TheScientist
MARCH 2019 | WWW.THE-SCIENTIST.COM
EXPLORING LIFE, INSPIRING INNOVATION

GOING UNDER

DISSECTING THE EFFECTS OF ANESTHETICS

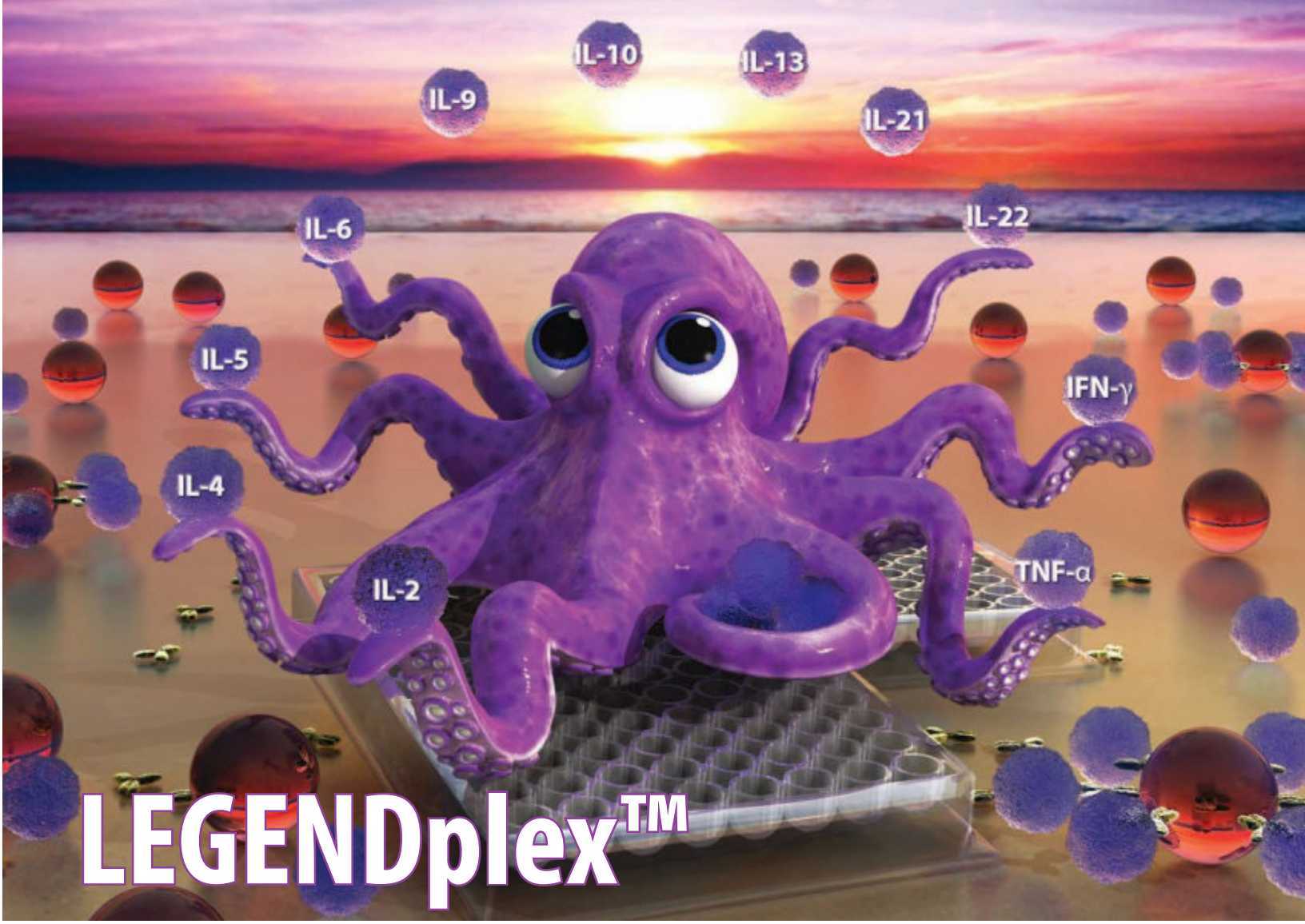
VIRUSES ON THE BRAIN

MALARIA PARASITES DEVELOP RESISTANCE

KNOCKING IN WITH CRISPR

PLUS

MANAGING AN AGING ACADEMIC WORKFORCE



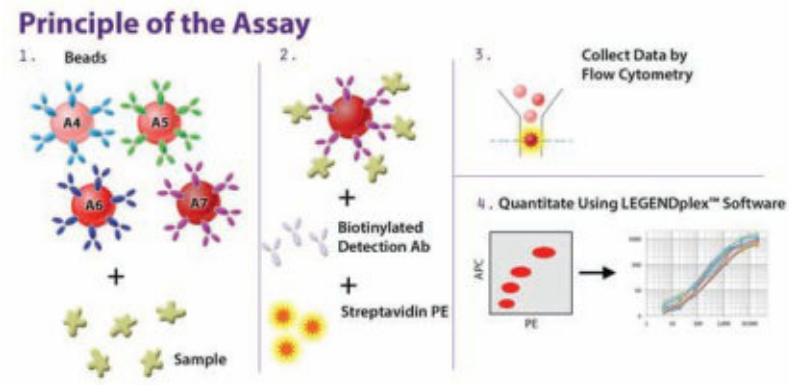
LEGENDplex™

One Assay, Multiple Analytes.

Quantifying multiple biomarkers should be easy.

LEGENDplex™ multiplex panels reliably and accurately generate more data, in less time, with just one assay.

- **Specifically designed for use with standard flow cytometers**
- **Free data analysis software included with every assay**
- **Human, mouse, rat, and non-human primate specificities**
- **Expanding list of analyte targets**
- **Flexible ordering formats**



Visit: biolegend.com/legendplex

BioLegend®

BioLegend is ISO 13485:2016 Certified

Toll-Free Tel: (US & Canada): 1.877.BIOLEGEND (246.5343)
Tel: 858.768.5800
biolegend.com

08-0075-11

World-Class Quality | Superior Customer Support | Outstanding Value

NEW ENGLAND BioLabs® Inc.

be INSPIRED
drive DISCOVERY
stay GENUINE

How low can you go?

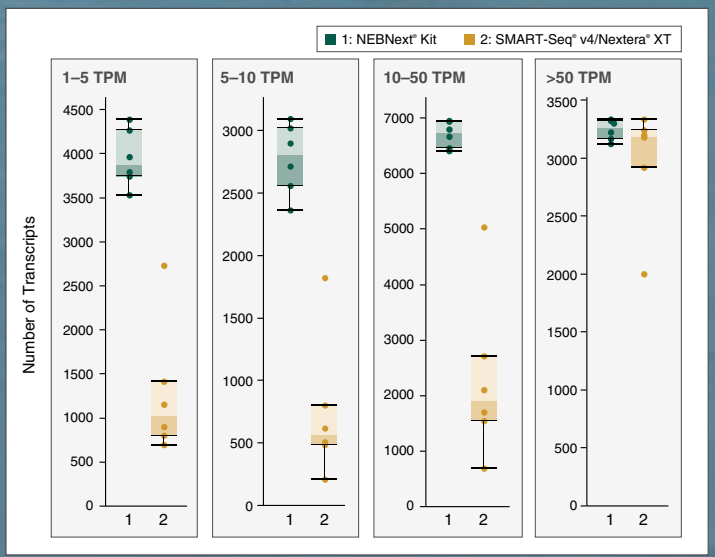
NEBNext® Single Cell/Low Input RNA Library Prep Kit

With this unique streamlined method, high-quality, full-length transcript sequencing libraries are made directly from single cells, or from as little as 2 pg – 200 ng of total RNA.

- Detect more transcripts, including low-abundance transcripts
- Obtain uniform, full-length transcript coverage, regardless of input amount or sample type
- Save time with a streamlined workflow, minimal handling steps and hands-on time

Visit **NEBNext.com** to request your sample today.

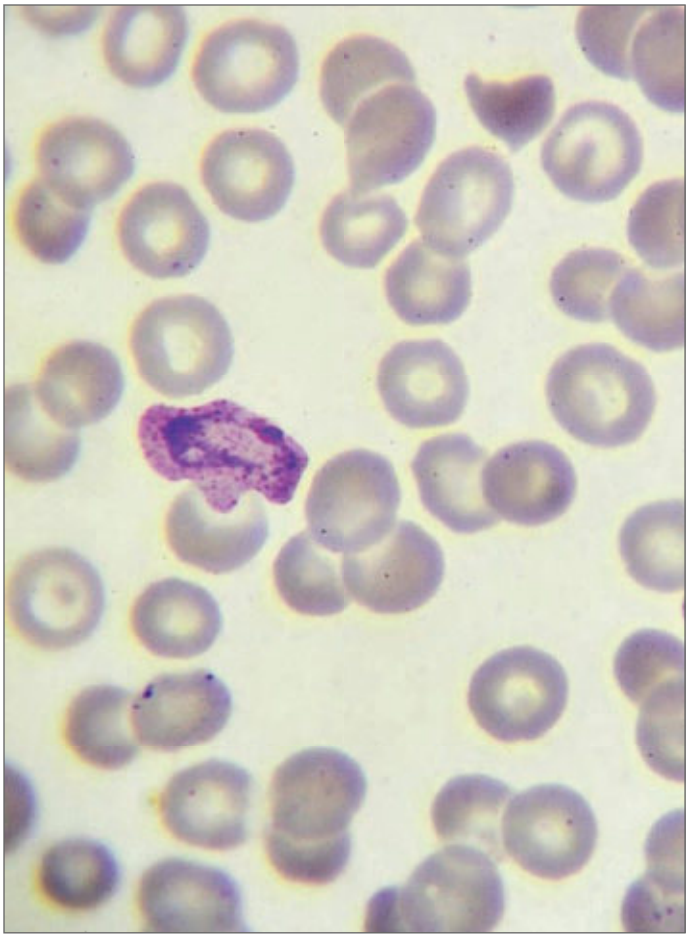
Superior transcript detection with the NEBNext Single Cell/Low Input RNA Library Prep Kit

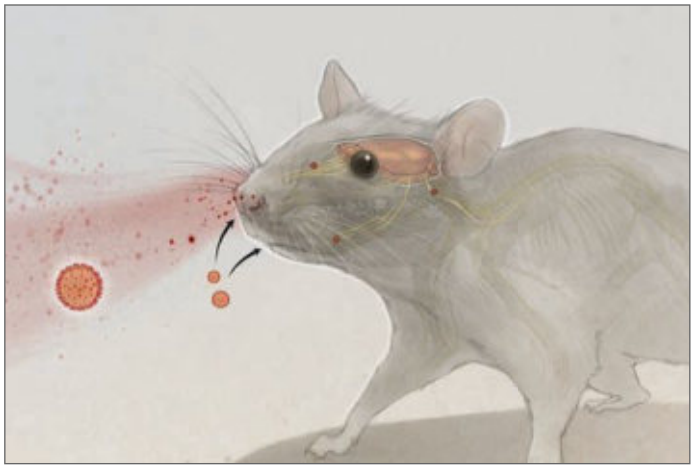


Sequencing libraries were generated from Jurkat single cells (6 replicates) using the NEBNext Single Cell/Low Input RNA Library Prep Kit, or the SMART-Seq® v4 Ultra® Low Input RNA Kit for Sequencing plus the Nextera® XT DNA Library Prep Kit. Libraries were sequenced on an Illumina® NextSeq® 500. Each dot represents the number of transcripts identified at the given Transcripts Per Kilobase Million (TPM) range, and each box represents the median, first and third quartiles per replicate and method. Salmon 0.6 was used for read mapping and quantification of all GENCODE v25 transcripts. Increased identification of low abundance transcripts is observed with the NEBNext libraries.

Contents

THE SCIENTIST | THE-SCIENTIST.COM | VOLUME 33 NUMBER 03





Features

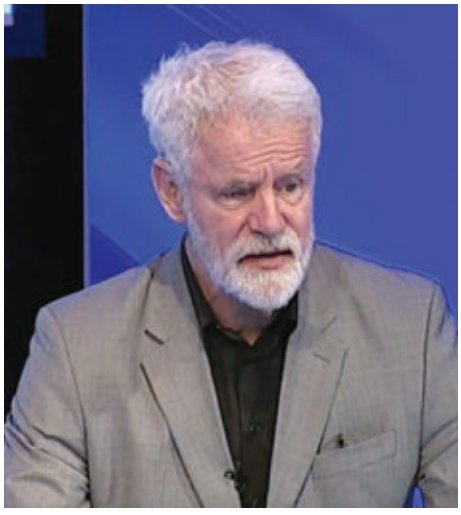
ON THE COVER: © ISTOCK.COM, STEEX

Department Contents

CORRECTIONS:
The February 2019 article "Follow Your Nose" inaccurately stated that Louisa Dahmani previously conducted research on both memory and olfaction. In fact, she reviewed the literature. *The Scientist* regrets the error.

PUZZLE ON PAGE 12

Online Contents



THIS MONTH AT THE-SCIENTIST.COM:

VIDEO
Diseased Duck
Watch the video that inspired Thomas Jefferson University researcher Richard Smeyne to explore the connection between viral infection and neurodegeneration.

VIDEO
Kári Stefánsson Speaks
See the Decode Genetics founder discuss the genetic basis of human diversity.

VIDEO
Innovation en Masse
Biotech entrepreneur and Reading Frames author Safi Bahcall speaks about the emergent nature of invention.

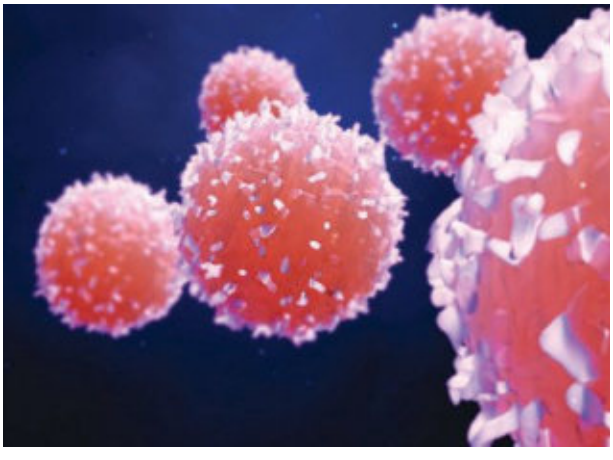
AS ALWAYS, FIND BREAKING NEWS EVERY DAY ON OUR WEBSITE.

