternal grandmother. His family was not "academic," says Elledge. He attributes his own interest in science partly to the Science Research Associates (SRA) reading program: reading-level-rated pamphlets on different subjects, including science. "This was during the Space Race era, so there was a real effort by the U.S. government to get kids interested in science." The final levels—bronze, silver, and gold—were physics and chemistry subjects on matter, atoms, and subatomic particles. "The one on how matter was built out of smaller building blocks really got my attention," says Elledge. "The idea that you could explain everything from smaller and smaller components really appealed to me. I remember sitting on this couch at my grandmother's that had fraying edges, where you could peel back layer after layer until you got to the wood, and thinking that this couch is built just like all matter."

A thing of beauty. By middle school, Elledge was checking out chemistry books from the library. In high school, he excelled in math and chemistry classes. He was on the chemistry team and participated in an interstate contest sponsored by the American Chemical Society, taking first place in the exam competition. "I just loved chemistry: the way the periodic chart self-

COURTESY OF STEPHEN ELLEDGE

STEPHEN J. ELLEDGE

Gregor Mendel Professor of Genetics and Medicine
Harvard Medical School
Geneticist, Brigham and Women's Hospital
Investigator, Howard Hughes Medical Institute

Greatest Hits

- Discovered that mRNA levels of the ribonucleotide reductase enzyme, which helps make DNA nucleotides, increased dramatically in response to DNA damage, resulting in the proposal that a signal transduction pathway senses the rate of DNA replication and adjusts DNA synthesis and repair for accurate genome synthesis
- Identified the *CDK2* gene that encodes the cyclin-dependent kinase 2 enzyme, and together with Wade Harper established how Cdk2 protein kinase is activated and functions to control the transition from the G1 to the S phase of the cell cycle
- Devised cloning technologies, including the first hybrid plasmid and bacteriophage vector and derivatives that could be expressed in either *E. coli* or *S. cerevisiae* and used for the two-hybrid system
- Identified *DUN1,* a gene that encodes a classic kinase activated by DNA damage, providing evidence for an intracellular signaling pathway activated directly by DNA damage
- Showed that the DNA-damage signaling pathway communicates with and influences many cellular functions beyond DNA repair, including senescence, apoptosis, and metabolism

assembles and predicts the properties of different elements, and the idea that there is a physical reality behind everything and everything has an explanation. I thought that was so beautiful," says Elledge.

Biology eye-opener. In 1974, Elledge entered the University of Illinois at Urbana–Champaign on a full scholarship and majored in chemistry. Thinking he would work in the chemical industry, he all but ignored biology. When his pre-med roommate told him he should pay attention to this "DNA makes RNA makes protein and that it's really cool, I just said, 'Yeah, yeah,' and ignored him." says Elledge. But learning about recombinant DNA in a senior-year biochemistry course opened his eyes to biology. "DNA was cool itself, but the fact that you could take it apart and put it back together and test ideas on genes—that totally blew my mind. I decided I wanted to do that," he says. Elledge applied to graduate school in biology, but was nervous about getting in because he had no lab research experience. He decided on the Massachusetts Institute of Technology (MIT), based on advice that it was the best place with plenty of good potential advisors.

EFFECTIVE ELLEDGE

Genetically inclined. Elledge entered MIT in 1978 as a biology graduate student. To compensate for his lack of biology knowledge, he overloaded on catch-up courses, taking 13 over three semesters. Despite thinking he would do enzymology research, Elledge joined Graham Walker's bacterial genetics lab, drawn to Walker, "who was a really nice person," and to the lab, thanks to a paper he had read about the RecA protein, a bacterial protease that's essential for DNA repair. In Walker's lab, Elledge worked on the *umuC* (unmutable C) gene that, when mutated, resulted in strains that couldn't produce genomic mutants even when the bacteria were grown in the presence of mutagens. Elledge initially cloned the *umuC* gene using a technique he developed himself because the standard plasmid library of *E. coli* DNA to complement the mutant phenotype didn't work with *umu* gene mutants. "I discussed a strategy of how to do it with Graham, but when I told him how I did it, he asked how I ever thought of that, because the method I had used was not the one he suggested. I had misunderstood him, and it turned out that the best idea of my thesis was one that no one actually had!" Elledge showed that *umuC* was really two genes, *umuC* and *umuD*, and they worked together to promote error-prone repair.

Genetic tools. Besides working on the DNA-damage signaling pathway, Elledge also focused on creating new laboratory methods. As a graduate student, he had already designed novel bacteriophage lambda cloning vectors. As a postdoc in Davis's lab, Elledge designed multifunctional lambda phage vectors that could be converted to plasmids for expression in yeast and *E. coli*. When Elledge started his own laboratory at the Baylor College of Medicine in Houston in 1989, one of his first experiments was to create a human cDNA library using his phage vectors. Elledge did an experiment—a repeat of one he heard Paul Nurse describe in a talk—to find human genes by complementing a yeast cell-division-cycle (CDC) mutant, *CDC28*. Elledge not only identified the same gene as Nurse, but also the *CDK2* gene, required for eukaryotic cells to proceed to the S phase of the cell cycle.

Revealing how cells cope with DNA damage. In 1993, Elledge and his first graduate student, Zheng Zhou, identified DUN1, a yeast kinase they showed was directly involved in the signal transduction pathway that controls the DNA-damage response. The work was the first to demonstrate that the damage response in eukaryotes is regulated by phosphorylation, providing evidence for Elledge's hypothesis that an intracellular signaling pathway monitors genomic integrity. Elledge's lab, together with that of biochemist Wade Harper, then also at Baylor, discovered cyclin-dependent kinase (CDK) inhibitors, including p21, which modulate the activity of the CDK-cyclin complexes that control transition to the S phase of the cell cycle. His lab continued to identify components of both the yeast and mammalian DNA-damage signaling pathway, components of which also interacted with the cell cycle machinery.

In 1995, Elledge's graduate student Jim Allen found yeast mutations that result in cell death if the cells are exposed to DNA damage. The mutations were in the *RAD53* gene, which encodes a protein kinase that acts upstream of DUN1, and revealed that a kinase signaling pathway is responsible for maintaining genomic integrity—alerting the cell that there is DNA damage and arresting the cell cycle until that damage is fixed. His lab also identified two yeast genes, *MEC1* and *TEL1,* which encode kinases that act upstream of Rad53. Upon DNA damage, Mec1 and Tel1 can transduce the DNA damage signal to Rad53, which ultimately results in arrest of the cell cycle. The team also discovered a role for the DNA damage response in regulation of *BRCA1*, a tumor suppressor gene that can lead to breast cancer when mutated.

Start signal. In 2003, Elledge moved his lab to Harvard University, where he continues to study DNA-damage signaling. That year, he and Lee Zou identified one of the ways that the pathway senses a damaged DNA replication fork—the accumulation of single-stranded DNA (ssDNA) coated with the replication protein A (RPA). "This is what happens at the top of the pathway that leads to all of the signal transduction: when there are stopped or stalled replication forks, you get longer stretches of ssDNA," says Elledge.

State of the cell. "Many biologists used to think that DNA damage was just about arresting the cell cycle, but the fact is that the DNA-damage pathway regulates about 5 percent of the genome, so it's really a global controller that throws many switches on and off," says Elledge. "These switches have to do with repair at the right time and the right place and with communication to other cells, to the immune system. It's remarkable that the cell can figure out whether and how its DNA is damaged and can then do something about it." To understand how broadly the DNA-damage-signaling pathway extends within the function of a eukaryotic cell, Elledge's lab set out to identify all the substrates of the kinases within the pathway. In 2007, they demonstrated the extent of the influence of the DNA-damage response on cell function—beyond mediating the cell cycle—by showing that two of the upstream kinases that mediate the response modify nearly 1,000 proteins, including ones involved in repair, but also in senescence, apoptosis, and metabolism. Many of these proteins, including the DNA-damage response and cell-cycle ones, are mutated in cancers.

LEADING EDGE ELLEDGE

Can't stop, won't stop. In addition to working on DNA-damage response, Elledge's lab is also developing tools to understand how the immune system is wired, including how immune cells and antibodies recognize their epitopes. His lab's first stab at studying HIV, a genetic screen, identified more than 250 proteins HIV needs for its life cycle in its human host, and they have performed similar screens for hepatitis C virus and influenza A. Elledge's lab also recently developed a blood test that can provide a personalized history of an individual's exposure to viruses by identifying the immune system's memory of the viral exposure, using antibodies in the blood. Of 600 individuals studied, the study found, the average person had been exposed to 10 viral species over his or her lifetime. Now, Elledge wants to study immunology.

Lesson learned. "I was really nervous about doing research in graduate school because I had no experience, and I learned a really valuable lesson right away. Someone gave me a plasmid and told me its concentration in the sample. I was supposed to transform it into *E. coli*. I tried and tried and thought my method and plates were bad. I finally figured out that the person told me the wrong plasmid concentration by a factor of 1,000. That's when I realized that you can't trust anyone else's reagents, a really valuable lesson I tell my graduate students all the time."

Partner in science. Elledge is married to Harvard geneticist Mitzi Kuroda. "I had to marry a scientist because no one else could put up with my passion for science unless they really understood it themselves, and I think that was a huge part of my success—I was able to follow my passion. I love and want to talk about it all the time, and to have a partner who shares that same passion is great," he says. ■

Harald Janovjak: Cellular Scion

Assistant Professor, Synthetic Physiology, Institute of Science and Technology Austria. Age: 38

BY AGGIE MIKA

When Harald Janovjak filed his first patent describing the cell growth–regulating receptors he had engineered to be activated by light, he stumbled upon his great-grandfather's 1920 patent for a device that projected color onto movie screens. Janovjak comes from an impressive line of engineers stretching back four generations. But he says he was pleasantly surprised to find that nearly a century later, "we're still tinkering with light-based things."

As a child growing up in Switzerland, Janovjak was always building things with his father. "We would inherit bicycles from my uncles or my older cousins, and we would take them apart and modify them." The acumen he developed for disassembling things, tweaking them, and putting them back together has served him well as a synthetic physiologist.

Janovjak embarked on a career in science as a third-year undergraduate at the University of Basel, after a survey course in biophysics introduced him to microscopist Daniel Müller. In his lab, Müller had pioneered an imaging technique using atomic force microscopy "to visualize single membrane proteins in their native membranes," says Janovjak. He was hooked, and recalls begging Müller for a spot on his team.