Coming in April

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

- Immunotherapy for solid tumors
- Contagious cancers
- Small molecules that target RNAs
- The epigenetic link between your grandfather's diet and your cancer risk

AND MUCH MORE



eppendorf

The Next Stage

The new Mastercycler® X50

Speed and the 2D-Gradient for enhanced PCR optimization make the Mastercycler X50 the ideal cycler for advanced research in molecular biology. The excellent block regulation gives rise to the next stage of PCR reproducibility.

- > Innovative 2D-Gradient for enhanced PCR optimization
- > Heating rate: up to 10 °C/s
- > Wide selection of blocks from a fast silver block to 384
- > Intuitive touch display
- > Connect up to 10 units to a network

www.eppendorf.com/mastercycler • 800-645-3050

TheScientist
EXPLORING LIFE, INSPIRING INNOVATION

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

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 Katie Prud'homme at katiep@the-scientist.com

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

Although his lifelong dream was to become a doctor, **Emery Brown** had originally intended to study the romance languages as an undergraduate at Harvard College before attending medical school. As a sophomore, he realized the power of statistics and ended up majoring in applied mathematics. Brown eventually earned his PhD in statistics along with his MD. He specialized in anesthesiology because he enjoyed it in medical school. "It was a lot of fun. It was very real time. You had to make decisions on the spot," says Brown.

Brown has been practicing medicine for nearly 30 years and is currently an anesthesiologist and researcher at Massachusetts General Hospital, Harvard Medical School, and Massachusetts Institute of Technology. Early in his career of putting people under, he kept a list of questions that puzzled him about what anesthesia does in the brain. After many years of watching the mystery of anesthesia at work, he realized that he possessed the know-how to research it. He discovered that the actions of anesthetics are strongly linked to the powerful rhythmic patterns they produce in patients' brain waves. When traveling abroad to teach and share this research, Brown sometimes lectures in Spanish or French.

Francisco Flores can trace his fascination with the effects of drugs on the brain back to his childhood in Chile. Growing up in the countryside, he saw people practicing forms of natural medicine with plants and herbs that would later feed an interest in what chemicals do in the body.

At the University of Santiago, Chile, Flores studied biochemistry but decided to home in on the brain after some classes in pharmacology. "How these different drugs can affect brain function in such dramatic ways is to me a very interesting topic," he says.

After finishing his PhD at the University of Chile, Flores started a postdoc in neuroscience working with Emery Brown at Harvard Medical School. Flores ended up staying at Harvard, where he currently holds a faculty position studying how anesthesia effects the brains of patients and larger questions of their experience. "From the lack of consciousness, we can understand the presence of consciousness," he says.

On page 38, Brown and Flores write about the current research on anesthetics and how they alter brain waves.

Carolyn Wilke, *The Scientist*'s current editorial intern, gave a career in science more than a fair shake. She earned her PhD in environmental engineering from Northwestern University, studying the effects of nanomaterial pollution on microorganisms, at the end of 2018.

But on the road to a terminal degree in science, Wilke also got a taste of what it was like to cover research and researchers as a reporter. In addition to writing stories for Northwestern's online science magazine, *HELIX*, a couple years into her PhD program, she spent three months at the *Sacramento Bee* as part of a AAAS Mass Media Fellowship in 2017. The latter experience really left an impression. "I sort of missed the lab," Wilke recalls. "But I also realized that this was really fun, and I would enjoy doing it full time."

Though she had decided that a career in science journalism better meshed with her talents and interests, Wilke still saw a benefit to finishing out her PhD work. She joined the *TS* editorial team in December, just as her degree program was wrapping up.

At *The Scientist*, Wilke has excelled at covering a suite of life-science topics, a flexibility she chalks up, in part, to her education. "I do actually feel like doing a PhD, where I had to teach myself about a lot of different things, helped me feel more comfortable diving into a new topic."

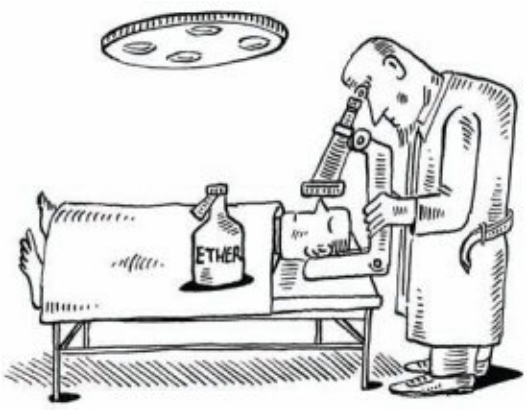
On page 49, Wilke writes about the oddities of archaeal mating.

DAVID ORENSTEIN; KRIS BREWER

Drugs, Developed

In an era of instant communication, we must be careful how word of new and untested treatments is shared.

BY BOB GRANT



ANDRZEJ KRAUZE

I am no fan of anesthesia. The feeling of being rendered unconscious to facilitate the manipulation of my body, only to be reanimated afterward, gives me, like many people (I assume), the heebie-jeebies. But alas, anesthesia is a medical necessity. It has made lifesaving surgeries and once-dreaded dental procedures pain-free and relatively routine for more than 150 years. My own medical care, not to mention that of billions of other people and animals, has benefited greatly from this chemical control of consciousness.

Beyond my personal misgivings, anesthesia's development into a widely accepted medical protocol illustrates an interesting, if outmoded, avenue of innovation—let's call it efficacy sans mechanism. As described on page 38 by scientists Emery Brown and Francisco Flores in their dispatch from the front lines of anesthesia research, in the mid-19th century, dentist William Morton successfully put a patient under general anesthesia (using ether vapor, in this case) in a public amphitheater at the Massachusetts General Hospital so that surgeons could remove a tumor from the patient's neck. How the anesthetic ushered the patient into an unconscious state, in which his body did not register the pain of the scalpel, wasn't known and didn't much matter. Over the following weeks and months, the approach revolutionized medical care as fast as communications of the day would allow. An account of the procedure reportedly made it aboard the paddle steamship *Acadia* heading from the US to England, where American physician Francis Boott shared it with his friend and neighbor James Robinson, a dentist, who became the first in England to administer ether for general anesthesia, just two months after the seminal demonstration in Boston.

As the new application for ether took the global medical community by storm, other anesthetics—many of them ether derivatives—were added to the surgeon's toolbox. But it wasn't until the 1980s that scientists began to parse the specific mechanisms of action for a variety of anesthetics, some of which had been part of standard medical practice for more than a century. Even today, more than 170 years after the first successful general anesthetic was administered, science is still uncovering the intricacies of how these drugs work in the brain.

The arc of discovery for anesthesia stands in stark contrast to our current framework for biomedical research. The time that stretches between the identification of potentially therapeutic compounds and their use in the clinic is now measured in decades, not months. Rigorous testing—for safety, efficacy, and dosage—lies between the bench and the bedside. Through this extensive study, a drug's mechanism of action is typically uncovered and dissected.

Even today, to be sure, understanding a drug's mechanism is not a prerequisite for approval, and there are established mechanisms for accelerating the clinical use of biomedical breakthroughs. (See "Picking Up the Pace," *The Scientist*, January 2016.) But could we imagine a modern scenario in which a drug was adopted as swiftly after its first successful clinical use as ether was? Likely not. And that's a good thing. The tale of the medical revolution sparked by general anesthetics via a long-abandoned model of drug development sounds quaint to our ears—even nostalgic. But it was an exception, not the rule. For every ether-soaked success story, history is littered with countless other tales of unproven medical treatments causing severe and widespread harm.

Even though modern researchers have tools, technologies, and biological insights that would have been utterly fantastical to their 19th-century counterparts, the danger of untested treatments is greater now than it was then. As the steamship has given way to the internet, word of untrialed medical approaches spreads faster than ever before, meaning that the potential to do harm is amplified. One has only to look to recent upticks in antivaccine sentiment or the rise of spurious supplements for illustrations of the corrosive power of spreading unverified scientific knowledge via modern modes of communication.

Even when interventions do work, it's important to understand the mechanism. In the case of general anesthesia, researchers have been hard at work digging into the nuts and bolts of the revolutionary drugs ever since that first successful application. Over the past several years, the resulting insights are feeding back into clinical practice, honing the application of modern anesthetics. This heartens me. Even if being put under still gives me the willies. ■

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

isoplexis

When Editing Immune Cells, The Small Things Make a Big Difference

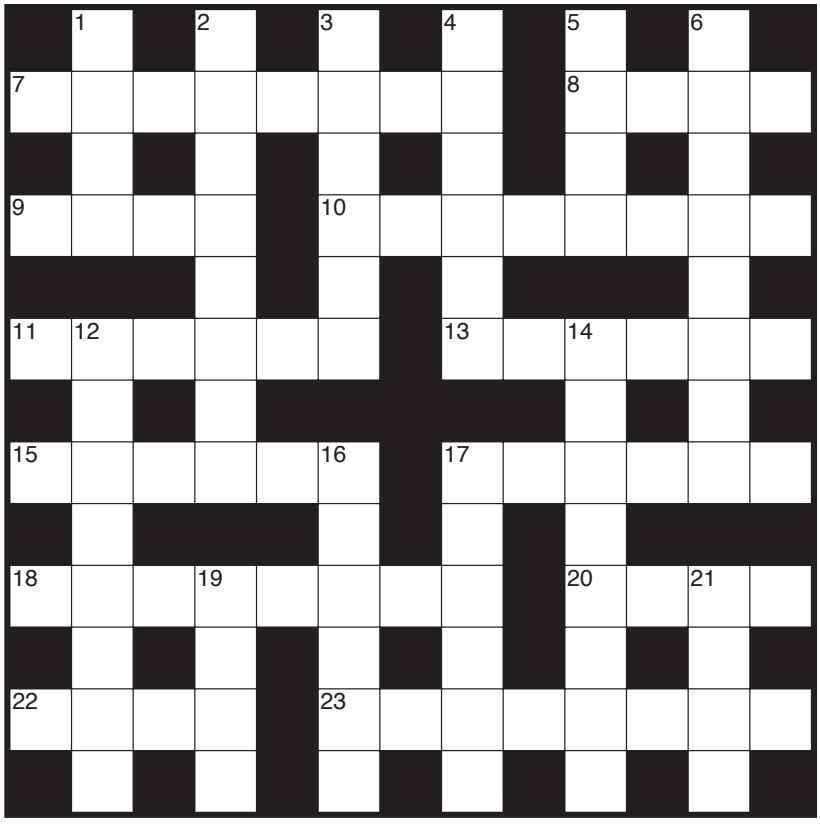
IsoPlexis is showing how small groups of gene engineered or edited cells make a big difference in cell product potency.

IsoPlexis, with industry leaders, has uniquely revealed differences between Cell Therapy Non-Responders (NR) versus Responders (R) from the cell therapy product itself.

See how we're revealing key differences in pre-clinical development, manufacturing, and biomarker discovery to enable what's next in cell engineering and therapy.

Follow our Immune Editing data at www.IsoPlexis.com/CellT-P
Let's discuss how we can work together!

Speaking of Science



Note: The answer grid will include every letter of the alphabet.

BY EMILY COX AND HENRY RATHVON

ACROSS

7. Carbon product obtained by pyrolysis
8. Communication via pen?
9. Big start of the 6-Down
10. Flower in the snapdragon family
11. Component of natural gas
13. Element in Einstein's mass-energy equation
15. Musk ox and narwhal's regional habitat
17. Lobes of a whale's tail
18. Articulation involving the femur (2 wds.)
20. Sound off in a lupine fashion
22. Little songbird with a complex repertoire
23. Microscopic life forms

DOWN

1. Seed rich in omega-3 fatty acids
2. What a male seahorse can become, oddly
3. Bean with an Ethiopian origin
4. Network of vessels or nerves
5. Galliformes or Anseriformes members
6. Cosmologist's allover concern
12. Period before the Quaternary, traditionally
14. The toxic stuff in poison ivy
16. What Jane Goodall studied
17. Unit important to oceanographers
19. Swiss pioneer of analytical psychology
21. Source of 7-Across

Answer key on page 5

After two years, it's clear that this administration values neither the work of federal scientists nor the health and safety of the public. Science is being silenced, in a truly unprecedented way—and we're all paying the cost.

—**Jacob Carter,** research scientist at the Union of Concerned Scientists and lead author of the organization's report entitled "The State of Science in the Trump Era" (January 28)

It almost feels like a hostage situation, like I am being held hostage—my life, my credit, my financial stability—for something that I never voted for [and] I don't believe in. It's really frustrating.

—**Amber Lucas**, cofounder and CEO of Impact Proteomics, the biotech she planned to launch late last year before her National Science Foundation Small Business Innovation grant was delayed by the month-long partial government shutdown that lasted until late January (*The Scientist*, January 16)

JONNY HAWKINS

Notebook

MARCH 2019

BLUE LAGOON: Oases scattered around the Cuatro Ciénegas Basin in Northern Mexico are home to ancient microbial life.

WIKIMEDIA COMMONS, CAZADORDEMOLINOS

A Lost Microbial World

Deep within Northern Mexico's Chihuahuan Desert lies the Cuatro Ciénegas Basin, a butterfly-shape valley where small turquoise lagoons dot the landscape. These hidden oases possess conditions remarkably similar to those of the planet's prehistoric past and house many organisms, including fish, diatoms, and bacteria, that cannot be found anywhere else on Earth. The aquatic system is also one of the few places where stromatolites—rock- or reeflike structures, built up by microbes, that once dominated the shores of ancient oceans—still live and grow, their surfaces made up of active microbial colonies.

The unusual features of the Cuatro Ciénegas Basin and its inhabitants have drawn many scientists to the area since it was first encountered by biologists in the late 1930s. Valeria Souza, a microbiologist at the National Autonomous University of Mexico, was introduced to Cuatro Ciénegas nearly two decades ago by Jim Elser, an ecologist who was investigating stromatolites in the basin. "It was obvious to me that working there wasn't viable without closer collaboration with Mexican scientists," Elser, who is now at the University of Montana, recalls. Souza has been studying the basin ever since.

Since the early days of her work in Cuatro Ciénegas, Souza has been interested in the microbial communities that inhabit the basin. The nutrient composition of the lagoons is quite different from that found elsewhere in the world, Souza says, probably due to the inflow of water from deep aquifers under the mountain—which harbors ancient sediments and clay—at the center of the valley. With high levels of sulfur and very low concentrations of phosphorus, the water in Cuatro Ciénegas is much more similar to oceans of the Precambrian era, which ended approximately 542 million years ago, than to modern-day seas. Most of Earth's contemporary life forms require more phosphorus in the environment to survive, so Souza and her colleagues were puzzled when they found a high level of microbial diversity in the area (*PNAS*, 103:6565–70, 2006). "This is probably [one of] the most diverse places on Earth," Souza says. "And I've been trying to understand why."

Given the prehistoric Earth–like conditions of the basin, Souza and her col-

leagues hypothesized that Cuatro Ciénegas may be a "lost world," a safe haven where ancient organisms could persist and evolve in isolation from the rest of the planet. To test this theory, the researchers recently gathered soil, sediment, and water samples from ten sites in Churince, a 300-meter-long lagoon in the basin. They then extracted DNA and conducted 16S ribosomal sequencing, which revealed more than 5,000 species of bacteria and archaea.

To uncover the evolutionary history of some of these organisms, the team zoomed in on one genus of bacteria, *Bacillus*. By comparing the sequences of approximately 2,500 species to those in two online databases, the team identified two lineages that were unique to Cuatro Ciénegas—one that appeared in sediment and another that was related to modern-day marine microbes (*eLife*, 7:e38278, 2018).

When the researchers dated the two groups using software that reconstructs evolutionary trees from molecular sequences, they discovered that the sediment lineage appeared approximately 650 million years ago—during the late Precambrian period—and that the marine one emerged around 160 million years ago, during the Late Jurassic. The researchers suggest that there were two events that led the lineages to set up shop in the basin: one, an abrupt change in the balance of nutrients at the end of the Precambrian era, and the other, the breakup of Pangea during the Jurassic.

"It's odd to think that there is a place where there are organisms that have not been anywhere else for [this long]," says Frederick Cohan, a microbiologist at Wesleyan University who did not take part in the study but was a reviewer of the paper. "It's like Jules Verne in a microscope," he adds, referring to the author of *Journey to the Center of the Earth*, where fictional explorers descend into a volcano and discover prehistoric animals hidden below the surface. "And that just blows me away."

"This idea of a lost world, where a certain environment is a niche for ancient microorganisms, is quite interesting," says Brendan Burns, a microbiologist at the University of New South Wales in Australia who was not involved in this work. "I think it might take some more digging to see whether other areas like this can be found, to see how unique what they discovered really is."

The water in Cuatro Ciénegas is much more similar to oceans of the Precambrian era than to modern-day seas.

Souza and her colleagues believe that the extreme nutrient conditions in Cuatro Ciénegas—and possibly a heightened ability of native bacterial species to fight off invading microorganisms—may explain why these ancient microbial lineages never left the basin. Cohan agrees that the uniqueness of the environment is likely a key factor, but doubts that rivalry between species played a big role. "My understanding is that antagonism in bacteria is primarily against fairly close relatives," Cohan says. "I think it would be difficult to imagine that this community is defended from everything that could come in."

More research is needed to fully understand the history of these ancient bacterial communities. But for scientists who wish to continue investigating the Cuatro Ciénegas Basin, there is some bad news: the wetlands in the valley have shrunk by 90 percent over the last 50 years. The Churince lagoon dried up in 2017. "The last time we saw it with water was in 2016," Souza says. When she and her colleagues returned to find the area bone dry in the fall of the following year, "we cried like crazy," Souza recalls.

One of the primary causes of the desiccation is agriculture—specifically, farmers diverting water from the valley's wetlands for their crops. To address this problem, Souza and her colleagues have started to engage the local community in conservation efforts. In 2011, for example, they set up a lab at the high school in the small city of Cuatro Ciénegas to educate students about the importance of the region—and have been taking them out into the field and getting them involved in collecting and analyzing samples. The efforts have resulted in positive change—for example, some irrigation systems in Cuatro Ciénegas have now been replaced with ones that use much smaller amounts of water—but Souza says their conservation work is far from complete.

"It's sad to think that this basin has been there with those living organisms for [almost] a billion years," says Cohan, "and yet with the short time of human disturbance, it [could] disappear."

—Diana Kwon

Avian Drifters

In 2011, an undergraduate/masters student at Bangor University in the UK brought physical oceanographer David Bowers an annotated map of the Irish Sea. The map showed the trajectories of colonial seabirds called razorbills (*Alca torda*) that had been fitted with GPS trackers. The student was interested in why the razorbills had gone to particular regions to feed. But Bowers noticed something else in the data.

"At nighttime, the birds were moving in a way that wasn't flying; they were going too slow," Bowers, now retired, recalls. "And crucially, they changed their direction when the tide turned from going one way to the other. . . . Straightaway I realized they were going with the flow."

The insight lay dormant until four years later, when Bowers suggested that a Bangor student named Matt Cooper, who was interested in the use of tides as a source of renewable energy, pick up on the observation. The island of Anglesey, which is home to Bangor's School of Ocean Sciences, juts out into the Irish Sea, creating strong tidal flows off the northern coast of Wales. Areas around the island have been earmarked by energy companies Morlais and Minesto as testing grounds for tidal power technologies. Identifying the best locations for these technologies requires monitoring the ocean's movements to find areas where tidal flow is strongest.

Oceanographers traditionally deploy large buoys to track tidal currents. But if the GPS-tracked razorbills were indeed

drifting with the tide, perhaps while sleeping or resting before foraging, the birds could provide another source of data, Bowers and Cooper reasoned.

The researchers tracked down the razorbill dataset from the Royal Society for the Protection of Birds, which had tagged the animals. Cooper, who graduated in 2016 with his master's in oceanography, compared the birds' movements during periods of apparent drifting with tidal data, and saw that patterns in the two datasets mirrored each other. As the tides sped up, so did the birds, and when the tide turned, the birds changed direction by 180° (*Ocean Sci*, 14:1483–90, 2018). "It's overwhelming evidence that they are moving with the tide," Cooper says.

Drifting seabirds could offer several advantages over traditional ocean monitoring. While buoys tend to converge in a few major oceanic streams, birds only drift for part of the day or night before picking up and flying to new locales. They also go places that buoys can't, such as close to shore, Bowers adds. And the best part: the data already exist. "People all over the world have tagged seabirds to see where they go," says Cooper. Using these animals to assess ocean currents—"it's recycling of data."

It's not the first time that researchers have turned to seabirds to monitor physical properties of the world's oceans. Willem Bouten, a computational ecologist at the University of Amsterdam, started tracking seabirds in the early 2000s and soon noticed patterns of movement that seemed to be linked with ocean currents. He and his colleagues confirmed the drifting behavior among lesser black–backed gulls (*Larus fuscus*) in the North Sea off the coast of the Netherlands. Comparing the birds' movements to a model of the tides, they found that "it matched perfectly," Bouten says (*Ibis*, 153:411–15, 2011). And in 2014, Nagoya University seabird biologist Ken Yoda showed that tagged streaked shearwaters (*Calonectris leucomelas*) could be used to track water currents off the northeastern coast of Japan (*Prog Oceanogr*, 122:54–64).

As living organisms, however, birds are "not a sensor that you can control," says Bouten. And in some cases, their behaviors may complicate the data. In Cooper's analysis, the speed at which the birds were moving was not quite the same as the tidal speed predicted by an oceanographic model. It could be that the models were wrong, says Bowers, or that the birds were paddling.

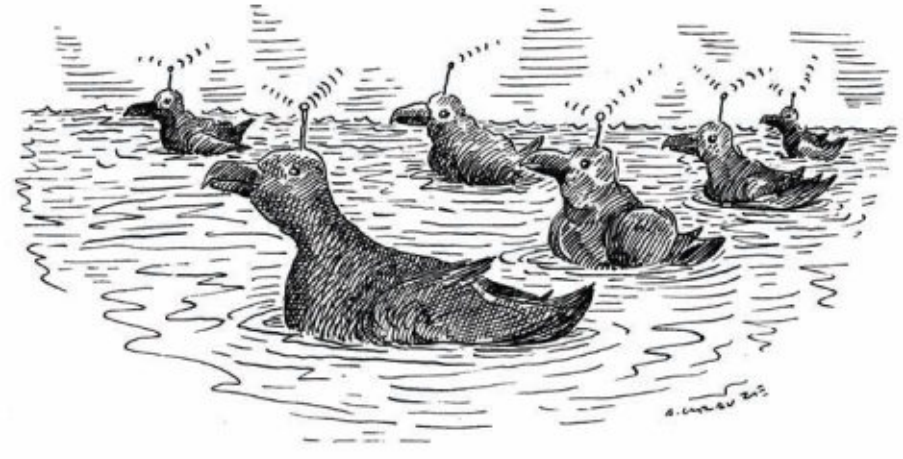
Another risk is that the birds may be pushed by the wind in a way that causes their path to deviate from the currents below the surface, a phenomenon known as slip. "We have to think about the degree of slip to estimate ocean currents," Yoda tells *The Scientist* in an email. "Nobody knows the strength of the effects." As a result, he adds, "the method is not an alternative to satellites, drifter buoys, or current-meter moorings."

Drifting seabirds could offer several advantages over traditional ocean monitoring.

Still, he and others argue that seabirds could provide a wealth of additional information on ocean currents. Bouten says that he and his collaborators have tracked some three or four hundred birds floating on the ocean's surface and that the data are available to oceanographers. To date, he hasn't gotten any requests. "I think that's because people are not used to this."

Bowers agrees. "I suspect that for some time oceanographers . . . won't trust the seabirds because they're not perfect drifters," he says. But, "they've got advantages. I suspect as time goes on, people will explore those advantages."

—Jef Akst



ANDRZEJ KRAUZE

Passing Marks

Brown and yellow mice nestle side by side in their cages in Anne Ferguson-Smith's molecular genetics lab at the University of Cambridge. The mice are Agouti Viable Yellow, naturally occurring mutants, which, though genetically identical, have coats that vary in color—a phenomenon that researchers have long studied as an example of epigenetic inheritance.

All of the mutant mice have a gene, *Agouti*, that influences coat color, and an adjacent transposable element—a DNA sequence that can move about the genome, creating or reversing mutations—that promotes the gene's expression. In the brown

EPIGENETIC MISMATCH: Agouti Viable Yellow mice share the same DNA sequence, but have different methylation patterns.

WIKIMEDIA COMMONS, RANDY JIRTLE AND DANA DOLINOY

mice, this element is methylated and, therefore, silenced. But in the yellow mice, it isn't methylated, meaning that these animals overexpress Agouti signaling protein in many tissues, leading to their yellow hue.

Importantly, Ferguson-Smith says, yellow mother mice tend to have yellow baby mice and brown mother mice tend to have brown baby mice, suggesting that the methylation mark—or lack of it—is passed down from generation to generation. This phenomenon has sparked scientists to hypothesize that other methylation marks on transposable elements can also be passed directly from parent to child, raising the possibility that parents' diet, behavior, and experiences might affect future generations via this route.

The conceptual framework has already made its way into genetics textbooks, Ferguson-Smith says. "But in fact, the evidence for that is virtually nil." In mammals, she explains, epigenetic marks are erased completely, and reprogrammed twice during the lifetime of an individual. The first wave of epigenetic erasure happens in primordial germ cells. Then the methylation comes back again in egg-specific marks and sperm-specific marks. And then, upon fertilization, that egg and that sperm meet and the marks are erased again. "So there's two rounds of epigenetic reprogramming that occur in the germline that basically prevent any epigenetic marks from being transmitted from one generation to the next," Ferguson-Smith explains. "People don't seem to appreciate this."

To dig into the problem experimentally, Ferguson-Smith and her colleagues decided to rigorously test the idea that transposable

elements act generally as gene promoters and that the methylation marks on these elements could be passed from one generation to the next. Researchers have postulated that transposable elements may have methylation marks resistant to reprogramming—so, in theory, these marks should be most likely to be inherited.

In a series of experiments examining the T cells and B cells of multiple generations of Agouti Viable Yellow mice, the researchers screened the animals' genomes searching for transposable elements that were methylated similarly to the one that sits next to the *Agouti* gene. The screen identified dozens of these transposable elements but revealed that only rarely do they work as promoters to control the expression of adjacent genes. The methylation marks on these transposable elements are also wiped clean and reprogrammed after fertilization, the team found, meaning they can't be directly passed from generation to generation (*Cell*, 175:1259–71.e13, 2018). "It's hard to imagine how a memory of methylation can be transmitted from one generation to the next if it's being erased and reestablished in each generation," Ferguson-Smith says.

"This study is an enormous technical tour de force," Dirk Schübeler, a molecular geneticist at the Friedrich Miescher Institute for Biomedical Research in Basel, Switzerland who was not involved in the study, tells *The Scientist*. In the past, researchers suggested that the epigenetically regulated Agouti trait was the tip of the iceberg for DNA methylation–based epigenetic inheritance, he says. "This study shows there is no iceberg."

The screen did identify one transposable element that, like the element abutting the *Agouti* gene, displayed a bit of memory, Ferguson-Smith says, "but our data suggested that memory is not being conferred by DNA methylation."

The researchers could see that methylation marks on this transposable element were erased between generations, and reestablished again in a form reminiscent of what was found in the parental generation. "So we asked, what might it be that causes that methylation to be reconstructed after erasure in the same way [in the next generation]?" Ferguson-Smith explains. "We think that it's conferred by genetics." The study results, she says, suggest that a particular sequence in the genome causes a specific methylation mark on a transposable element to reconstruct itself in the offspring in the exact same way it existed in the parent, and that such genetic sequences are adjacent to the transposable elements in the genome. So "non-genetic inheritance could, in fact, be genetic in origin," she says.

Epigenetic marks are erased completely, and reprogrammed twice during the lifetime of an individual.

Schübeler says the idea is perfectly possible, but more work needs to be done to understand exactly how the genetic mechanism underlying these epigenetic marks might work.