Janovjak's drive as a graduate student impressed Müller, who is now at ETH Zurich. "He was really burning for what he was doing; it was incredible," says Müller. "He never stopped thinking about the science." Janovjak's graduate work centered on developing methods to better understand how membrane proteins fold and stabilize. To study protein dynamics, "We had to do quite a bit of method development," Janovjak recalls. In one of the ten papers Janovjak published during his two and a half years in graduate school, he characterized the energy necessary to stabilize a bacteriorhodopsin protein by assessing the amount of force it takes to break it apart.[1]

Müller identifies Janovjak's key strength as a talent for distilling complex biological phenomena into manageable queries. "[Biologists] don't often see the trees in the forest," says Müller. "Harald has no problem understand[ing] very complex theory or biological scenarios—and condensing [them] to a very simple question."

In 2006, Janovjak moved stateside to do a postdoc in the University of California, Berkeley, lab of Ehud Isacoff. By combining genetic components from viruses, bacteria, and rodents, the two engineered a "Frankenstein" version of a light-controlled excitatory glutamate receptor, but with a twist: they converted it into an inhibitory receptor, making it suppress, rather than excite, neuronal activity.[2]

Harald was "the kind of person you want to have as a postdoc," says Isacoff. "Smart and disciplined and not afraid of anything."

As a principal investigator at the Institute of Science and Technology Austria in Klosterneuburg, Janovjak has developed methods aimed at commandeering cell growth using light.[3] His group is taking cells that aren't light responsive and reengineering them to grow using light-activated growth receptors. While the possibilities are vast, he aims to apply this technology to regenerate specific cell populations in diseases caused by cell death, such as type 1 diabetes and Parkinson's.

Janovjak recently accepted a position at the Australian Regenerative Medicine Institute at Monash University, where he will move this December. "We think we have an amazing environment for our work," he says. "The goal has to be that we keep pushing this until we end up with something that is to the benefit of people." ■

© STEFAN FUERTBAUER

REFERENCES

1. H. Janovjak et al., "Probing the energy landscape of the membrane protein bacteriorhodopsin," *Structure*, 12:871-79, 2004. (Cited 86 times)
2. H. Janovjak et al., "A light-gated, potassium-selective glutamate receptor for the optical inhibition of neuronal firing," *Nature Neurosci*, 13:1027-32, 2010. (Cited 94 times)
3. M. Grusch et al., "Spatio-temporally precise activation of engineered receptor tyrosine kinases by light," *EMBO*, 33:1713-26, 2014. (Cited 55 times)

Drugging the Disorderome

Strategies for targeting intrinsically disordered proteins

BY AMBER DANCE

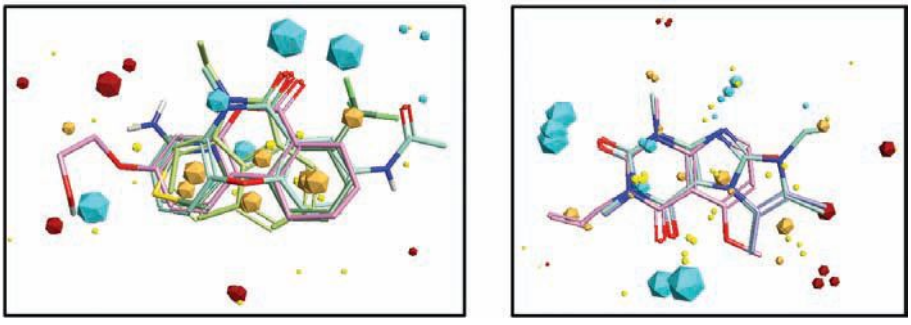
Scientists know a lot about drugs for proteins that settle down into nice, stable conformations and stick with them. Not so for the set known as intrinsically disordered proteins, or IDPs. Their amino acid chains rapidly cycle between multiple conformations, sometimes within microseconds. They're continually striking different poses, like a nanoscopic, superspeed version of Madonna in her music video "Vogue."

As many as one-third to one-half of human proteins are partially or fully disordered, and those proteins are common in signaling and disease pathways. Normally tightly regulated, IDPs are multitasking molecules that can be swiftly repurposed and reconfigured to play roles in multiple regulatory cascades. But when their expression is altered, IDPs can be implicated in conditions such as cancer, cardiovascular disease, and neurodegeneration. Their flexibility stymies drug developers, who are accustomed to defined protein structures, with clear binding pockets where small molecules can dock.

"There's hundreds [of IDPs] that we would like to drug, and we just don't yet know how to do it," says Richard Kriwacki of St. Jude Children's Research Hospital in Memphis, Tennessee.

But he and others think it's worth a shot. Small molecules might, theoretically, nip into a transient binding pocket, stabilize IDPs, and limit their range of possible poses. Or drugs could, perhaps, hover at the edges of the rapidly swirling peptide structures to shut IDPs down. The challenge is to identify or design those small molecules, which will likely bind only weakly to their fluctuating protein targets.

As things stand, drugs that target the so-called "dark proteome" are a future prospect. For now, scientists are conducting proof-of-principle studies that show they can observe that binding, and that such IDP-aimed drugs might be possible. Here, *The Scientist* profiles five approaches to drug the disorderome.



PROMISING PAIRS: An NMR screen for inhibitors of the cell cycle regulator $p27^{Kip1}$ yielded a number of hits, which clustered into two groups that bind to different parts of the protein. Here are representative small molecules that bind to the two different regions. The colors of the polygons reflect the binding site characteristics favored by inhibitors (blue, electropositive; red, electronegative; gold, hydrophobic; yellow, van der Waal) and the polygons' sizes indicate the favorability of the contact interaction.

SPECTROSCOPIC SCREEN

Kriwacki used nuclear magnetic resonance (NMR) spectroscopy to hunt for inhibitors of the cell cycle regulator $p27^{Kip1}$. He and his colleagues first got interested in the protein because it's highly expressed in inner-ear hair cells, preventing them from regenerating in people who've lost hearing due to loud noises or chemotherapy treatment. The protein is also involved in diabetes, obesity, and breast cancer.

Kriwacki's team performed a fragment screen, looking for drug-like moieties that might bind to $p27^{Kip1}$. (See "Piece By Piece," *The Scientist,* June 2013.) But a standard 1,100-fragment library yielded only two hits, and Kriwacki suspected the usual-size fragments were simply too small to grab onto the flapping IDP. The key to success, he says, was creation of a specialized library, with fragments a bit larger. After screening a further 1,222 compounds from that library for interactions with $p27^{Kip1}$, the researchers identified seven more hits. By computationally modeling those molecules and their interactions with the target $p27^{Kip1}$, they found characteristics that allowed them to identify other possible interactors, which they confirmed with NMR. That brought the total number of hits to 36.

The researchers tested one of their hits using in vitro functional assays, and showed it was able to partially disengage $p27^{Kip1}$ from its cellular target, Cdk2/cyclin A—a displacement that activated the kinase. Theoretically, in a cell, this activation would lead to cell cycle progression (*Sci Rep*, 5:15686, 2015).

"The affinity is super-low; it's really just a proof-of-principle experiment," says Kriwacki. By synthesizing larger second- and third-generation compounds, he says, the team is already seeing higher affinity of the small molecules for the IDP. Kriwacki says these compounds bind $p27^{Kip1}$ by a novel mechanism he expects to publish soon.

PROS

- One-dimensional NMR is sensitive enough to identify weak binding, says Kriwacki.
- Two-dimensional NMR can identify the binding sites for the hit molecules.

CON

- The method is expensive and time-consuming; Kriwacki estimates the team used a month or more of continuous NMR time.

SCI REP, 5:15686, 2015

COMPUTER-AIDED DESIGN

To find a drug, one first needs a drug pocket, reasons Lisa McConlogue of the University of California and the Gladstone Institutes in San Francisco. She and her colleagues performed a computational screen to find potential pockets in α-synuclein, a protein that aggregates in Parkinson's disease (*PLOS ONE*, 9:e87133, 2014).

The team started with a set of 40,000 theoretically possible α-synuclein conformations, based on NMR constraints for the protein's disordered ensemble. They picked 22 of them, mostly compact ones they figured were likely to contain a good binding pocket. Then, they used a computer algorithm to predict how various fragments of drug-like molecules might interact with the surfaces of these structures. Based on that information, the researchers identified eight potential binding sites where fragments were most likely to latch on. Using those sites as binding pockets, they then computationally attempted to dock 33,000 small molecules into those spots. This yielded 89 hits.

The authors selected one compound, ELN484228, for further experiments. Overexpression of α-synuclein is known to interfere with phagocytosis, and ELN484228 fixed this defect in cultured cells. It also protected cultured brain cells from the neurotoxic effect of mutant α-synuclein. It's still not clear what ELN484228 is doing to α-synuclein; McConlogue suspects it may bind to beneficial conformations, promoting the protein's healthy activity.

PRO

- Low cost
- High throughput

CON

- Computational screens are risky, says collaborator Gergely Tóth of the Hungarian Academy of Sciences in Budapest and the University of Cambridge in the U.K. They may yield false results that don't hold true in real-world studies.

SKIMMING THE SURFACE

McConlogue, Tóth, and colleagues used a physical binding screen to look for small molecules that interact with tau, which aggregates in Alzheimer's and related conditions (*Curr Alzheimer Res*, 12:814-28, 2015).

In collaboration with Graffinity Pharmaceuticals, now part of NovAliX in Heidelberg, Germany, the researchers began with Graffinity's in-house library of 110,000 small drug-like compounds. They attached these to microarrays, then used surface plasmon resonance (SPR) imaging to determine which were bound by tau protein. SPR uses the angle of light reflected by an array of molecules to detect the increase in mass when one of those molecules is bound by one of another set of molecules, floated over the array in liquid.

This protocol flipped the typical SPR method, in which the proteins are immobilized on the array and the small molecules are flowed over them. The traditional technique wouldn't work for IDPs, Tóth reasoned, because the binding of small molecules would be so weak, it wouldn't change the resonance signal of the large protein. But with the small chemicals immobilized, even weak binding by a large tau protein changed their physical characteristics enough to register. Plus, that way the IDP tau was unbound, and so sure to be in its native conformation. Placing many drug-like molecules in the array was the only way to perform a high-throughput SPR screen, adds McConlogue.

The scan yielded 834 hits. Of these, the team tested 70 for impact on tau aggregation. The authors were pleasantly surprised to see that, in cultured cells, at least two-thirds of the compounds blocked tau aggregation to some extent, and three molecules the authors investigated in more detail did so in a dose-dependent manner. Tóth suspects they bind and stabilize the protein monomer. He is now CEO of Sunnyvale, California–based Cantabio Pharmaceuticals, a company using these techniques to identify small molecules that act as "chaperones" to stabilize IDPs and other proteins involved in neurodegeneration.

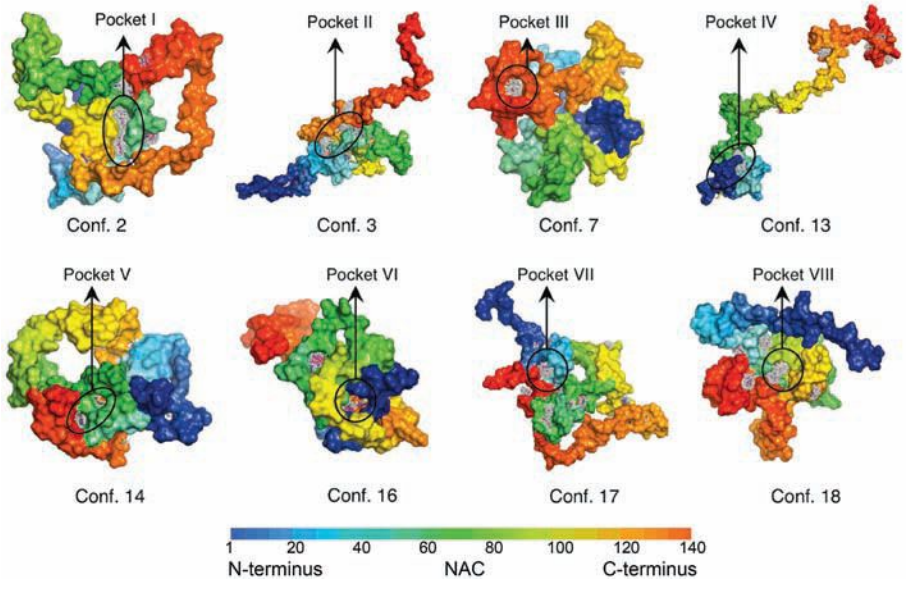
PRO

- High throughput
- Sensitive to weak interactions
- Effective at near-physiological concentrations of protein; the team used 100–500 nM tau.

CON

- Compounds that bind under microarray conditions, immobilized on a surface, may not do so in other circumstances.

A COZY FIT: Scientists identified eight potential drug pockets in α-synuclein by computationally fitting small, drug-like molecules into different places on the protein's diverse conformations.



PLOS ONE, 9:E87133, 2014

ALL ABOUT ALLOSTERY

The protein tyrosine phosphatase PTP1B used to be considered undruggable, says Navasona Krishnan, a research investigator in the Nicholas Tonks lab at Cold Spring Harbor Laboratory in New York. Pharmaceutical experts would love to inhibit it, anticipating treatments for diabetes and obesity as well as breast cancer. But scientists have struggled to slide a small molecule into the highly charged active site. The resultant drug candidates tend to be poor at entering cells, or at reaching target tissues when administered orally.

So Krishnan and Tonks went looking for an allosteric inhibitor, which would deactivate the enzyme by binding outside the active site and altering its conformation. They found it in an appetite suppressant called MSI-1436 (trodusquemine). Krishnan and his colleagues tried, and failed, to obtain a crystal structure of the protein's regulatory carboxyl terminus, where MSI-1436 binds. What was the problem? NMR confirmed that the binding site was intrinsically disordered.

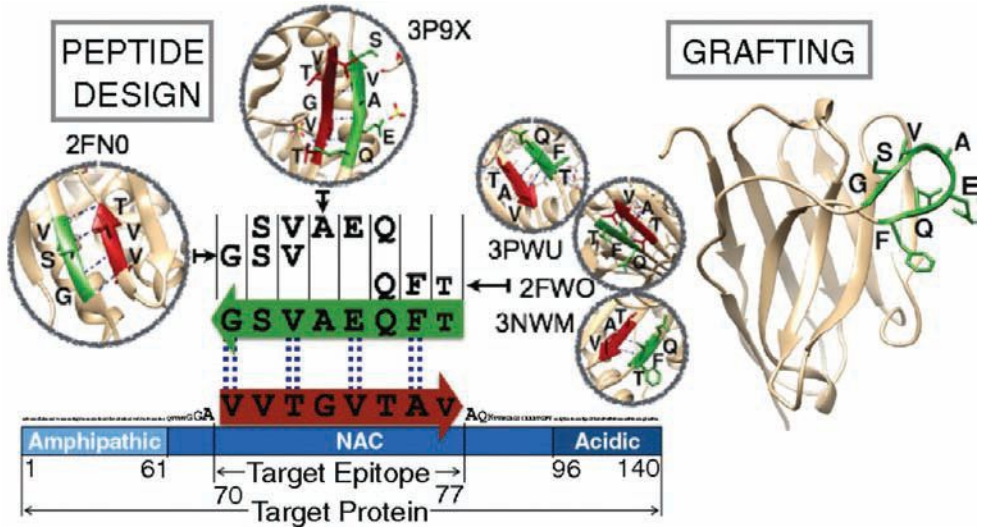
MSI-1436, the researchers discovered, binds to a short helix that only forms when the compound is present. This helix migrates to the backside of the catalytic domain and recruits a second MSI-1436 molecule to that spot. The pair hold the enzyme in an open conformation, so it can't dephosphorylate its substrates (*Nat Chem Biol*, 10:558-66, 2014). In mouse models of breast cancer, MSI-1436 prevented tumor growth and metastasis; it's already in a Phase 1 clinical trial for breast cancer. It crosses cell membranes, and while it's not orally bioavailable, the scientists have made analogs that are, says Krishnan.

PRO

- Allostery can work when the active site creates pharmacological complications.

CON

- When working with IDPs, scientists must check that their compound is selective for the target protein, since there will be other IDPs about in the cell, cautions Krishnan.



PATCHWORK ANTIBODIES: To make an antibody against this particular epitope of the α-synuclein protein, a computer program overlapped short amino-acid sequences from three different kinds of proteins in the Protein Data Bank. Then, scientists fit the genetic code for that amino-acid sequence into the gene for an antibody, resulting in one that should bind α-synuclein at that site.

DESIGNER ANTIBODIES

While many scientists aim to bind IDPs with small molecules, Michele Vendruscolo of the University of Cambridge has developed a way to engineer large antibody binders that might serve in research or the clinic.

It can be difficult to generate antibodies to IDPs using standard methods, so Vendruscolo's team came up with an *in silico* technique (*PNAS*, 112:9902-07, 2015). The procedure starts with a desired epitope of eight residues. Then, the researchers seek strings of amino acids that would likely bind to that epitope. As candidates for those amino acid strings, they cull peptide sequences from the Protein Data Bank, picking any short peptides known to interact with at least three residues of the target epitope. The computer program then combines those snippets to build up larger peptides, which ought to cover the entire epitope.

All the scientists have to do then is clone a stretch of DNA for that peptide into the third variable complementarity-determining region (CDR) of an antibody heavy chain. The antibody they chose as the scaffold was one that's easy to purify from bacteria and known to function well despite insertions into that variable portion. The authors call the resulting computer-designed antibodies DesAbs.

In this manner, the team made antibodies against α-synuclein, as well as against amyloid-β, which is associated with Alzheimer's disease, and against islet amyloid polypeptide (IAPP), which is involved in diabetes. Most are weak binders to the IDP monomers, says Vendruscolo, though they are more likely to bind aggregates. He thinks these kinds of antibodies will probably find a use in diagnostic or imaging tests in the clinic, as well as in labs.

PROS

- Inexpensive
- The team can generate an antibody within a few weeks.
- Reliable; the group has tried their method on about 30 different epitopes of α-synuclein, amyloid-β, and IAPP, and it "nearly always works," Vendruscolo says.

CON

- The technique is new, and Vendruscolo says there's still plenty of optimization to do.
- Antibodies are difficult to apply as treatments, because they're large proteins. It's hard to deliver them to the brain, for example, because they don't naturally cross the blood-brain barrier. ■

PNAS, 112:9902-07, 2015

Designer DNA

Computational tools for mapping out synthetic nucleic acids

BY RACHEL BERKOWITZ

When James Watson and Francis Crick announced in 1953 that they had determined the double-helical structure of DNA, the letters G, T, A, and C were forever embedded in the collective mind of the biology world. The arrangement of these four nucleotide bases in a strand of DNA dictates the sequence of an organism's every protein.

These days, synthetic biologists can treat those four bases as the programming language underlying protein design. The field is grappling with how best to manipulate this blueprint that "makes a hummingbird into a hummingbird and not into a cow," says Claes Gustafsson, cofounder of a bioengineering company called ATUM (formerly DNA2.0).

Scientists have known for decades how to manufacture DNA in the lab, in principle allowing them to manipulate life in ways that Watson and Crick couldn't have imagined—inserting genes into bacteria, yeast cells, or algae to produce enzymes from different organisms, or encoding proteins that fold into shapes not found in nature.

But in practice, discerning the precise DNA sequence that gives rise to a certain protein, or predicting how a sequence will behave when expressed in a host organism, has been a tedious, manual activity. In recent years, however, a new crop of open-source computational tools has emerged, allowing researchers to improve the accuracy and efficiency of designing synthetic DNA.