University of California, Santa Cruz geneticist Susan Strome, who was also not involved in the study, notes that even if the DNA methylation mode of non-genetic inheritance is rare, as Ferguson-Smith's team suggests, it doesn't mean all other modes of non-genetic inheritance are also rare. Modifications to histone tails, which Strome's lab studies in worms, and small RNAs are passed down between generations and have epigenetic effects in at least some organisms, she says. "I would not extrapolate from the Ferguson-Smith paper to say that epigenetic inheritance is nearly non-existent."

—Ashley Yeager

High Time

Even a seasoned mystery novelist might find it difficult to come up with a more tantalizing string of clues than those left by the Denisovans. Paleontologists found the only remains of the ancient hominin species in a Siberian cave in 2008. Yet a genetic analysis published early last year reported that, among modern-day human populations studied, the largest proportion of Denisovan-derived genes, about 5 percent, is found a watery hemisphere away—in residents of Oceania, in the South Pacific (*Cell*, 175:P53–61.E9). Another study found that a distinctive variant of the gene *EPAS1* that helps Tibet-

DIG AT DEVU: Excavations on the Tibetan Plateau have revealed thousands of fragments of Paleolithic tools.

YINGSHUAI JIN

ans cope with high-altitude conditions is similar to an allele found in the Denisovan genome, and likely was acquired many millennia ago through interbreeding with the now-extinct species and spread through the population thanks to natural selection (*Nature*, 512:194–97, 2014).

Just when and where did the Denisovans mate with *Homo sapiens*, and what migration routes did their mixed offspring follow? The remains in the Siberian cave date to between 30,000 and 50,000 years ago (*Nature*, 468:1053–60, 2010), and a 2016 comparison of Denisovan with modern human genomes estimated that admixture occurred 44,000–54,000 years ago (*Curr Biol*, 9:1241–47). But a study published in November of last year could provide a fresh clue—or a red herring.

In it, researchers with Beijing's Institute of Vertebrate Paleontology and Paleoanthropology and their colleagues excavated an archaeological site 4,600 meters up on the Tibetan plateau, unearthing more than 3,000 stone tool fragments, most of them from double-sided blades, scattered through three layers of sediment. Using a technique called optically stimulated luminescence, which measures the photons emitted by minerals and can be used to estimate the last time sunlight reached a sample, the team calculated the age of the tools at between 30,000 and 40,000 years old. This suggested that the artifacts are far more ancient than what had previously been the oldest human settlement found on the higher, central part of the plateau, which was dated to around 7,400 years old (*Science*, 362:1049–51, 2018; *Science*, 355:64–67, 2017).

"[W]e did not initially expect to find archaeological evidence of humans in this high and challenging environment any earlier than the Last Glacial Maximum [LGM] of roughly 18–22,000 years ago," notes John Olsen, an anthropologist at the University of Arizona and a member of the research team, in an email to *The Scientist*. "As the name implies, the LGM was a period of relative cold and aridity within the Pleistocene (Ice Age) and the common assumption was that initial human occupation of the high plateau must have occurred after the LGM . . . [but this] seems not to have been the case."

In an opinion article published alongside the study, geochronologist Jia-Fu Zhang of Peking University and archaeologist Robin Dennell of the University of Exeter—neither of whom were involved in the research—note that the site, Nwya Devu, is near a ridge of black slate that would have provided the raw materials for the tools. "Because of the proximity of the site to a large source of flakable stone, the site is likely a workshop where tools were made that were then used on hunting expeditions at other locations," Zhang and Dennell argue. Furthermore, they write, although no human remains were found at Nwya Devu, 40,000-year-old *Homo sapiens*

TIBETAN TOOLS: Most of the 3,000 fragments were from double-sided blades.

remains have been found in a cave in northern China, suggesting that the people who made the tools were not Denisovans, Neanderthals, or another relative, but belonged to our species. Thus, the finding appears to push back the date of *Homo sapiens* presence in one of Earth's harshest environments.

Yet the study raises more questions than it answers. For one, if the ancient blade-makers really were *Homo sapiens*, were they the ancestors of today's Tibetans, or a people who later vanished from the plateau? Rasmus Nielsen, a geneticist at the University of California, Berkeley, who led the study that identified the Denisovan origins of *EPAS1* but was not involved in the current study, says that work on ancient DNA indicates that two key altitude-coping genes did not come under selective pressure in the Tibetan genome until around 4,000 years ago, or even later, making it unlikely that Tibetans' ancestors had settled at high altitudes many millennia before that. But without human remains at the site, "what these people were, and how they're related to modern Tibetans . . . we don't know about that," he adds.

The team calculated the age of the tools at between 30,000 and 40,000 years old.

Olsen says the team plans to address this gap in the human fossil record; its members will continue their work at Nwya Devu and other sites on the plateau this summer. One question Olsen is especially interested in addressing is where the toolmakers came from. "The tools from Nwya Devu most closely resemble contemporaneous artifacts from Xinjiang (in northwest China), Mongolia, and southern Siberia in Russia," he writes to *The Scientist*. "Is this pattern indicative of the directionality of the initial settlement of the Tibetan Plateau? We just don't know the answer to that question yet, and it will require a much more robust sample size than one site located in the middle of such a large plateau."

It's also not clear whether early humans on the plateau settled there, or visited intermittently. Mark Aldenderfer, an archaeologist at the University of California, Merced, who was not involved in the study but has collaborated with Olsen in the past, says some archaeologists believe the plateau could only have been permanently occupied beginning around 5,200 years ago, once humans had agriculture to help sustain them (*Science*, 347:248–50, 2015). Aldenderfer has disputed this, arguing that archaeological evidence, and some currently available genetic evidence, points to permanent occupation of the plateau at sites more than 7,000 years old. For example, a 2013 analysis of Y-chromosome and mitochondrial DNA lineages in present-day Tibetans found evidence for two waves of migration to the area, with the first dated to about 30,000 years ago (*Mol Biol Evol*, 30:1761–78).

Humans would not have had much success sustaining pregnancies and establishing a long-term population atop the plateau unless they had genes to help them adapt—which is where Denisovans may or may not come in. Nielsen says that if *Homo sapiens* were braving the plateau as early as the new study suggests, they might even have interbred with Denisovans there, although he doesn't consider that the most likely scenario. That's because Denisovan alleles are also found in some other East Asian populations, suggesting they were acquired before the plateau's colonization.

The authors of the paper suggest a different possibility, citing the similarity of the Nwya Devu tools to others found in Siberia, and the prevalence of Denisovan DNA in modern Melanesians: that the Tibetan Plateau might have been one area through which genetic traces of the Denisovans made their way south across the *Homo sapiens* population, conferring tolerance to high altitude along the way. Whoever the people at Nwya Devu were, Zhang and Dennell write, their discovery "provides a graphic example of how successful our species has been as a colonizing animal."

—Shawna Williams

XING GAO

More Than the Sum of Its Parts

The study of evolution requires consideration of organisms' microbiomes.

BY ITZHAK MIZRAHI AND FOTINI KOKOU

It is widely accepted that all animals and plants host diverse microbial communities that are vitally important for their functioning and survival. In many cases, these microbiomes can be at least partially heritable, being passed from parent to offspring. Thus, when environmental changes occur, we would expect to see alterations not only in hosts' physiology over subsequent generations, but also in their microbiomes.

Husband-and-wife team Eugene Rosenberg and Ilana Zilber-Rosenberg of Tel Aviv University in Israel were the first researchers to propose this concept (*FEMS Microbiol Rev*, 32:723–35, 2008). A host organism and its resident microbes—the so-called holobiont—functions as a whole on multiple levels, they argued, from the gene and chromosome to the organism's anatomy and physiology, and acts as an independent unit of selection.

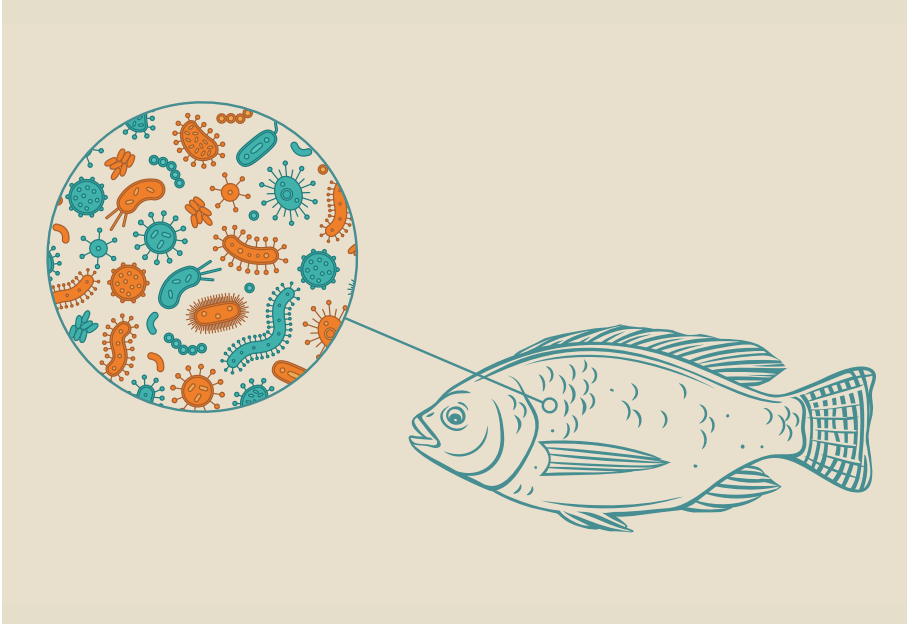
A famous example of this concept is the relationship between corals and their symbionts, the zooxanthellae. Researchers have demonstrated that some corals can evolve to tolerate higher water temperatures by changing the makeup of their symbiont communities. Because microbes have much shorter generation times than coral polyps, the genetic composition of the symbiont populations can evolve much more rapidly than that of their hosts, and these changes can confer higher tolerance on the holobiont unit.

Over the last decade, it has become evident that the idea of the evolutionary concept of the hologenome, which views the holobiont as the unit of selection, can be applied across the tree of life, with examples cropping up in plants and insects. This revelation motivated us to explore the relevance of the microbiome to the adaptation of so-called poikilothermic animals, which are unable to maintain a stable body temperature using internal mechanisms. Specifically, we set out to answer whether host selection for an environmental stressor such as cold exposure results in selection of fishes' associated microbes.

We bred tropical blue tilapia, which are typically found in marine environments with high water temperatures of 24–28 °C. Over three generations, we selected for fish whose siblings had high survival rates in low-temperature conditions. We then compared the gut microbiomes of genetically cold-resistant fish to those of cold-sensitive fish. Despite having never experienced low-temperature environments themselves, these two groups had different gut microbiomes as a result of the selection. Moreover, when we challenged all these fish in low-temperature conditions, cold-resistant fish's gut microbiomes were more stable, as were the fish's transcriptomes. Thus, our selection regime shaped both the host and its associated microbiome to be more resilient to drops in temperature (*eLife*, 7:e36398, 2018).

These findings are no doubt just one example of coordination between a host and its microbes. As the evolutionary concept of the hologenome matures, researchers will likely document many more plant and animal communities that evolve with their microbiomes. It remains to be determined whether a microbiome's compositional changes directly affect its host's physiological response to changing environmental conditions. But the hologenome concept will undoubtedly influence our understanding of the evolution and ecology of all organisms. ■

Itzhak Mizrahi is an Associate Professor at Ben-Gurion University in Israel. Fotini Kokou is a postdoctoral fellow in his group.



MODIFIED FROM © ISTOCK.COM; *THE SCIENTIST* STAFF

Stick-On Immune Cell Monitor

A microneedle-containing skin patch offers researchers a noninvasive way to survey immune responses in mice.

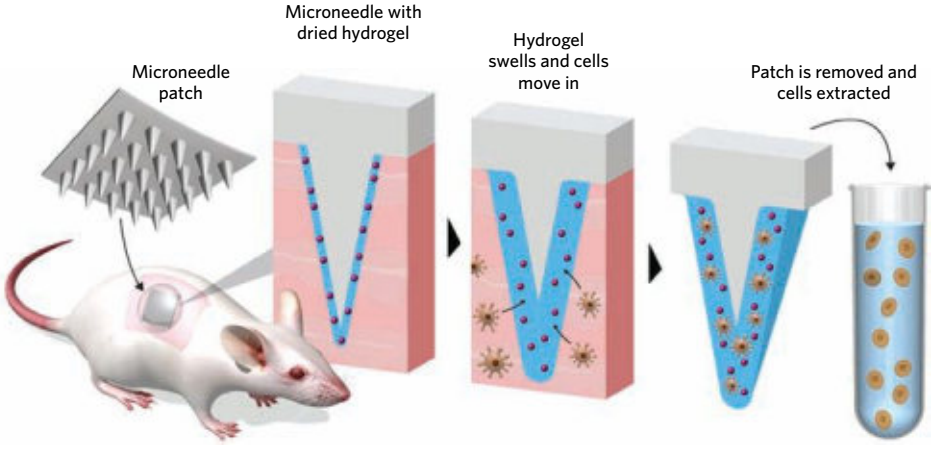
BY RUTH WILLIAMS

The progression of an immune response can be thoroughly studied in the blood thanks to the ease of drawing the fluid from animals and humans. But "it's becoming increasingly clear that tissue-level responses don't look like blood-level responses," says immunologist Sarah Fortune of the Harvard T.H. Chan School of Public Health. "And we haven't had very good ways to monitor tissue responses."

A researcher could examine an immune response in, for example, the skin by taking a punch biopsy, which is invasive, or by measuring redness and swelling, which reveals nothing about the cells and factors involved in the response.

But new microneedle patches devised by MIT's Darrell Irvine and colleagues provide information about the cellular response without having to remove tissue for analysis.

The patches measure approximately 1 cm^2, contain an array of roughly 80 solid polymer microneedles (each one around 250 μm^2 at its base and 500–600 µm long), and are coated in a biocompatible dried hydrogel. When applied to the skin, the needles puncture the epidermis, the hydrogel swells as it contacts fluids in the tissue, and immune cells can migrate inside. While the gel can be used as is, the team found that including nonspecific immune-boosting agents (adjuvants) increased cell recruitment twofold. Specific antigens can also be included to assess different immune dynamics, such as the development of antigen-targeted cells following a vaccination.