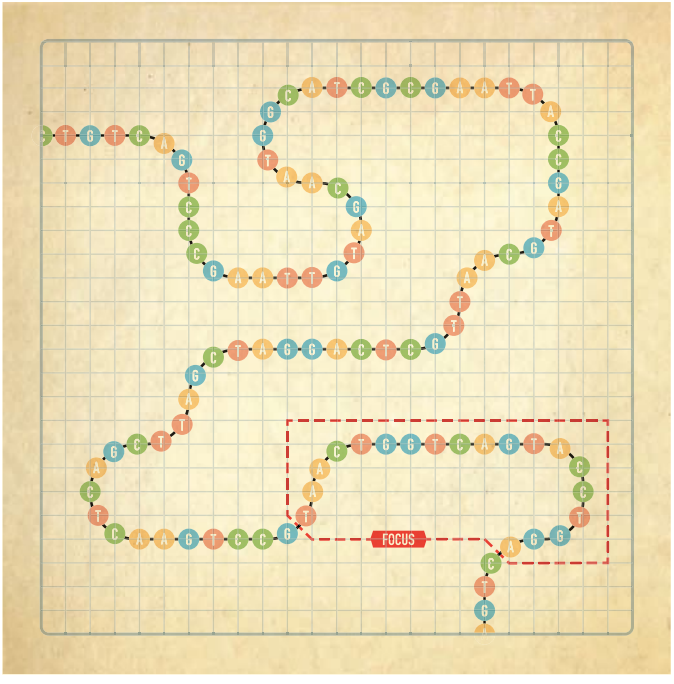
The Scientist explores some of the tools available to synthetic biologists for gleaning function from sequence, predicting protein structure, reducing synthesis errors, and designing complex systems.

MAPPING SEQUENCE TO FUNCTION

RESEARCHERS: Claes Gustafsson, cofounder and Chief Commercial Officer, and Alan Villalobos, Vice President, ATUM

PROBLEM: As the cofounder of a bioengineering company, Gustafsson was spending a lot of time helping customers extract information from sequences that they had compiled through computational methods but did not fully understand. So he started to put together glossaries of functional elements. Using them, he developed software that determines which segments of a sequence encode which features, including gene promoters and markers. "In the old days, it would take a day just to sort out what was in the user's file. Now I can take the sequence, dump it into DNA ATLAS, and it takes half a second to get exact, detailed meta-info," he says.

TOOL: DNA ATLAS allows researchers to efficiently track, annotate, visualize, interrogate, and predict sequence-function correlations. The user inputs a DNA sequence as a text file, and gets a graphical plasmid map representation of the features encoded in that sequence. The underlying cloud-based dictionary of several thousand genetic elements lets users annotate any DNA sequence with the push of a button, and annotations reflect changes in knowledge as the database grows.



FUNCTIONALITY: Recently, DNA ATLAS helped a research group sort through years' worth of unexamined sequences from an old database. In the early days of DNA synthesis, people would typically record sequences with a name that "meant something for the person who wrote it, when they wrote it," says Gustafsson. "The amount of information lost was staggering." But Gustafsson's tools used sequence data alone to identify genes and map them to function—a wealth of information that current group members use.

TIPS: If DNA ATLAS returns very few feature hits, it's likely the system is unfamiliar with the specific sequence in the input file. Users can manually add sequences to DNA ATLAS, expanding its knowledge base.

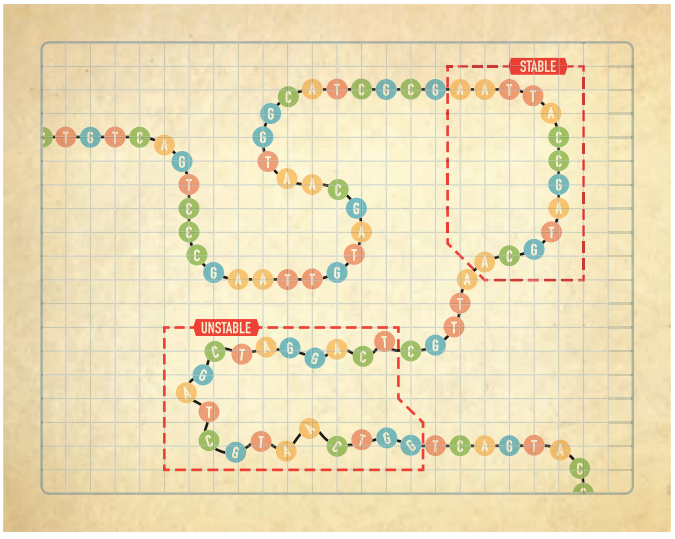
FUTURE PLANS: Villalobos and Gustafsson plan to add visualization and data exploration tools that customers can use on their function data. The company's internal version is also integrated

with wet-lab data and machine-learning tools to draw understanding from sparse data sets.

DECODING STABLE SEQUENCES

RESEARCHERS: Gabriel Rocklin, Postdoctoral Fellow, and David Baker, Professor of Biochemistry and Director, Institute for Protein Design, University of Washington

PROBLEM: De novo proteins are designed to have novel structures not found in nature and offer vast potential for creating useful new functionality. But when designing these proteins, not all



amino acid sequences fold into the desired structures. "De novo protein design, for decades, has involved making ten proteins and testing them, hoping that a few of them work," says Rocklin. Current computer simulations cannot reliably determine whether a given sequence will fold into a stable structure. This led Rocklin and Baker to develop a method for generating thousands of possible sequences that could encode novel protein shapes, and identifying those that yield stable folded structures.

TOOL: A user specifies a desired structure—for example, a helix 13 residues long and connected to another helix or to a β-sheet with a defined length. The open-access prediction and design software ROSETTA, developed along with colleagues at 40 universities, generates a 3-D model of the protein and proposes thousands of sequences that could fold into that structure. Then, researchers convert the optimized list of amino acid sequences into DNA sequences and synthesize those genes thousands at a time as an oligonucleotide library. By inserting the synthesized genes into yeast cells, which then produce the proteins, and introducing enzymes that digest only the unstable proteins, Rocklin and Baker can ferret out the sequences that achieved stable structures (*Science*, 357:168-75, 2017).

FUNCTIONALITY: The duo's proof-of-concept analysis identified more than 2,500 stable designed proteins, enough to figure out important design principles for small proteins and to improve their success rate by a factor of eight.

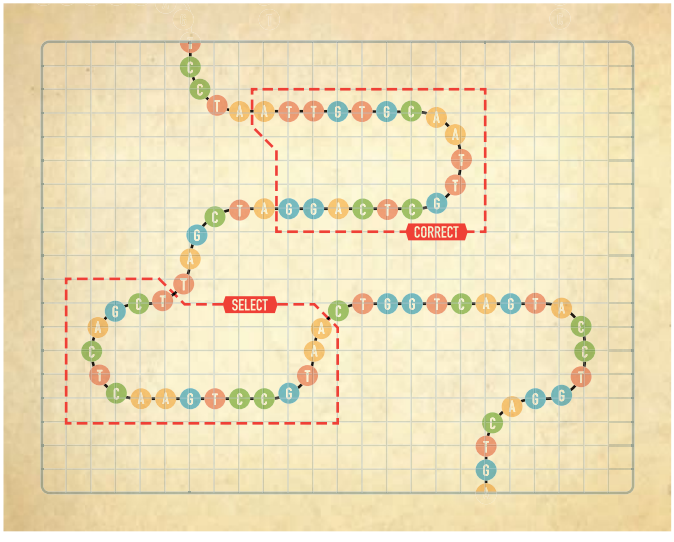
TIPS: For designing new protein structures with ROSETTA, Rocklin advises starting with structures that have properties comparable to those that his study identified as stable. Some important properties that lead to stability include the amount of buried hydrophobic surface area and the compatibility between local sequence and secondary, folded structure.

FUTURE PLANS: Rocklin and Baker plan to move beyond stability to design small proteins with other useful functions. For example, a protein designed to target a specific binding partner may act as a therapeutic compound. (For methods aimed at drugging intrinsically disordered protein, see article on page 62.) As DNA synthesis technology improves, they also envision expanding their high-throughput approach to larger, more complex protein structures.

REDUCING ERRORS

RESEARCHERS: Ernst Oberortner, Jan-Fang Cheng, Samuel Deutsch, and Nathan Hillson, Department of Energy Joint Genome Institute (JGI)

PROBLEM: DNA synthesis companies can't always manufacture the sequences that investigators submit to them, especially when they contain long stretches of the same base or repetitions of the same sequence. That's in part because available computational tools do not sufficiently consider the limitations of synthesis technologies when designing sequences, says Deutsch. Sending researchers back to the drawing board to redesign their sequence can significantly increase a project's cost and time line. Oberortner, Deutsch, and their colleagues sought to streamline the transition from design to synthesis. They created a DNA synthesis design tool



that incorporates knowledge of features that simply don't work in the DNA manufacturing process, thus fully automating the detection and resolution of synthesis constraints and producing ready-to-build sequences that code for proteins that should be functional.

TOOL: Different DNA synthesis companies are limited in different ways, depending on their manufacturing processes and analytical techniques. With an understanding of these limitations, the JGI team built a suite of software, called the Build Optimization Software Tools (BOOST), which automates the once-manual process of fixing problematic DNA sequences. The user uploads up to 1,000 sequences per run, in some cases adding information specific to the host organism. The software detects amino acid codons, corrects errors, verifies against manufacturing constraints, and separates the sequence into synthesizable portions. The open-access software is easily integrated into pre-existing design pipelines, or can be used as an independent web-based user interface (*ACS Synth Biol,* 6:485-96, 2017).

FUNCTIONALITY: A known enzyme, such as one that fixes CO_2, might not fold properly when introduced into a different host. The best approach to finding one that works in the model organism is to try hundreds of similar enzymes that have a similar function. This was impossible before computer-aided design; even now, some designed sequences can't be synthesized. BOOST helps to ensure that every sequence results in a testable experiment, avoiding the problem of selecting a sequence that cannot be manufactured.

TIPS: Given the different constraints on individual DNA synthesis vendors, and the continuous evolution of the field, researchers should be careful to select the appropriate vendor-specific constraints in the software to make sure BOOST generates suitable output.

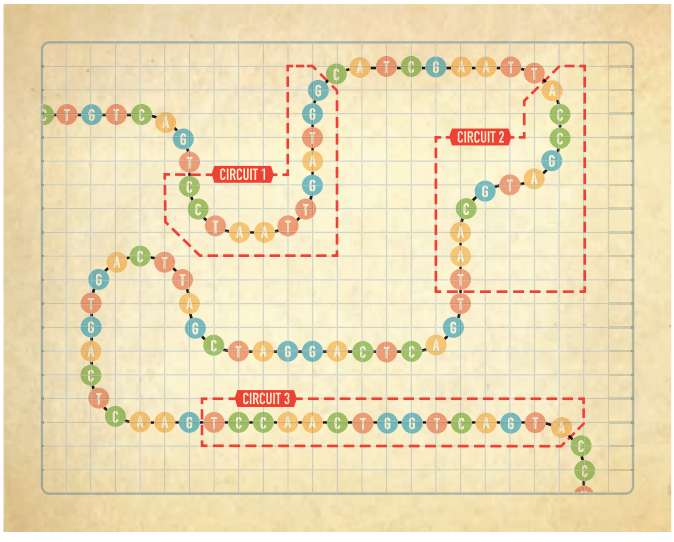
FUTURE PLANS: BOOST is currently a gene-centric algorithm, but synthetic biologists want to build entire circuits that entail multiple proteins working in conjunction to dictate cell behavior. Future versions will automatically correct a complete signaling pathway.

COMPOSING GENE CIRCUITS

RESEARCHER: Christopher Voigt, Professor of Biological Engineering, MIT Synthetic Biology Center

PROBLEM: Designing reliable and complex circuits encoded in DNA—sets of genes that work together to carry out a desired function—is a central problem in synthetic biology. Complex systems require control over the timing and conditions dictating when each gene gets turned on. Tired of manually piecing together DNA sequences cataloged in a Microsoft Word file, Voigt, along with Douglas Densmore of Boston University, developed the open-source software Cello to automatically transform a desired circuit function into a DNA sequence.

TOOL: Cello is based on a text-based programming language called Verilog, which engineers use to design electronic chips. Users input the desired function, such as a logic operation, and these can be connected to genetic sensors—for example, to build a cell that responds to light and a signal from a neighboring cell. They also upload the genes that they want to be triggered to effect a given response, such as producing a certain metabolite. Cello parses the Verilog text input, creates the circuit diagram, and determines the DNA sequence that will take the specified inputs and yield the desired output (*Science,* 352:aac7341, 2016). Once the DNA sequence is generated, you can synthesize it yourself or outsource the process, Voigt says.



WHAT IT CAN DO: Voigt has designed genetic circuits to make cells in a fermenter optimize their own production in response to specific cellular conditions. He has also designed circuits that make bacteria deliver therapeutics in response to conditions encountered in the human body.

CHALLENGES: "We're working with very small circuits compared to what you have in electronics," says Voigt. Designing more than nine regulatory genes to work together becomes difficult. In electronic circuits, a logic gate is built once and then replicated, but with proteins, newly added logic gates can conflict with others. Additionally, boosting a cell's protein expression can cause toxicity because it taxes the cell's resources.

FUTURE PLANS: Cello currently operates on Boolean logic. Now, Voigt is designing versions that use more-complex, sequential logic and that can make different sensors operate at different times. Along with his former student Alec Nielsen, who developed the technology, he has started the company Asimov to commercialize Cello. ■

Navigating a Rocky Landscape

The battle for control of the intellectual property surrounding CRISPR-Cas9 is as storied and nuanced as the technology itself.

BY AGGIE MIKA

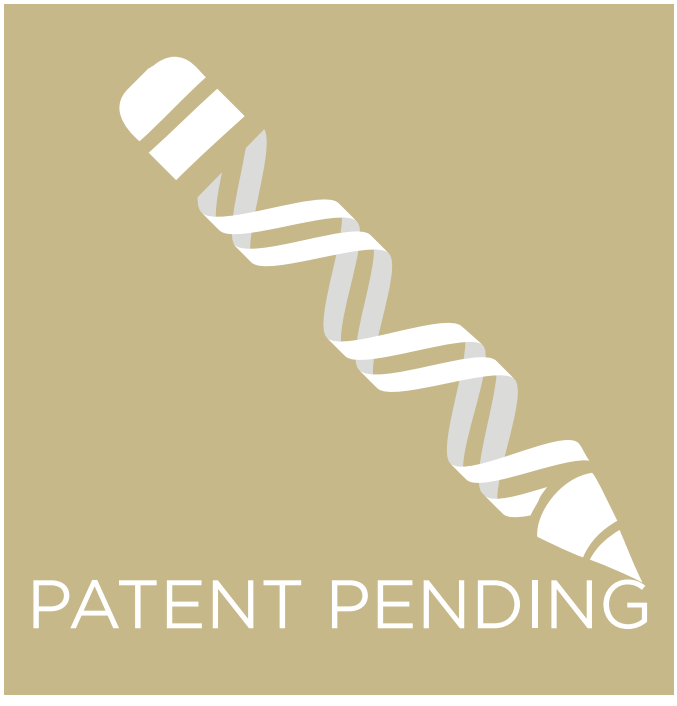
In May 2012, the University of California, Berkeley, filed a patent application for biochemist Jennifer Doudna and the University of Vienna's Emmanuelle Charpentier, then of Umeå University in Sweden, based on their seminal observation that the bacterial CRISPR-Cas9 gene-editing system can be used to target different sequences of DNA by reprogramming the system's small homing guide RNAs. The Broad Institute of MIT and Harvard followed suit that December with applications for bioengineer Feng Zhang and colleagues covering CRISPR's use in eukaryotic cells. When the US Patent and Trademark Office (USPTO) granted Zhang's patent in April 2014, thanks to an expedited review process, a now-infamous dispute was born.

The University of California (UC) group quickly filed for a patent interference hearing, which the USPTO's Patent Trial and Appeal Board (PTAB) granted in December 2015. In February of this year, however, the PTAB three-judge panel ruled that the Broad's innovations are patentable separately from the UC team's original discovery. Not wanting to be limited to gene editing in bacteria, the UC side appealed the ruling in April, claiming that their original application covers the use of this technology in all cells—plant, animal, and human, in addition to bacterial. "There's a lot of uncertainty right now about who is going to own what rights," says Lisa Larrimore Ouellette, a law professor at Stanford University.

While the dispute has little bearing on the use of CRISPR-Cas technology by academic researchers—who have unimpeded access so long as they don't try to sell the fruits of their labor—those hoping to develop this technology for commercial uses find themselves navigating a challenging IP landscape. "[Some companies] have begun to think about licensing from both the Broad and UC Berkley, just to hedge their bets," says Arti Rai, a law professor and codirector of the Duke Law Center for Innovation Policy at Duke University.

As scientists harness CRISPR for a growing list of medically relevant tasks, including germline editing and CAR T-cell therapy, the stakes continue to rise. "Human therapeutics is where it's at, commercially," says bioethicist and former patent attorney Christi Guerrini of Baylor College of Medicine. "That's the prize."

Applications constituting more than 1,720 patent families—applications filed for the same invention in multiple countries are considered part of the same patent family—have been submitted worldwide by the Broad, UC, and hundreds of other institutions, companies, and researchers, according to Fabien Palazzoli, an analyst at IPStudies. "Around 100 new patent families on CRISPR are published each month," says Palazzoli in an email to *The Scientist*. Less than 10 percent of these patents have been granted thus far, more than one third of them in the U.S., according to Palazzoli. Along with their collective commercial licensees, these patent holders are now making moves that will affect how this technology will be used and who will have access for years to come.



It's a risky business to be in.

—Lisa Larrimore Ouellette, Stanford University

One-stop shop

In April, Denver, Colorado–based MPEG LA, LLC announced the formation of what is known as a patent pool for CRISPR-Cas9 technologies, inviting relevant parties to submit their patented tools to a single consortium that would simplify the licensing process—one license would grant licensees access to the pool's slew of patents. In theory, the pool would promote access through nonexclusive agreements while still profiting the patent owners through royalty payments. It would especially serve the smaller fish, says MPEG LA's executive director of biotechnology licens-

ing Kristin Neuman. Licensing every relevant patent can be costly and time-consuming, restricting the number of licenses a smaller company can obtain. This could potentially leave out patent holders with "just one piece of the puzzle," says Neuman. As part of a pool, however, patent holders "would be able to achieve a broader scope of licensing of their IP rights."

Already, the Broad, Rockefeller University, Harvard, and MIT have submitted 22 of their granted and pending CRISPR-Cas9 patents for consideration. MPEG LA wouldn't divulge whether it has successfully wooed other patent holders, but is "very pleased with the results of our call for patents," Neuman says. Whether UC will join is still unclear. Its decision will likely depend on the outcome of the UC team's appeal of the patent interference ruling, notes Rai. If UC is granted its broad-reaching patent on appeal, it will command the lion's share of CRISPR IP, and could likely negotiate for the bulk of the pool's royalties. But, "historically, the king doesn't join the pool," says Rai. "If Berkeley wins, that will create quite a situation." UC has declined to comment on its plans to enter the pool while the litigation is ongoing.

Regardless of how the situation with UC plays out, these are "early days for the pool," Neuman stresses, and its key patent holders have yet to define the terms. But, she says, the pool will likely entail nonexclusive licenses, allowing patent holders to enter into agreements with outside parties, should they choose to. It's hard to predict how the pool would affect existing licensing agreements, she says. "MPEG LA is not privy to such agreements, which are between private parties, so we have no way of knowing how preexisting licenses [would be] affected (if at all) by subsequent patent pool formation," she writes in an email. As per the pool's nonexclusive licensing model, patent holders that have entered into exclusivity agreements would be barred from entering the pool with the same exclusively granted patent rights.

Human therapeutics is where it's at, commercially. That's the prize.

—Christi Guerrini, Baylor College of Medicine

Nonexclusive licenses have their pitfalls, notes New York Law School attorney Jacob Sherkow: they don't lend themselves well to the development of human therapeutics. "You need some form of exclusivity for companies to conduct clinical trials," he says, as a market monopoly incentivizes companies to take on the costs and liability of developing CRISPR-based treatment for disease. Otherwise, he says, it is unclear if anyone will pony up the resources.

CLAIMS OF GRANTED U.S. PATENTS

Of the patent applications that have been granted, who owns what piece of CRISPR? The gene-editing technology is like an onion: the layers can be peeled back one by one, says Christi Guerrini, a bioethicist at Baylor College of Medicine. In the chart below, multiple patents are listed within the same claim category if they claim different pieces of the same technology. For instance, multiple patents exist for editing and tagging bacteria, though they differ in their precise means. Likewise, the same patent can fall under multiple claim categories, as patents often list more than one claim.