PATCHWORK IMMUNE MONITORING: To monitor an animal's immune response to a given stimulus, researchers can apply a stick-on patch, containing polymer microneedles coated with a dried hydrogel, to the skin, puncturing the epidermis. The hydrogel swells upon contact with fluids in the tissue, and skin-resident immune cells can migrate into it. Immunogenic molecules and antigens incorporated into the gel help to attract and retain immune cells either specifically (via antigen recognition) or nonspecifically (via innate pathways). After 24 hours, removal of the patch and processing of the hydrogel enables recovery of the cells for further analysis.

The team used such adjuvant- and antigen-loaded microneedle patches to analyze skin-resident memory T cells in mice after vaccination with an immunogenic protein. They found that while both the skin and blood had an abundance of these cells in the early weeks following vaccination, after a few months their numbers had dwindled in the blood but remained high in the skin—possibly because the skin is a first line of defense, says Irvine.

Fortune, who was not involved in the study, says the patches are "an extremely useful immune monitoring technology that opens up new opportunities for understanding tissue-level responses to infection and vaccination." (*Sci Transl Med,* 10:eaar2227, 2018) ■

AT A GLANCE

SKIN IMMUNE CELL MONITORING	PROCEDURE	IMMUNE CELL ANALYSIS	INVASIVE?	APPROVED FOR USE IN HUMANS?
Biopsy	A small section of epidermis with underlying dermis is removed with a metal punch	Histological techniques, such as cell staining with labeled antibodies or flow cytometry	Yes	Yes
Microneedle patch	The patch is pressed onto the skin and held in place for 24 hours with medical tape. The hydrogel-coated microneedles puncture the epidermis and upper dermis and immune cells migrate into the gel.	Flow cytometry of cells extracted from the dissolved hydrogel	Minimally	No

The Resilient Parasite

The microorganism that causes malaria has evolved resistance to medicine's front-line therapies before. Can it do it again?

BY NATALIE SLIVINSKI

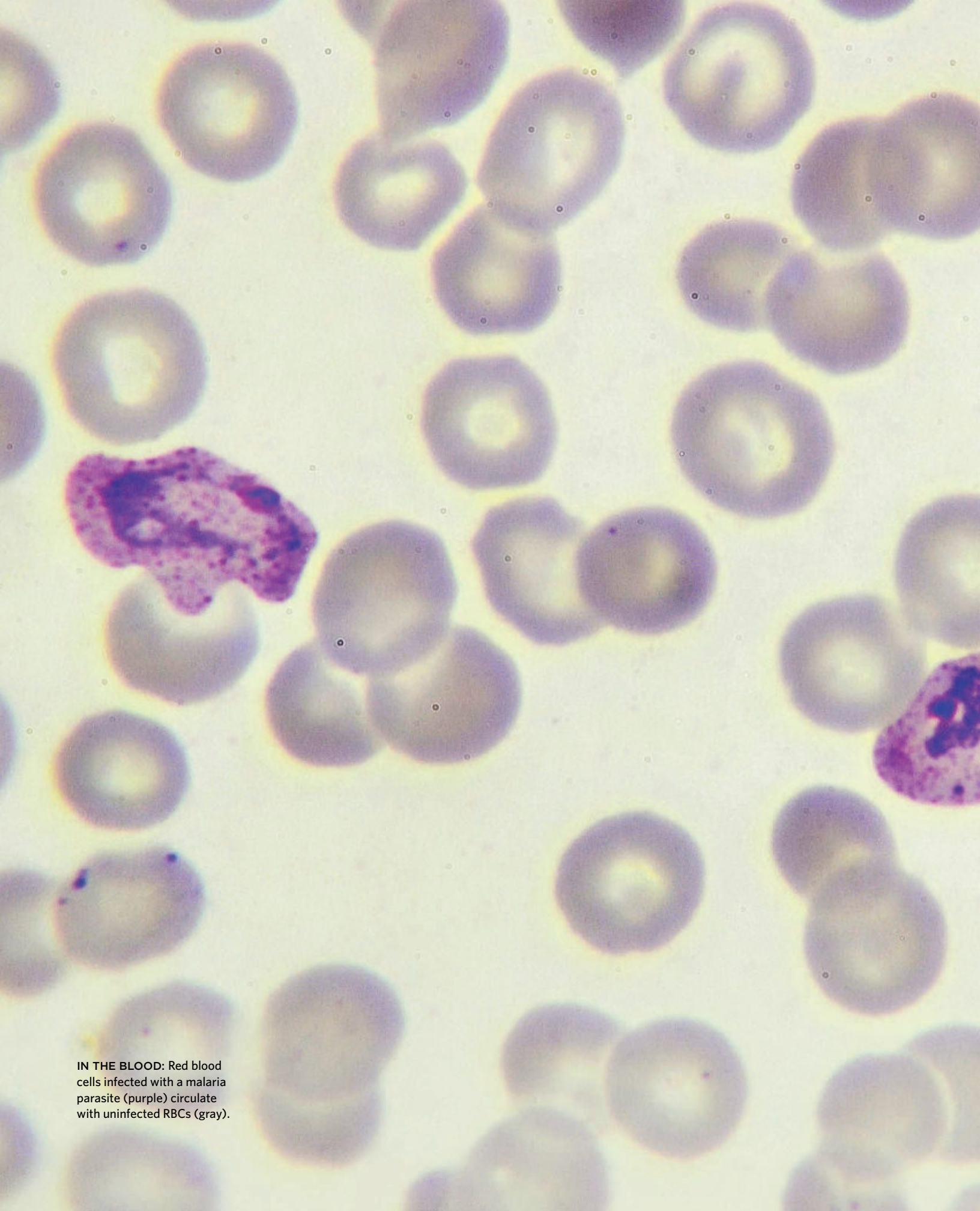
It's not clear why, but the Greater Mekong Subregion—Cambodia, southern China, Laos, Myanmar, Thailand, and Vietnam—is a major source of malaria drug resistance. Each time a drug has been deployed in the area, resistance mutations in local *Plasmodium falciparum*, the parasite that causes the mosquito-borne disease, have followed close behind. Parasites there seem more adaptable than *P. falciparum* in other regions, says Thanat Chookajorn, an assistant professor of biochemistry at Mahidol University in Thailand, who studies the molecular genetics of malaria parasites that thrive in the Greater Mekong.

"It sounds kind of self-centered to say, 'My parasite's the worst in the world,'" Chookajorn says. "But I would say that there's definitely something funny going on with this population."

Resistance to chloroquine, the first widely used antimalarial drug, first arose in the Greater Mekong shortly after World War II. Chloroquine-resistant strains eventually spread to Africa, which carries more than 90 percent of the global malaria burden. This explosion of drug resistance contributed to an alarming climb in worldwide mortality rates in the second half of the 20th century.

In the 1990s, artemisinin—a compound derived from the wormwood plant that was used for centuries in natural medicine to treat pain and fever—was released globally as a new malaria treatment.[1] The drug was a boon to malaria scientists, who were able to pair brief pulses of aggressive, short-acting artemisinin derivatives with longer-acting partner drugs to make artemisinin-based combination therapies (ACTs). These extremely effective treatments—plus intensive programs for implementing rapid diagnostic tests and insecticide-impregnated bed nets—slowed the parasite's progress. Between 2010 and 2015, global malaria mortality dropped almost 30 percent. Compared to nearly 1 million annual malaria-related deaths in the late 1990s, today only about 400,000 of the 220 million cases per year end in the patient's death, according to the World Health Organization (WHO).

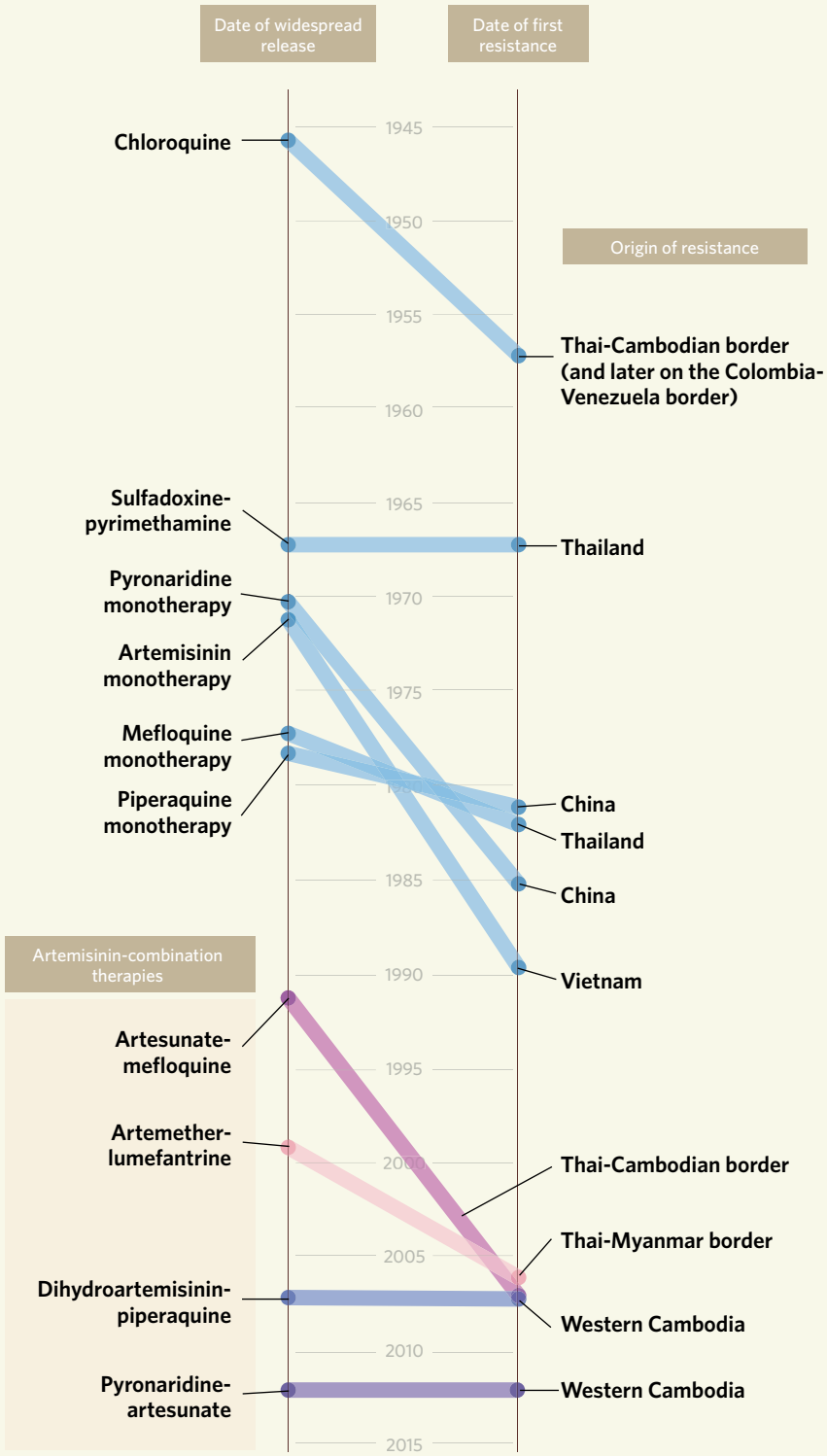
Starting around 2007, however, ACT resistance slowly began creeping into parasite populations, especially in the Greater Mekong. These days, there is at least one *P. falciparum* mutant resistant to each ACT partner drug, and a handful have begun to show partial resistance to artemisinin itself, with the drug taking longer to clear the parasites from the bodies of infected people. "This truly is a wily parasite," says biochemist David Kaslow, director for the PATH Malaria



IN THE BLOOD: Red blood cells infected with a malaria parasite (purple) circulate with uninfected RBCs (gray).

THE RISE OF MALARIA DRUG RESISTANCE

Plasmodium falciparum resistance to artemisinin-based combination therapies (ACTs) started to crop up around 2007. Infections, especially in the Greater Mekong area of Southeast Asia, seemingly survived treatment. This was largely due to the pairing of artemisinin derivatives with older drugs that had existing resistance problems. But some experts think the emergence of partial resistance to artemisinin itself—which allows parasites to persist for longer in the body following treatment—could also play a role.



Vaccine Initiative, a nonprofit dedicated to malaria prevention and eradication.

With no viable alternative to artemisinin, increasing numbers of malaria infections with delayed clearance following artemisinin treatment are concerning, experts say—and if resistance develops in Africa, the results could be disastrous. Strains with partial artemisinin resistance have drawn a great deal of attention, even being dubbed "super malaria" by some media outlets. "I really caution us against complacency," says Kaslow. "I don't think there's evidence today to say that resistance is going to explode in Africa. But tomorrow, it could. I would not put anything past this parasite."

Some scientists are less concerned over the danger of ACT resistance, arguing that the threat is overblown. Currently, the ACT arsenal remains strong enough to defeat the parasite, says Sanjeev Krishna, a molecular parasitologist at St. George's University of London. "If you have the right combination partner, then your treatment is curative."

Steve Meshnick, a professor of epidemiology at the University of North Carolina at Chapel Hill, agrees that partial artemisinin resistance is, for now, a mere blip in the fight against malaria. "I'm not saying it's not a problem," he says, "but I think it gets too much attention."

A history of resistance

Resistance against malaria drugs has been a battle since day one. Soon after chloroquine's international release in the late 1940s, parasites began to fight back, particularly in Colombia, Thailand, and Cambodia,[2] which were subjected to mass chloroquine treatments, often at low doses that promoted the evolution of resistant parasites. The drug was even added to table salt in some countries in a desperate attempt to curb infections.[3]

Sure enough, *P. falciparum* strains in these areas developed multiple mutations in the transmembrane protein PfCRT.[4] This allowed the parasite to reduce the accumulation of the drug in its digestive vacuole, where it normally kills the parasite by binding to subunits of oxidized heme, thereby

preserving the toxicity of this byproduct of the parasite's digestion of hemoglobin. Normally, the parasite polymerizes heme subunits into harmless clumps, but the binding of chloroquine prevents this aggregation, preserving the heme's toxicity. Mutated PfCRT appears to limit chloroquine's access to the digestive vacuole, allowing resistant parasites to clean up the dangerous scraps of their hemoglobin dinner without interruption. (See illustration on page 26.)

Before long, chloroquine was rendered too unreliable to use as a regular treatment against *P. falciparum*. Researchers developed synthetic alternatives, but these too encountered resistant parasites soon after their release.[5] Some drugs also carried risks of severe side effects such as bad skin reactions and liver problems; or, they had complex dosing instructions that reduced compliance, thereby limiting their use as frontline treatments. Mortality rose at an alarming rate, especially in Africa. In Senegal, for example, death rates climbed as much as sixfold in children under 10.[6]

Enter artemisinin, which would come to be known as a "miracle drug." Semisynthetic antimalarial formulations of the natural compound, originally developed in China in the 1970s, proved incredibly effective at swiftly killing *P. falciparum* with minimal side effects. When activated by iron released by the hemoglobin-digesting parasite, the drug is thought to pummel *P. falciparum* at multiple targets, including ATPases and enzymes important for the redox cycle, the core of fundamental biological processes such as metabolism and cellular respiration. A short, three-day pulse of artemisinin wipes out the vast majority of infecting parasites. When paired with slower-acting partner drugs that mop up any stragglers, artemisinin derivatives such as artemether, artesunate, and dihydroartemisinin (DHA) became unstoppable. By the early 2000s, such ACTs were the go-to malaria treatment. Mortality rates slowed globally, then dropped by the millions.

The problem was that some of the partner drugs had already been around for decades as chloroquine alternatives. That meant various *P. falciparum* strains had already developed resistance to some of these partner drugs. Because of this, researchers began to see malaria infections resurface after ACT treatment.

The rise of ACT resistance

The first widely used ACT paired the existing drug mefloquine, which had been around since the late 1970s, with a blast of artesunate. In the early 1990s, artesunate-mefloquine (ASMQ) was used to treat malaria in provinces on the Cambodia-Thailand border, where existing mefloquine therapies were failing. With an initial dose of artesunate to wipe out the bulk of offending parasites, ASMQ succeeded in curing almost 100 percent of infected patients in the area.[7]

Resistance against malaria drugs has been a battle since day one.

By the mid-2000s, however, it became clear that artesunate could no longer compensate for the ever-increasing ability of *P. falciparum* to resist mefloquine. By upregulating the multidrug resistance gene that encodes the mutated transport protein PfMDR1, the parasite likely pumps the drug away from its cytosolic target and into the digestive vacuole to be destroyed. Many parasites that lingered after the artesunate treatment were thus able to survive the mefloquine mop-up. By 2008, ASMQ was failing in about 20 percent of patients in that region of Southeast Asia[8]

A new ACT, which combines DHA with the existing drug piperaquine (PP), hit the market in 2007. DHA-PP worked well against ASMQ-resistant parasites, but soon failed against strains from a lineage called PLA1 that somehow resisted PP. Like chloroquine, PP inhibits heme detoxification, but it's also thought to target plasmepsins 2 and 3, which are proteases the parasite requires to digest hemoglobin for its peptides.[9] Without these proteases, *P. falciparum* starves. By making multiple copy numbers of plasmepsin 2 and 3 genes, PLA1 parasites can overcome the effects of the drug.[10] By 2013, 25 percent of patients in western Cambodia weren't responding to DHA-PP.[11]

In addition to resistance to the partner drugs in ACTs, *P. falciparum* has also shown signs of evading artemisinin derivatives. Around the same time that DHA-PP was released, researchers in western Cambodia noticed that it was taking patients longer to clear parasites following the initial pulse of artesunate, DHA, and other artemisinin-derived drugs.[12] *P. falciparum* were found in the blood three or four days after treatment, whereas it normally took just one or two days for the drugs to reduce the infection to below-detectable levels of parasite. Malaria scientists dubbed this phenomenon "partial" or "emerging" artemisinin resistance, because although the treatment was taking longer to work, it was still effective, with most resilient parasites being cleared within a week.[13]

Investigations into delayed-clearance mechanisms pointed to various mutations in the *kelch13* gene, which codes for a poorly understood kinase-binding protein. In vitro studies show that the parasite's resistance is effective at a specific life stage—the early ring stage, which occurs soon after *P. falciparum* enters a red blood cell but before it starts replicating. Artemisinin derivatives get metabolized by the body very quickly, so "resistant" parasites appear to evade the drug by lingering longer in the ring stage, biding their time.[14] (See illustration on page 28.)

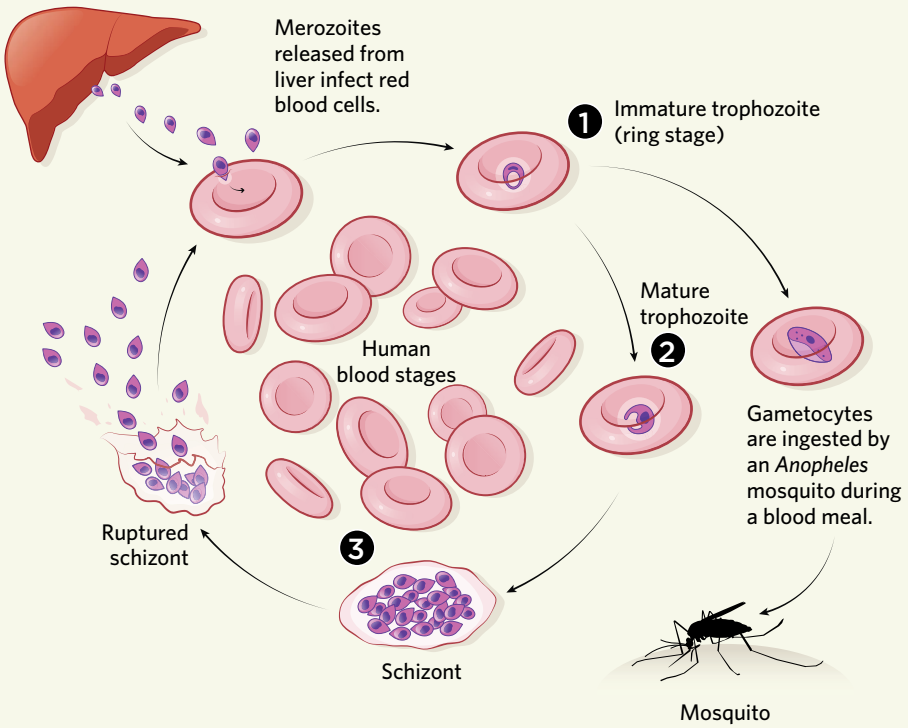
Initially, cases of delayed clearance in Western Cambodia were limited to small, discrete geographic regions where the parasites carried various different *kelch13* mutations, none of which appeared to have an advantage over the others, says Roberto Amato, a population geneticist at the Wellcome Sanger Institute. It wasn't

ACT OF RESISTANCE

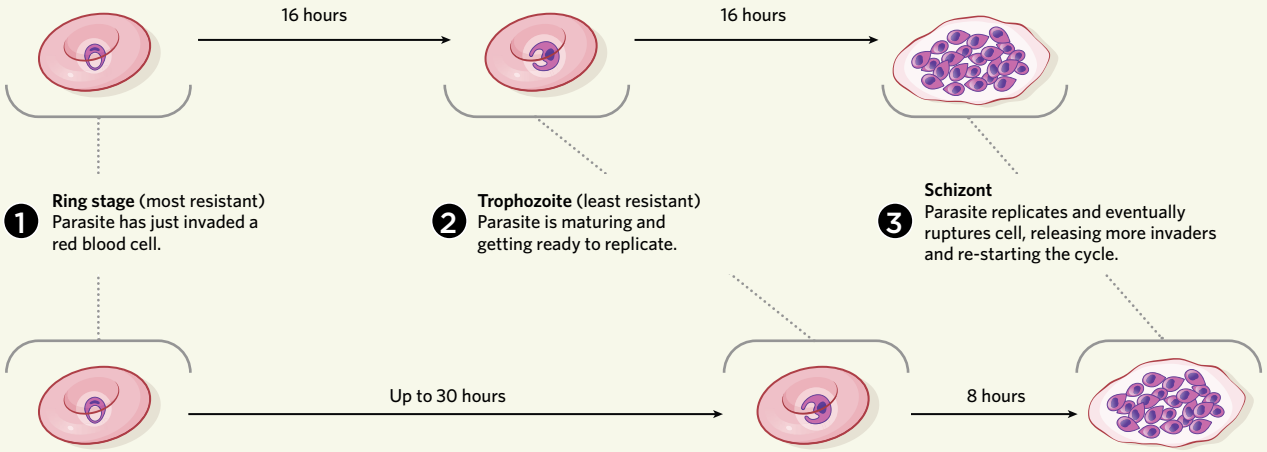
Plasmodium falciparum resistance to artemisinin-based combination therapies (ACTs) started to crop up around 2007. This largely arose from pairing artemisinin derivatives with older drugs that had existing resistance problems. But the emergence of partial resistance to artemisinin itself—which allows parasites to persist for longer in the body following treatment—may also play a role.

PARTIAL ARTEMISININ RESISTANCE

Researchers have linked partial resistance to artemisinin-derived drugs with several mutations in the *kelch13* gene, which encodes a binding protein whose role in the parasite's ability to persist is still unclear. Delayed parasite clearance has also been linked to a prolonged ring stage, which appears to be the only part of the parasite's lifecycle during which it is able to partially survive artemisinin derivatives such as artemether, artesunate, or dihydroartemisinin. A single dose of these ACT ingredients stays in the body for only a few hours, and patients are typically treated with an artemisinin derivative for only the first couple of day of malaria therapy, so it's thought that the prolonged ring stage may help the parasites survive the therapy. Preliminary evidence suggests that resistant parasites also rush through the subsequent trophozoite stage, which appears to be the most susceptible to artemisinin.



WILDTYPE PARASITE

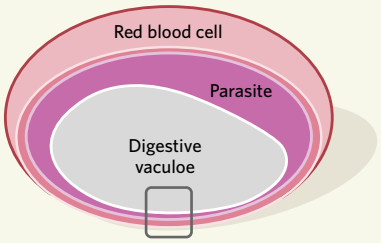


PARASITE WITH PARTIAL ARTEMISININ RESISTANCE

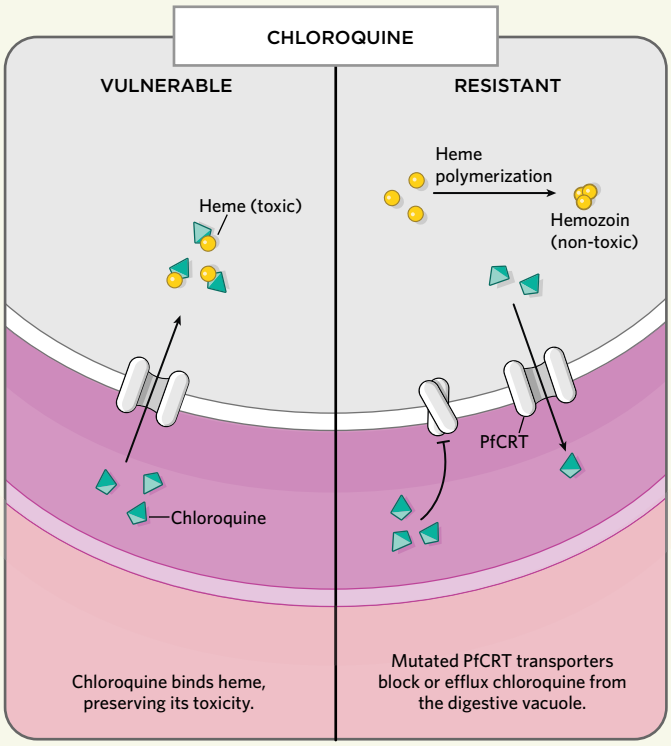
Data taken from *Antimicrob Agents Chemother*, 59:3156–67, 2015. Times are approximate.

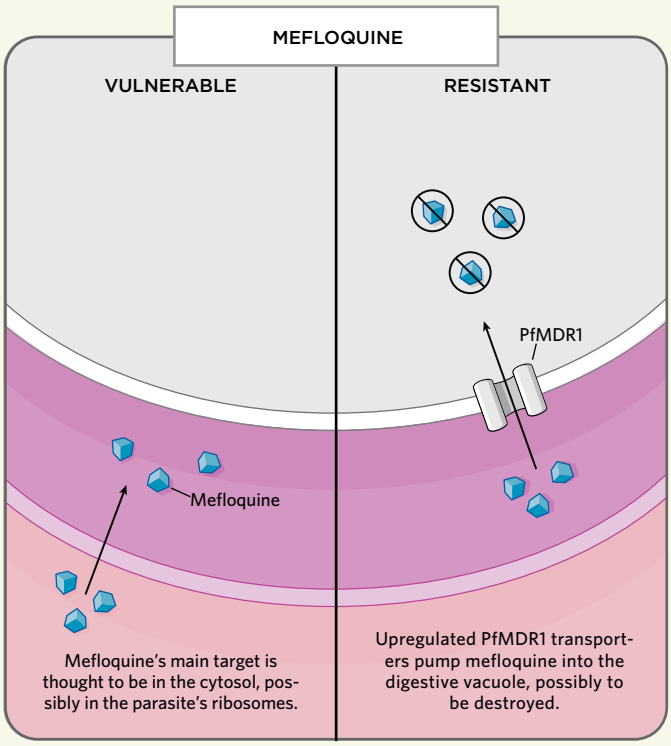
PARTNER DRUG RESISTANCE

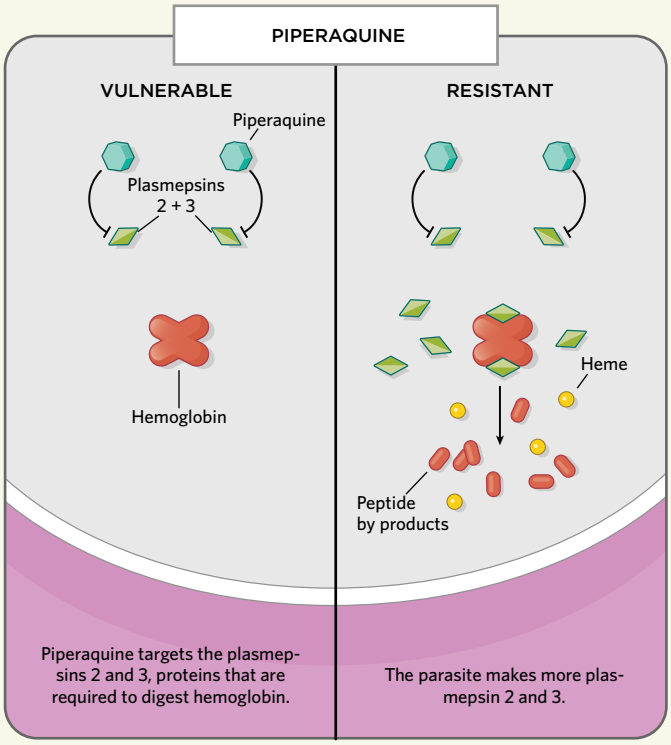
In red blood cells, *P. falciparum* digests human hemoglobin to feed itself. In addition to amino acids, this releases toxic heme. Normally, the parasite polymerizes the heme into harmless clumps of hemozoin or degrades it through a handful of poorly understood pathways. But most ACT partner drugs inhibit detoxification. Some partner drugs also attack the parasite through other mechanisms. Here are examples of how *P. falciparum* strains resist these drugs.