Overarching category	Detailed category	Examples
System components	Endonuclease	Cas9, Cas6, Cpf1, split-nexus Cas9-associated polynucleotides, Cas9 nickase, RNA-guided FokI nucleases, mutant Cas9 proteins, chimeric CRISPR enzymes
	CRISPR RNA (crRNA)/ trans-activating crRNA (tracrRNA)/guide RNA (gRNA)	Extended DNA- and RNA-sensing gRNAs, switchable gRNAs, truncated gRNAs, chimeric gRNAs, DNA-guided CRISPR systems
	Vectors	Large targeting vectors, viral vectors, plasmid vectors, nanoparticles
Methods and applications	Target cell	Optimization of CRISPR system for expression and function in eukaryotic and prokaryotic cells
	Gene editing uses	Correcting genetic mutations in cell lines, producing knock-outs and knock-ins, and regulating transcription
	Other methods	Processing a target RNA, delivering proteins inside cells
	Therapeutics	Correcting mutations in proteins associated with Alzheimer's disease, treatments for cancer cachexia

Neuman is familiar with this criticism. "That's largely why we haven't seen patent pools operating in human therapy," she says. But according to her, a patent pool could set the stage for developing CRISPR-based therapeutics. For example, offering foundational CRISPR patents that are not focused on a specific therapy or gene on a nonexclusive basis might stir competition and accelerate innovation by giving "patent owners the opportunity to maximize return and minimize risk on their technology investments from many developers in many fields," she explains in an email. Narrow, disease-specific patents would be precluded from the patent pool, and thus would allow companies licensing those technologies to maintain exclusivity in a given market.

But Sherkow argues that this strategy doesn't address the crux of the issue, and is likely to prompt an innovation impasse. "The issue isn't whether the target-specific patents are available for exclusive licensing," he writes to *The Scientist* in an email. "It's what to do about the foundational patents currently being parceled off in large swaths."

The technology and scope of the pool's patents are also still up in the air—this would depend on which patent holders end up joining the pool and the terms they negotiate—although the pool could lay claim to applications in agriculture, industrial biotechnology, and human therapeutics. Depending on how this shakes out, and given the complexity of CRISPR technology, "we might have to do a modular approach" and offer license subsets for specific purposes, Neuman says. The first meeting of the pool's patent holders is slated for some time between October and the end of the year.

Adding to the uncertainty, vast numbers of CRISPR patent applications are still pending, Neuman adds. "We're just starting to see what the patent landscape looks like." Even after it's formed, the pool will continue to evolve as it keeps up with the ever-changing landscape.

The lay of the land

According to Palazzoli, there are approximately 78 patents with claims pertaining to CRISPR in the U.S. Northeastern universities are in the lead, with 13 inventions affiliated with Zhang's lab and held by the Broad and its collaborators, including MIT and Harvard, while 14 are held by Harvard and list David Liu and/or George Church as inventors. Although Doudna's foundational patent is still in dispute, four other patents related to the endoribonuclease Csy4 are affiliated with her lab and held by UC. Of the nonacademic patent holders, Caribou Biosciences, cofounded by Doudna, has seven, while several other companies, including Recombinetics, Regeneron, and Agilent have also carved out their own pieces of CRISPR IP. (See chart at left.)

Inventions that tweak CRISPR's various molecular components in an effort to optimize the technology shape the patent landscape. In this regard, guide RNAs (gRNAs)—the components of the CRISPR system that lead the Cas9 enzyme to precise locations within target DNA—can be altered to home in on an infinite number of genes and to perform more-selective cuts. Similarly, inventors have made modifications to the Cas9 enzyme or adopted other bacterial endonucleases. Different approaches for modifying eukaryotic cells for the treatment of various diseases can also be separately patentable. (See chart on page 69.)

CRISPR PATENTS GRANTED IN THE U.S.

Currently, according to IPStudies, more than 200 patents have been granted worldwide that cover the components, applications, and delivery of this technology. The U.S. is a leader in this space with almost 80 granted patents with CRISPR claims. The chart below lists some of the major institutions and commercial entities that hold granted US patents with claims to CRISPR-Cas systems.

Applicant/assignee	Granted patents
Agilent Technologies Inc	1
Caribou Biosciences	7
DuPont Nutrition & Health	4
Feldan Bio Inc.	1
Institut Pasteur	1
Larix BioScience LLC	1
Pioneer Hi-Bred International	3
President and Fellows of Harvard College	14
Recombinetics Inc.	1
Regeneron Pharmaceuticals	4
Snipr Technologies Ltd	1
System Biosciences LLC	2
The Broad Institute of MIT and Harvard and collaborators	13
The General Hospital Corporation	3
The Regents of the University of California	4
University of Arkansas for Medical Sciences	1
University of Georgia Research Foundation	3
Vilnius University	1

Most of the patent holders listed above are included based on their claims to CRISPR and/or Cas9 inventions and were identified using the USPTO's full-text patent database. However, a few that did not explicitly state CRISPR-Cas in their claims were included based on their inventions' association with or intended use of the CRISPR system.

Earlier this year, Sherkow and University of Utah law professor Jorge Contreras published a detailed snapshot of the CRISPR licensing landscape (*Science*, 355:698-700). A complete picture, however, is nearly impossible to ascertain due to the lack transparency surrounding licensing. For instance, it's common for entities to license technology based on pending or unpublished patent applications, says Rai. In fact, it happens all the time, although nothing can be enforced until the patent is granted.

When it comes to commercialization of human therapeutics, it's clear that the patent holder's licensees are in control. Both UC Berkeley and the Broad have granted broad, exclusive rights for human therapeutics to their respective commercial enterprises—Berkeley to Caribou Biosciences, Intellia Therapeutics, and CRISPR Therapeutics, and the Broad to Editas Medicine. By granting such far-reaching, unrestrictive licenses, these patent holders have effectively passed off the responsibility of deciding who gets to commercialize CRISPR in the therapeutics space, Sherkow explains, as other entities seeking to commercialize now have to approach these companies for sublicensing. For instance, Editas has an exclusive agreement with Juno for CRISPR-based CAR T-cell technology, whereas CRISPR Therapeutics has granted Vertex rights to use CRISPR for cystic fibrosis therapeutics.

We're just starting to see what the patent landscape looks like.

—Kristin Neuman, MPEG LA

Sherkow argues that this power should be retained by the patent-holding institutions, however, which should grant only narrow and restricted licenses in the first place. This would "maximize competition" and expedite the development of promising products, he says. Under the current model, however, a company with the most appropriate expertise and resources for a particular therapy may be unable to obtain the necessary licenses or sublicenses to pursue it. The lack of oversight on how academic institutions, and their commercial licensees, are dishing up licenses and subsequent sublicenses is concerning, says Larrimore Ouellette. "There is not enough attention being paid to whether research from public institutions, funded by public money, is licensed in a manner that serves public interest."

Guerrini, on the other hand, feels that the Broad has a thoughtful approach to licensing CRISPR and commends it for negotiating certain restrictions with various commercial entities. For instance, Monsanto holds a nonexclusive license for the use of CRISPR in agriculture, but cannot use the Broad's technology for commercializing gene drives or tobacco products. Similarly, Editas and its sublicensees cannot venture into germline editing or use CRISPR to grow organs for human transplantation. "Their decisions demonstrate caution and delay where appropriate, and reflect the general ethical consensus on the use of this technology for these purposes," Guerrini says.

The value of the licenses and sublicenses that have been granted so far remains to be seen, however, pending the outcome of the ongoing legal dispute in the U.S. Some of the license agreements that have enabled the development of CRISPR-based CAR-T treatments, among other therapeutics, stem from the foundational CRISPR patents that are embroiled in the dispute between UC Berkeley and the Broad, for example. Editas, the exclusive licensee of the Broad's patents that are involved in the dispute, is confident that the PTAB's no-interference ruling in the Broad's favor will stand, according to Editas communications head Cristi Barnett. "[T]he appeal does not in any way impact our plans to continue investing in this technology," she writes in an email to *The Scientist*.

Regardless, "it's a risky business to be in," says Larrimore Ouellette. "[Researchers are] basing their companies on rights that might not end up existing." ■

CRISPR PATENTS WORLDWIDE

It's typical for inventors to apply for the same patent in multiple countries. Overseas, major CRISPR players from the U.S., including the University of California, Berkeley, and the Broad Institute of MIT and Harvard, have done so in order to extend their status as key patent holders. China's State Intellectual Property Office (SIPO) has granted the UC team its controversial patent with claims to CRISPR in both prokaryotic and eukaryotic cells—the same one that's embroiled in the dispute with the Broad in the U.S. The European Patent Office (EPO) has also granted UC Berkeley this wide-ranging patent. In Europe, there are currently more than 25 CRISPR-related patents, according to IPStudies, 10 of which belong to the Broad and its collaborators.

This summer, the EPO announced that it will also move forward with granting MilliporeSigma's CRISPR patent application for the use of the technology in adding genetic information into eukaryotic cells—claims that line up with the Broad and UC Berkeley's use of CRISPR in eukaryotes, according to patent attorney Catherine Coombes of HGF Limited. This overlap is bound to create uncertainty about who owns what, she notes in an email to *The Scientist*. "[A] company seeking to utilize CRISPR-Cas9 (e.g. for a particular therapeutic purpose) may ultimately need to seek a license from numerous patent holders in Europe."

Lost Minds

Modern technology can offer a window into the cognition of extinct species.

BY GREGORY BERNS

Earlier this year, there was a flurry of excitement in Queensland, Australia, over the renewed search for the iconic Tasmanian tiger—a.k.a. thylacine. Never mind that the last documented thylacine died in the Hobart Zoo in 1936, and that the animal was declared extinct 50 years later in accordance with international conservation standards. Sightings of the Tassie tiger have continued with regularity not only in Tasmania, but also on the mainland, where they haven't lived for 4,000 years. And yet, a tiger was reported at the northernmost tip of the Cape York Peninsula this year, spurring an army of camera trappers into action to prove they're still out there.

Clearly the dog-like appearance of the marsupial thylacine is a case of convergent evolution, but it made me wonder: If thylacines looked like dogs, did they think and behave like dogs do, too? Alas, the animal's mind seemed lost forever. Thylacines had been extinguished just as scientists had begun looking seriously at animal behavior.

I became obsessed with the thylacine. But rather than trying to find one hiding in the Tasmanian bush (although I did that, too), I spent two years searching for and studying the one artifact that might actually tell us what it was like to be a thylacine: its brain.