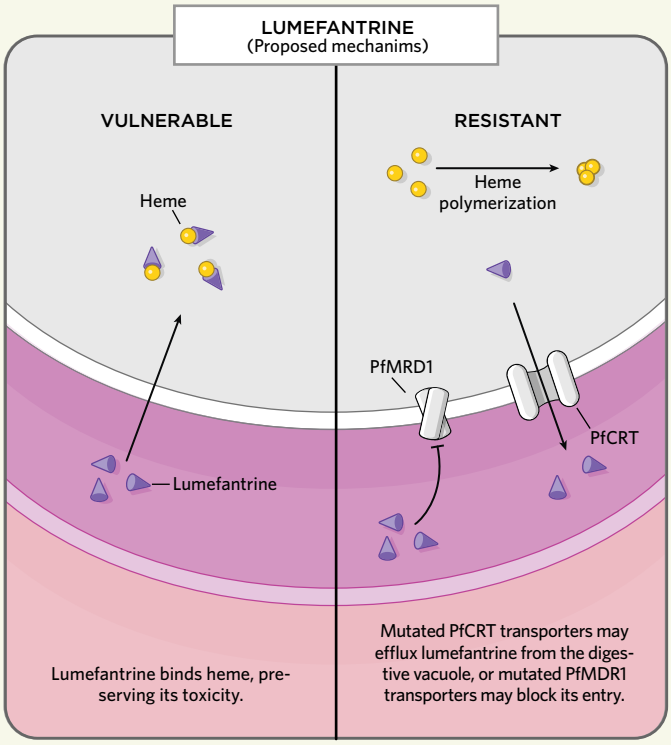


During the blood stages of malaria infection, the parasite resides within red blood cells, digesting hemoglobin to support its growth and maturation.









until DHA-PP–resistant PLA1 parasites emerged in 2008 that things started to get bad, he says.

Of all the *kelch13* mutants, one haplogroup, called the KEL1 lineage, appeared to have the potential to spread more aggressively than others. When parasites started picking up resistance alleles from both KEL1 and PLA1 together, those KEL1/PLA1 parasites came to dominate the landscape in Cambodia. The so-called co-lineage soon spread to Thailand, Laos, and Vietnam.[15] Frequencies of both the PLA1 and KEL1 parent lineages shot up—KEL1, from 4 percent in 2007 to 63 percent in 2013; PLA1, from zero to a whopping 79 percent in that same period. By 2013, more than 90 percent of DHA-PP resistant parasites carried resistance alleles from both lineages.[16] "That's when people really started shouting at each other," says Amato, with some arguing that ACT resistance could pose serious problems in the fight to eradicate malaria, and others maintaining that the concern was overstated.

By 2017, DHA-PP treatment failure had reached 30 percent in Vietnam and a staggering 90 percent in western Cambodia.[17] It's thought that the KEL1 lineage allowed parasites to persist in patients two or three days longer after the artemisinin pulse than in other endemic areas, while the PLA1 lineage made them resistant to PP. Currently, Amato says, "KEL1/PLA1 has basically replaced the indigenous population [of *P. falciparum*]. So the situation is not great."

Misplaced concern?

Experts caution against sensationalizing partial artemisinin resistance, as there is some debate over the role that it plays in ACT failure. Indeed, there are no known cases in which delayed clearance has led to treatment failure on its own, says Meshnick at the University of North Carolina—resistance to the partner drug, not artemisinin, is the primary driver for an ACT failing. For this reason, he adds, the term "resistance" should not be conflated with delayed clearance. "I think it's misleading. When you say everybody's 'artemisinin resistant.' . . . All it means is that everybody has delayed clearance times, but they're still responding to the drug." If the therapy uses an effective partner drug, it will still be successful—and currently, if one ACT fails, another will still be effective.

It is also reassuring that, so far, *P. falciparum*'s ability to resist artemisinin treatment is restricted to the ring stage, says Krishna. Ongoing research into the effects of adjusting the artemisinin dose could yield a solution, adds Meshnick. "I've heard people talk about giving artemisinin for longer periods of time, or maybe giving it twice a day versus once a day," he says. "I think all of those things should be on the table."

Cases involving delayed clearance in Southeast Asia and South America are on the rise, but actual clearance times have not increased further since they first appeared, says Pascal Ringwald, who leads the Drug Efficacy and Response unit for the WHO Global Malaria Program. Thus, the parasites do not appear to be moving toward more complete resistance—at least not yet, he says. "We are concerned, we are watching it, and we are trying to find alternatives. But we can still cure 100 percent of people if we have a good partner drug."

Ultimately, most experts agree that ACT resistance needs to be monitored, but when it comes to extinguishing malaria—which has stubbornly maintained a constant global burden since 2015—there are bigger fish to fry. Drug resistance has thankfully not played a role in the current stall, says Pedro Alonso, the director of the WHO Global Malaria Program: it seems ACTs are enough to keep resistance from affecting mortality rates for the time being, including in Africa. Despite occasional reports of artemisinin resistance markers popping up there, none have stuck around, suggesting there is no selective pressure for the parasites to survive the initial blast of the compound's derivatives. Since the mid-2000s, there have been signs of selection for resistance to lumefantrine, the partner drug of Africa's frontline ACT, but for now it remains 100 percent effective when combined with a blast of artemether. "There is no drug resistance of any significance in the entire continent," says Alonso.

Rather, Alonso says, logistical challenges of delivering ACT therapies, diagnostic tools, and other life-saving interventions such as insecticide-treated bed nets have caused declining mortality rates to stall at about 400,000 per year.

> I really caution us against complacency.
>
> -David Kaslow, PATH Malaria Vaccine Initiative

Furthermore, funding has not increased apace with population growth, meaning that bed nets and on-site clinics are in short supply. "We're probably seeing the limits of what can be achieved with the tools and the funding that we have." He and other experts also express greater concern about the evolution of DDT-resistant mosquitoes that could thwart additional efforts to prevent transmission.

Some, however, continue to sound the alarm. Even if delayed clearance doesn't directly lead to treatment failure, it puts more pressure on partner drugs to succeed in mopping up lingering parasites, says Nicholas White, a professor of tropical medicine at Mahidol University and the University of Oxford. Parasites even developed resistance to lumefantrine, which was never used as a monotherapy before its release in an ACT with artemether in the late 1990s, in the Greater Mekong within just five or six years. And the newest ACT, which pairs artesunate with the partner drug pyronaridine, was already failing in 10 percent of cases in western Cambodia at the time of its release in 2012, and artemisinin clearance times were already two to three days longer.

Even with ample ACT options, changing up the treatments typically given in a particular area is easier said than done, says Amato. For many patients in difficult-to-access regions of endemic countries with poor access to clinics, simply rolling out rotating partner drugs every time ACT resistance pops up is not always practical. In cases of delayed clearance, researchers are experimenting with tweaking the dose of artemisinin derivative, but this can be equally tricky to implement—for instance, if the derivative can't be easily separated from its ACT partner because it normally comes in a single pill or blister pack. "There are various strategies on the table," he says. "The question is, which ones are actually logistically implementable?"

And while the evidence says that partial artemisinin resistance doesn't cause ACT failure on its own, Alonso acknowledges that, with an ever-evolving parasite, the situation could change tomorrow. "In public health, one learns to never say never." ■

Natalie Slivinski is a freelance science journalist living in Seattle, Washington.

References

1. L. Cui, X. Su, "Discovery, mechanisms of action and combination therapy of artemisinin," *Expert Rev Anti-Infect Ther*, 7:999–1013, 2010.
2. T.E. Wellems, C.V. Plowe, "Chloroquine-resistant malaria," *J Infect Dis*, 184:770–76, 2001.
3. D. Payne, "Did medicated salt hasten the spread of chloroquine resistance in *Plasmodium falciparum*?" *Parasitol Today*, 4:112–15, 1988.
4. M. Chinappi et al., "On the mechanisms of chloroquine resistance in *Plasmodium falciparum*," *PLOS ONE*, 5:e14064, 2010.
5. C. Roper et al., "Intercontinental spread of pyrimethamine-resistant malaria," *Science*, 305:1124, 2004.
6. J.-F. Trape et al., "Impact of chloroquine resistance on malaria mortality," *C R Acad Sci III* , 321:689-97, 1998.
7. F. Nosten et al., "Effects of artesunate-mefloquine combination on incidence of *Plasmodium falciparum* malaria and mefloquine resistance in western Thailand: a prospective study," *Lancet*, 356:297–302, 2000.
8. R. Leang et al., "Therapeutic efficacy of fixed dose artesunate-mefloquine for the treatment of acute, uncomplicated *Plasmodium falciparum* malaria in Kampong Speu, Cambodia," *Malaria J*, 12:343, 2013.
9. A. Mukherjee et al., "Inactivation of plasmepsins 2 and 3 sensitizes *Plasmodium falciparum* to the antimalarial drug piperaquine," *Antimicrob Agents Chemother*, 62:pii:e02309–17, 2018.
10. K. Thriemer et al., "Delayed parasite clearance after treatment with dihydroartemisinin-piperaquine in *Plasmodium falciparum* malaria patients in central Vietnam," *Antimicrob Agents Chemother*, 58:7049–55, 2014.
11. R. Leang et al., "Efficacy of dihydroartemisinin–piperaquine for treatment of uncomplicated *Plasmodium falciparum* and *Plasmodium vivax* in Cambodia, 2008 to 2010," *Antimicrob Agents Chemother*, 57:818–26, 2013.
12. H. Noedl et al., "Evidence of artemisinin–resistant malaria in western Cambodia," *N Engl J Med*, 359:2619–20, 2008.
13. K. Thriemer et al., "Delayed parasite clearance after treatment with dihydroartemisinin-piperaquine in *Plasmodium falciparum* malaria patients in central Vietnam," *Antimicrob Agents Chemother*, 58:7049–55, 2013.
14. A. Hott et al., "Artemisinin-resistant *Plasmodium falciparum* parasites exhibit altered patterns of development in infected erythrocytes," *Antimicrob Agents Chemothe*r, 59:3156–67, 2015.
15. M. Imwong et al., "The spread of artemisinin-resistant *Plasmodium falciparum* in the Greater Mekong subregion: A molecular epidemiology observational study," *Lancet Infect Dis*, 17:491–97, 2017.
16. R. Amato et al., "Origins of the current outbreak of multidrug-resistant malaria in southeast Asia: A retrospective genetic study," *Lancet Infect Dis*, 18:P337–45, 2018.
17. L. Roberts, "Drug–resistant malaria advances in Mekong," *Science*, 358:155–56, 2017.
18. E.A. Ashley et al., "Spread of artemisinin resistance in *Plasmodium falciparum* malaria," *N Engl J Med*, 371:411–23, 2014.

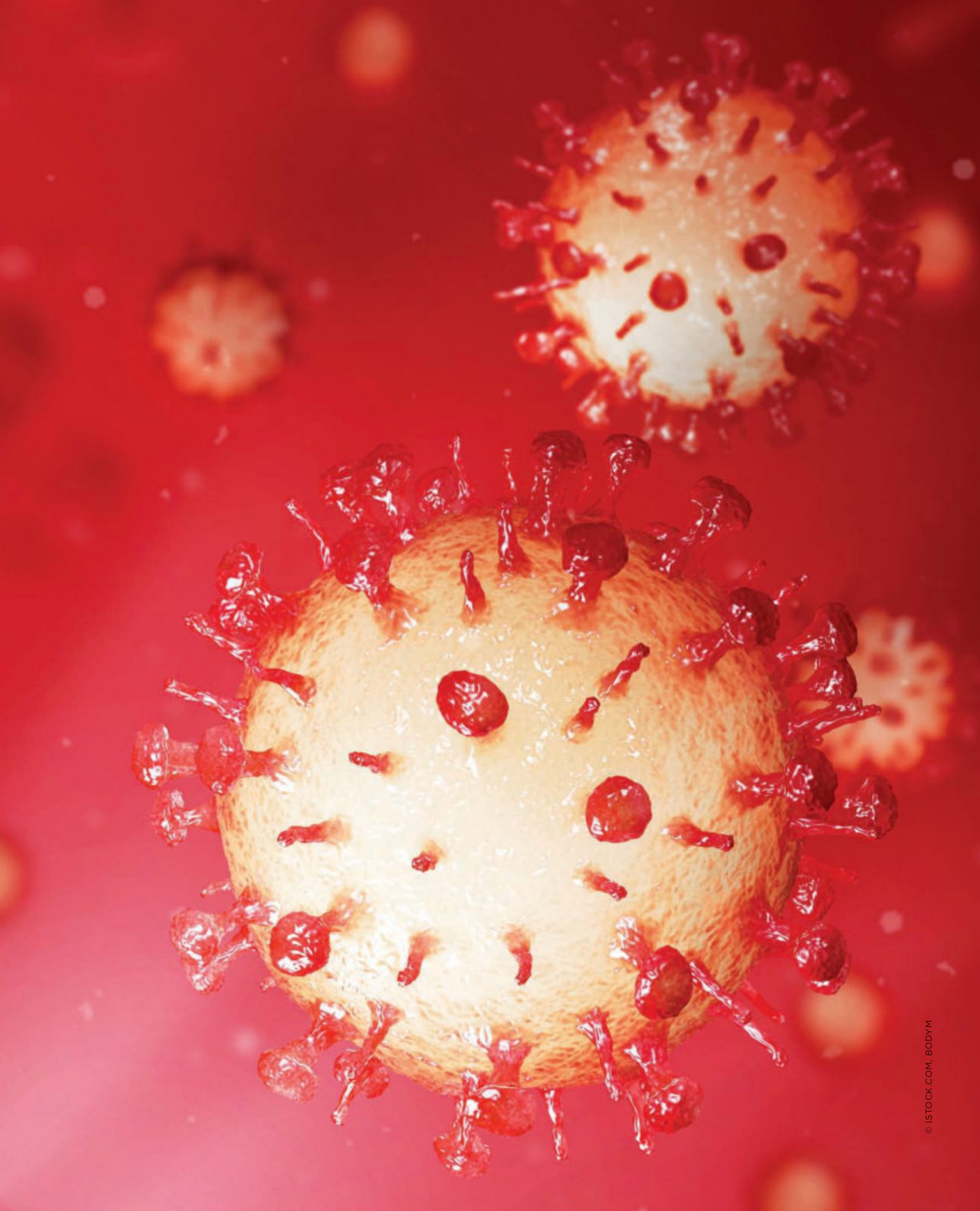
THE SEARCH FOR A BETTER ANTIMALARIAL

While experts debate the relevance of ACT resistance to the fight against malaria, researchers are always looking for alternatives. One option is to combine existing drugs in new ways. For instance, a Phase 2 clinical trial monitoring more than 2,000 patients throughout the Greater Mekong, southern Asia, and the Democratic Republic of the Congo is investigating whether the partner drug mefloquine can be combined with the ACT dihydroartemisinin-piperaquine (DHA-PP), as the mechanisms by which *P. falciparum* evades mefloquine and piperaquine appear to be mutually exclusive.