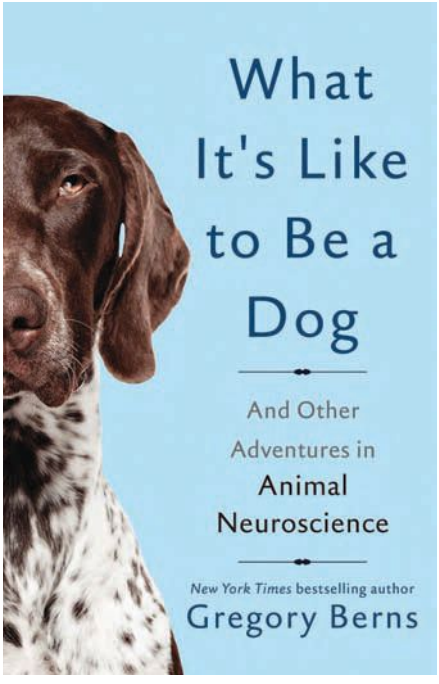
I describe this research in my latest book, *What It's Like to Be a Dog: And Other Adventures in Animal Neuroscience.*

There are four known thylacine brains sitting in jars of preservative in museums around the world. One is at the Smithsonian Institution in Washington, DC, one is in the Australian Museum in Sydney, while the two others reportedly suffered severe cuts when they were extracted from their skulls a century ago. My colleagues and I performed a type of MRI scan called diffusion tensor imaging (DTI) on the two good ones to forensically reconstruct the neural pathways in these century-old specimens. By comparing the architecture of the thylacine's brain with that of canids as well as with the brain of another living carnivorous marsupial, the Tasmanian devil, we hoped to learn something about the mental life of this iconic animal.

Despite its outward doglike appearance, the thylacine's brain looked very different from a dog's brain. I should know. I've also trained dogs to go in MRI scanners, awake and unrestrained, so that we can figure out what they're thinking. The marsupial tiger's brain suggested a cunning creature capable of outsmarting its prey, perhaps depending on smell even more than dogs do. It would have lacked many of the social characteristics that make dogs so endearing. The thylacine was all business and would not have made a good pet.

Like all marsupials, the thylacine did not have a corpus callosum to connect the left and right hemispheres of its brain. Those connections were instead carried in a bundle of fibers called the anterior commissure, which is relatively small in canids and other placental mammals, including humans. But the thylacine did have a frontal lobe proportionately about the same size as a dog's and bigger than that of the Tasmanian devil. This suggests an animal with the shrewd mentality of a predator.

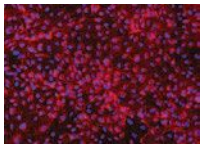
Although I hope some thylacines are still out there, hiding in the Tasmanian bush, the odds are against it. But there is still a lesson to be learned from their brains. Currently, the vast discipline of neuroscience has focused on a handful of species: humans, monkeys, rats, and mice, and a few fish and worms. Within the mammals alone, there are 5,000 other species. And the large ones—the megafauna—are disappearing at an alarming rate. The proximate cause is loss of habitat. By studying their brains before they're gone, we may learn something about other animals' mental lives and how they have evolved cognitive adaptations to live in their environmental niches. Some may have cognitive architectures that make them more adaptable to climate change, while others may need more help. ■



Basic Books, September 2017

Gregory Berns is a professor of psychology at Emory University. Read an excerpt of What It's Like to Be a Dog: And Other Adventures in Animal Neuroscience *at the-scientist.com.*

Endothelial Cell Research Support



Our new endothelial cell application center provides whitepapers, research publications, and product information to help advance your research. This handy online tool can be accessed anytime and offers support with applications including inflammation and cancer. The center also contains resources for setting up more complex *in vitro* cultures for blood-brain barrier modeling and tissue engineering.

Visit the application center here:
http://www.lonza.com/endothelial-cell-application-center

LONZA WALKERSVILLE, INC.
1-800-521-0390
scientific.support@lonza.com

Barrier Function of Endothelial Cells

When endothelial cell monolayers are grown in tissue culture, ECIS (Electric Cell-substrate Impedance Sensing) can be used to electrically probe changes in the paracellular pathways between the cells (red arrows in the diagram). ECIS has been used to monitor the effects of molecules effecting barrier function including VEGF, thrombin, TNF alpha, histamine and sphingosine-1-phosphate.

These measurements are carried out in real time and without the use of labels.

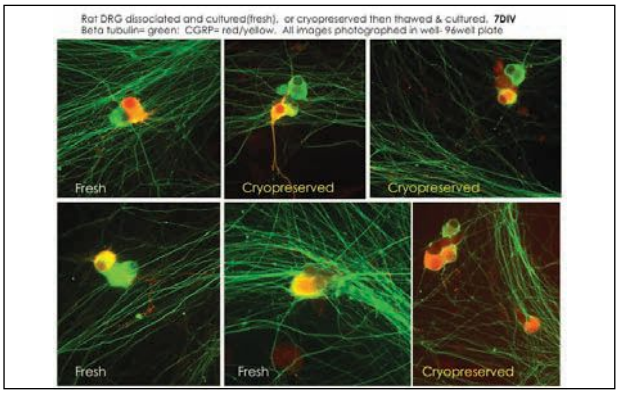
APPLIED BIOPHYSICS, Inc.
518-880-6860
www.biophysics.com

Microplate Reader & Washer Duo 800™ TS & 50™ TS

- Are improved editions of BioTek's legacy reader and washer, the ELx800 absorbance reader and ELx50 washer
- The 800 TS absorbance reader features a color touchscreen interface with easily programmed onboard software, 6- to 384-well microplate reading, and temperature control and shaking
- The 50 TS washer also features easy programming through its color touchscreen, and is compatible with 96-, 384-, and 24-well microplates

BIOTEK
www.biotek.com

High-Quality Dorsal Root Ganglion Neurons for Research



The dorsal root ganglion (DRG) contains cell bodies of primary sensory neurons and is located at the posterior root of each spinal nerve. Neurons of the DRG are considered pseudounipolar since they have two axons instead of a single axon and dendrite. One of these axons extends towards the spinal cord, while the other extends towards the skin or muscle.

Since primary sensory neurons are responsible for transmitting information from the periphery to the spinal cord, they are involved in many different **neural research applications**, including:

- Neurotoxicity and drug screening
- Neurodegenerative disease
- Neuropathic pain
- Receptor signaling
- Tissue engineering
- Gene expression
- Intracellular transport
- Electrophysiology
- Metabolic pathway

With years of experience in primary cell isolation, Lonza offers only the highest quality neural cells for your research. Lonza's ready-to-use cryopreserved neurons exhibit the same growth cone, axon growth, morphology, and neurochemistry when compared to freshly isolated cells.

In addition, Lonza offers Primary Neuron Growth Media (PNGM™) specifically developed for culturing embryonic primary rat and mouse neurons. Researchers culturing DRG neurons can expect lot-to-lot consistency, maximal cell growth and performance when using this optimized neural culture media.

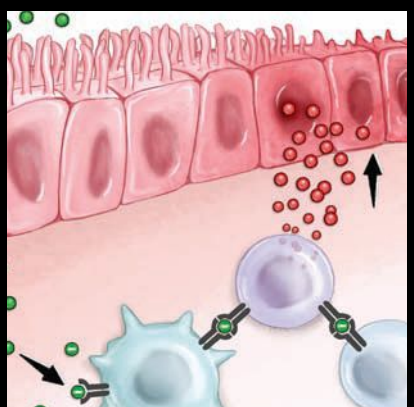
Lonza's **PNGM™ Primary Neuron Growth Media BulletKit**:

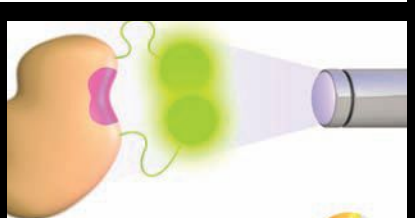
- Ensures long-term neuron survival and growth
- Maintains pure cell populations
- Eliminates the need for astrocyte feeder layers
- Is serum free

To find out more about Lonza's high-quality neural cells and supporting media, visit: www.lonza.com/neural-cells

LONZA WALKERSVILLE, INC.
1-800-521-0390
scientific.support@lonza.com

Lonza





BioProcess International West

March 19-22, 2018
Hilton San Francisco Union Square
San Francisco, CA

THE LEADING PHASE-BASED BIOPROCESSING EVENT TO ADVANCE BIOLOGICS FROM BENCH TO BEDSIDE

Single Use/ Flexible Facilities

Early Stage Process Development and Clinical Manufacturing

Late Stage Process Development & Commercial Launch Preparation

Commercial Manufacturing & Beyond

Register with VIP code **SCIENTIST10** by November 10 and Save an **Extra 10% Off** Early Bird Rates

www.BioProcessWest.com

Bathtub Bloodbath, 1793

BY SHAWNA WILLIAMS

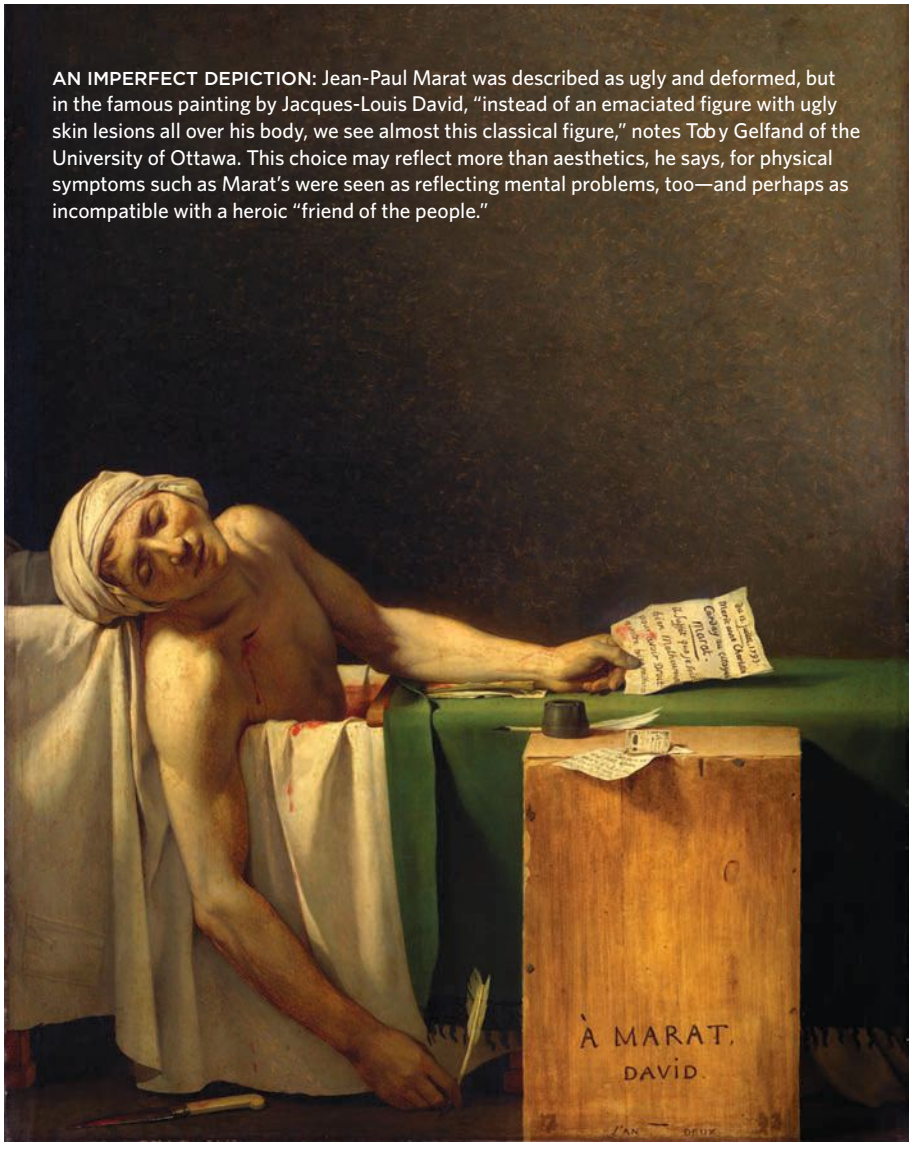
French radical Jean-Paul Marat famously died in his bathtub in 1793, stabbed by Charlotte Corday to put an end to his revolutionary activities. "I killed one man to save 100,000," Corday told a court before she was executed just days after murdering him.

But before he became a revolutionary and then a martyr, Marat was a well-regarded physician and scientist. Born in 1743 in what is now Switzerland, he studied medicine in France before moving to England to begin practicing, despite not having earned a degree. It was there that he published medical papers on gonorrhea and eye diseases—which won him a medical degree from the University of St. Andrews in Scotland—as well as his first political work, *Chains of Slavery*.

In 1776, Marat returned to Paris, where he became a physician to the aristocracy. He also began to conduct scientific experiments—some together with Benjamin Franklin—and published books on the nature of fire, light, and electricity. Eventually, he gave up his work as a doctor to focus on these subjects. He argued, for instance, that fire was not a form of matter, as then believed, but an "igneous fluid."

The French Academy of Sciences gave Marat's research a lukewarm reception. In a letter to a friend, he wrote: "To admit the truth of my experiments was to recognize that they [the academy's members] had worked for forty years on wrong principles. . . . Accordingly, it formed a veritable cabal against me."

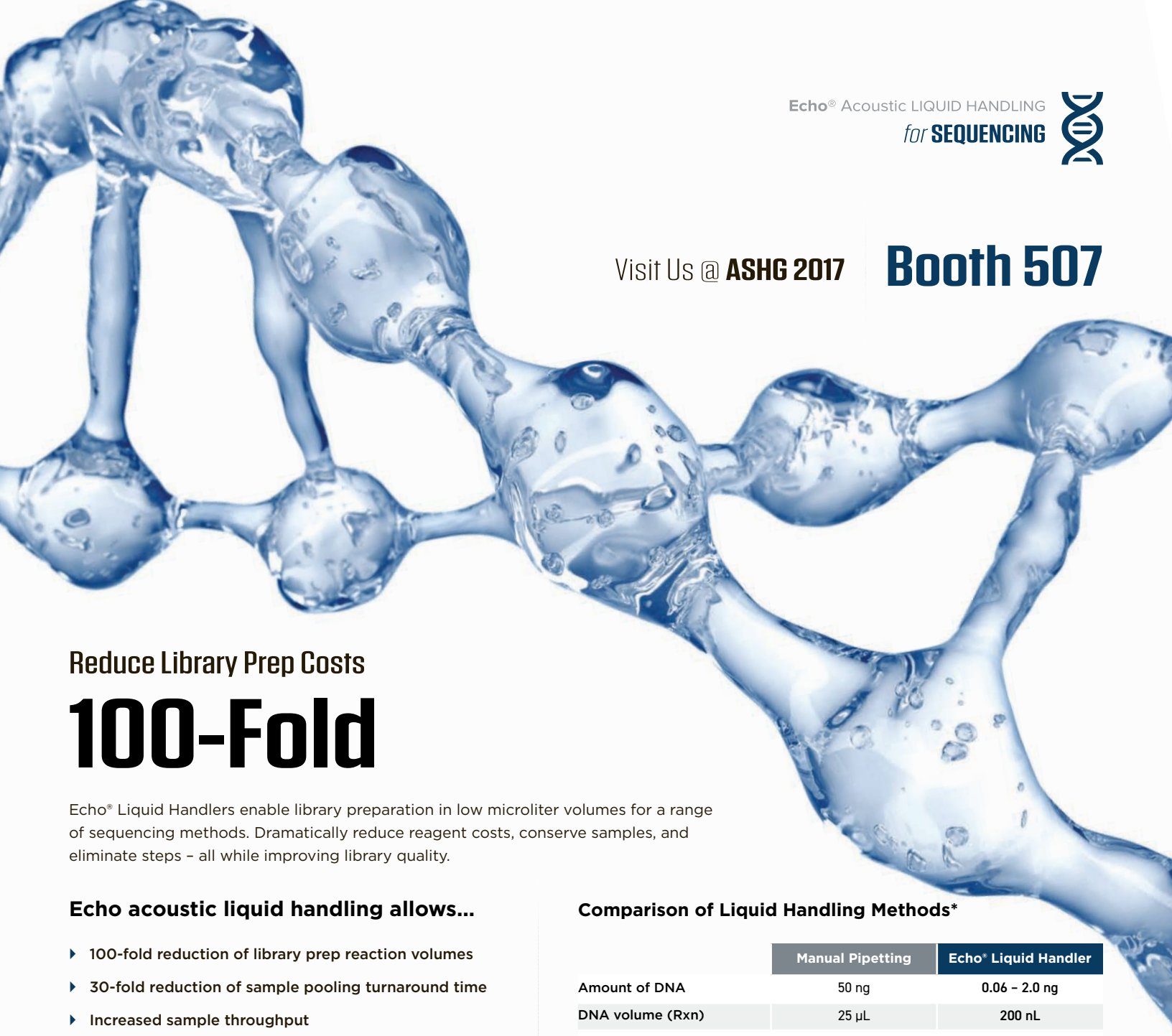
In 1788, Marat changed careers again, this time giving up experimentation in favor of politics. He started his own newspaper and emerged as one of the most radical voices of the French Revolution. Around this time, Marat was struck with a skin disease, "either concurrent with or aggravated by the times he spent hiding from his political enemies in the sewers of Paris in 1790," Josef Jelinek, a dermatologist at New York University Langone Health, wrote in *The American Journal of Dermatopathology* in 1979. Jelinek hypothesized that Marat was afflicted with dermatitis herpetiformis—a condition characterized by an itchy rash that would not be described for another century.



AN IMPERFECT DEPICTION: Jean-Paul Marat was described as ugly and deformed, but in the famous painting by Jacques-Louis David, "instead of an emaciated figure with ugly skin lesions all over his body, we see almost this classical figure," notes Toby Gelfand of the University of Ottawa. This choice may reflect more than aesthetics, he says, for physical symptoms such as Marat's were seen as reflecting mental problems, too—and perhaps as incompatible with a heroic "friend of the people."

During Marat's lifetime, medicine as practiced in France was still widely humoralistic—based on the belief that disease arose from an imbalance in bodily fluids, says Toby Gelfand, a medical historian at the University of Ottawa. Treatments sometimes aimed to encourage the body to expunge fluids, such as pus, saliva, or blood. This may have been the reason why Marat was known to use baths as a treatment for his skin disease; two possible ingredients would have been mercury and sulfur, Gelfand says. Marat was taking one such medicinal soak when Corday stabbed him to death. ■

PAINTING BY JACQUES-LOUIS DAVID, WIKIMEDIA COMMONS



Echo® Acoustic LIQUID HANDLING
for SEQUENCING

Visit Us @ **ASHG 2017** **Booth 507**

Reduce Library Prep Costs

100-Fold

Echo® Liquid Handlers enable library preparation in low microliter volumes for a range of sequencing methods. Dramatically reduce reagent costs, conserve samples, and eliminate steps – all while improving library quality.

Echo acoustic liquid handling allows...

- 100-fold reduction of library prep reaction volumes
- 30-fold reduction of sample pooling turnaround time
- Increased sample throughput
- Automation of workflow to easily prepare thousands of samples
- Improved accuracy of results

Comparison of Liquid Handling Methods*

	Manual Pipetting	Echo® Liquid Handler
Amount of DNA	50 ng	0.06 – 2.0 ng
DNA volume (Rxn)	25 µL	200 nL
Library prep volume (Rxn)	25 µL	300 nL
Total volume	50 µL	0.5 µL
Reactions per kit	96	9600
Cost per reaction	$72.91	**$0.73**

For more information, visit www.labcyte.com/**sequencing.**

** Low-Cost, High-Throughput Sequencing of DNA Assemblies Using a Highly Multiplexed Nextera Process. Shapland et al. ACS Synth. Biol., 2015*

LABCYTE™
The Future of Science is Sound

@LabcyteInc info-us@labcyte.com

GENOME EDIT DETECTION

FAST. SIMPLE. PRECISE.
DROPLET DIGITAL™ PCR

Accelerate genome editing with ddPCR™ Genome Edit Detection Assays.

Droplet Digital PCR (ddPCR) enables rapid quantifi cation of genome editing events. Design and order ddPCR Assays to detect NHEJ and HDR edits, for any target of interest, on Bio-Rad's easy-to-use Digital Assay Site. Use these assays to detect editing events with ultra-high sensitivity using minimal amounts of input DNA or to easily distinguish between homozygous and heterozygous edits in clonal populations.

Find out how Droplet Digital PCR can accelerate your genome editing research at bio-rad.com/info/GenomeEdit1

BIO-RAD