Simply recombining existing drugs may not be sufficient, however, and the search for antimalarial drugs with new mechanisms of action—such as damaging the parasite's digestive vacuole membrane or disrupting its ability to stick to red blood cells—is also underway. A dozen or so drugs, including both alternative partner drugs and novel artemisinin derivatives, are currently in early- to mid-stage clinical development. One new combination therapy, which has shown promise in Phase 2 trials against parasites that exhibit partial resistance to artemisinin, partners the chloroquine-like drug ferroquine with artefenomel, the first potential synthetic alternative to artemisinin. Artefenomel has a much longer half-life than artemisinin-derived drugs, allowing it to be given as a single dose. It has also shown high activity against the resistance-prone ring stage of the parasite.

Most of the antimalarials in development attack blood-stage schizonts, the stage that causes illness. However, a handful instead target the gametocyte stage picked up by the mosquito. Such drugs would not cure an existing infection, but could control transmission, a critical aspect of malaria elimination. Two of these, tafenoquine and primaquine, have advanced through Phase 3 trials, but they can't be used broadly, because they can cause severe damage to red blood cells in patients with a genetic condition called G6PD deficiency that is common in Africa. Another leading candidate, KAF 156, which doesn't carry the same risk, is currently being tested in Phase 2b trials in combination with the partner drug lumefantrine.

With a parasite that continues to evade many existing antimalarial treatments, says David Kaslow, director for the PATH Malaria Vaccine Initiative, "we've got to continue to invest in new drugs."



THE VIRAL BRAIN

Scientists may need to seriously reconsider the cast-aside hypothesis that pathogens can play a part in neurodegenerative diseases.

BY ASHLEY YEAGER

A little more than 10 years ago, when neurobiologist Richard Smeyne was working at St. Jude Children's Research Hospital in Memphis, he saw a video of a duck acting strangely. The white-feathered, orange-billed bird was standing slightly apart from its flock on a farm in Laos. It walked in circles and flipped up a wing, then lost its balance and fell over. It got up, tried to flap both wings, and fell over again.

Smeyne saw the video while attending a seminar being given by then-postdoc David Boltz and Boltz's advisor, a "flu hunter" named Robert Webster, who headed the influenza research program at the hospital. The duck, Boltz and Webster explained, was infected with the H5N1 bird flu virus that had sickened thousands of birds and killed hundreds of people in 2006 and 2007. Smeyne, who had been studying the neurobiology of Parkinson's disease in mice, recognized the animal's motor issues. *That duck has Parkinson's*, he thought.

He told Webster this after the seminar, and Webster laughed, Smeyne recalls. "He said, 'Well, it's a sick bird.'" But Smeyne was curious about the neural mechanisms underlying the duck's abnormal behavior.

He wondered if healthy ducks infected with H5N1 in the lab would show Parkinson's-like neurodegeneration. In St. Jude's biosafety level 3 lab, he and his colleagues infected ducks with the virus, then sacrificed the birds and removed their brains, storing them in formaldehyde for three weeks to kill the active virus.

When Smeyne began to dissect the once-infected duck brains, he focused on a region called the substantia nigra, which is often damaged in Parkinson's patients. "When I opened it up, when I cut the brain, the substantia nigra was devastated. All the neurons were completely gone," Smeyne says. He went back to Webster, he recalls, and said, "I wasn't wrong. Your duck does have Parkinson's disease."

Because the bird had had the flu, Smeyne wondered whether there was a connection between the viral infection and the extensive neurodegeneration he observed. He asked Webster about the symptoms experienced by people infected with H5N1. Webster's answer—inflammation of the brain that leads to tremors and other motor malfunctions—didn't sound like "full-blown Parkinson's disease," Smeyne says, "but it was parkinsonism," a subset of symptoms of the disease.

Looking into the literature, Smeyne found more hints of influenza's ability to damage the brain. One of the earliest links between influenza and neural dysfunction was a correlation between the 1918 Spanish flu, caused by a subtype called H1N1, and an epidemic of Parkinson's a few decades later. In the 1940s and early 1950s, diagnoses of the neurodegenerative disease appeared to increase abruptly, from 1–2 percent of the US population to 2.5–3 percent, then fell back down to 1–2 percent, Smeyne says. "Basically, 50 percent more people in those years got Parkinson's."

The evidence to suggest that influenza infection caused the neurodegenerative disorder was tenuous, to say the least, but the correlation was enough for Smeyne to investigate further. With his colleagues, he shot nonlethal doses of H5N1 or H1N1 up the noses of six- to eight-week-old mice, then tracked how the viruses spread through the animals' nervous systems. The results were startling, he says: some viruses weren't blocked from entering the brain by the blood-brain barrier—a semipermeable layer of cells that separates the central nervous system from the body's circulation. H5N1, for example, could easily infiltrate nerve cells in the brain and kill them, and it appeared to especially target the dopamine-producing neurons in the substantia nigra.[1] And while the H1N1 flu strain couldn't cross the blood-brain barrier, it still caused central nervous system immune cells called microglia to flow into the substantia nigra and the hippocampus, causing inflammation and cell death in the area.[2]

"So these were two different flus, two different mechanisms, but the same effect in a sense," says Smeyne, who moved to Thomas Jefferson University in Philadelphia in 2016. "They were inducing inflammation and death in the parts of the brain that we see degenerate in Parkinson's disease."

Smeyne's experiments aren't the only ones to suggest that viral infections can contribute to neurodegenerative disorders, and the connection is not limited to influenza. Several different viruses, including measles and herpes, can give rise to symptoms of multiple sclerosis (MS) in rodents, for example.[3] And levels of herpesvirus are higher in the brains of people who died from Alzheimer's than in those without the disease,[4] while some HIV patients develop dementia that appears to be associated with the infection.

"Viruses are often ignored in relation to neurodegenerative diseases," Yale University neurobiologist Anthony van den Pol tells *The Scientist*. "That's in part because there's no clear sign that a virus causes a neurodegenerative disease. But it might."

Invading the brain

As far back as 1385, doctors in Europe recorded connections between influenza infection and psychosis. That link between the flu and the brain became much more apparent during and after the 1918 Spanish flu epidemic. More direct evidence for the virus-brain link came in the 1970s, when researchers led by Eugenia Gamboa, then a neurologist at Columbia University, and colleagues found viral antigens in the brains of deceased people who had been afflicted with a condition known as encephalitis lethargica.[5] Having symptoms such as fever, headache, and double vision, encephalitis lethargica was associated with—and, some thought, caused by—the 1918 Spanish flu, and researchers speculated that the condition could be a precursor to Parkinson's symptoms. Then, in 1997, a team of scientists reported that rats exposed to Japanese encephalitis virus developed a disease with symptoms similar to human Parkinson's disease.[6]

But the connection between viral infection and brain disease has been hotly contested. And when researchers from the Armed Forces Institute of Pathology in Washington, DC, used PCR to look for fragments of the H1N1 genome in the preserved brain tissue of victims of encephalitis lethargica in the early 2000s, they found no signs of the virus.[7]

Such was the state of research when Smeyne uncovered the severe Parkinson's-like brain damage in the H5N1-infected ducks. No one had directly tested the virus's ability to cause Parkinson's disease until he infected mice with H5N1 and documented severe damage to the substantia nigra. His results also revealed a possible pathway for the virus to spread from the body into the brain. The substantia nigra, Smeyne says, wasn't the virus's initial target; it infected neurons in the gut first. "Then, the virus went into the vagus nerve and basically used the vagus nerve as a back door into the brain." (See illustration on opposite page.)

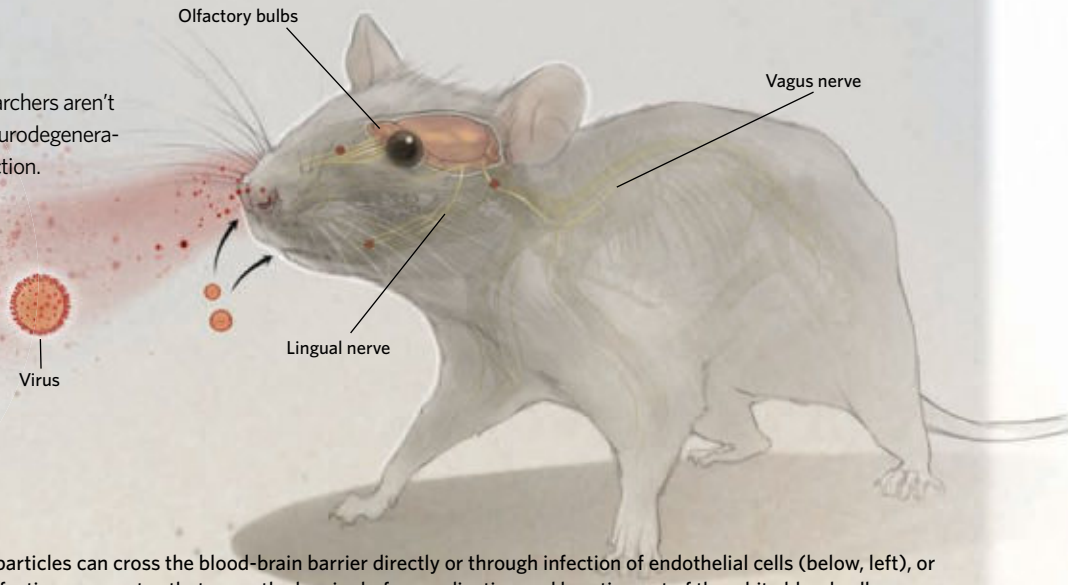
> They were inducing inflammation and death in the parts of the brain that we see degenerate in Parkinson's disease.
>
> —Richard Smeyne, Thomas Jefferson University

The pattern is strikingly similar to how Parkinson's disease appears to work its way through the human body, Smeyne says. According to a widely accepted hypothesis first proposed by German neuropathologist Heiko Braak in 2003, Parkinson's disease starts in the gut, manifesting as digestive issues, and then moves into the brain. "The progression of the disease from the gut to the forebrain, that takes place over maybe 25 or 30 years in a human," Smeyne says. But mice live much shorter lives. In the rodents, the flu virus can travel the same course and create signs of Parkinson's in a few weeks, he notes. And as Smeyne and his colleagues found with H1N1-infected mice, viruses unable to make it into the brain can still play a part in neurodegeneration, by triggering severe inflammation.

Some research has failed to find a link between viral infection and damage to the brain, however. For example, when researchers at the US Centers for Disease Control and Prevention in Atlanta, Georgia, studied the effects of the influenza strain that caused the 1918 Spanish flu epidemic, they didn't see any signs of inflamma-

VIRUSES ON THE BRAIN

Viral infections might cause brain damage. Researchers aren't exactly sure whether the injuries play a role in neurodegenerative diseases, but some studies suggest a connection.



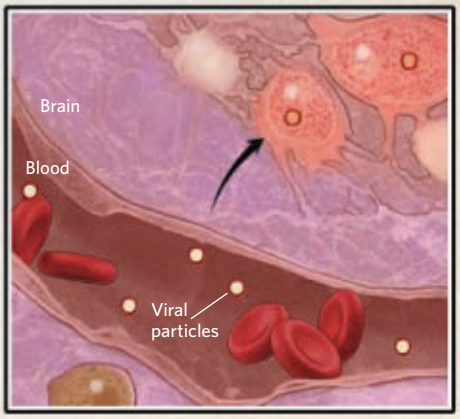
ROUTES OF PASSAGE

Some viruses can enter the body through the nose and mouth and move to the brain by replicating and spreading through the olfactory bulbs; the lingual nerve, which runs down the jawline and into the tongue; or the vagus nerve, which travels through the neck and thorax to the stomach.

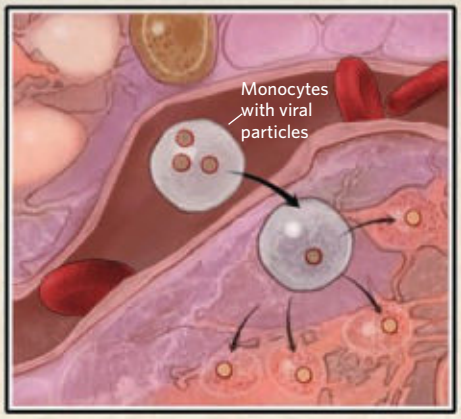
CROSSING BLOOD-BRAIN BARRIER

When interacting with the nervous system, viral particles can cross the blood-brain barrier directly or through infection of endothelial cells (below, left), or they can use a Trojan horse approach (center), infecting monocytes that cross the barrier before replicating and bursting out of the white blood cells once inside the brain. Alternatively, some viruses do not cross the blood-brain barrier but invoke an immune response that may spur cytokines or chemokines to breach the divide (right).

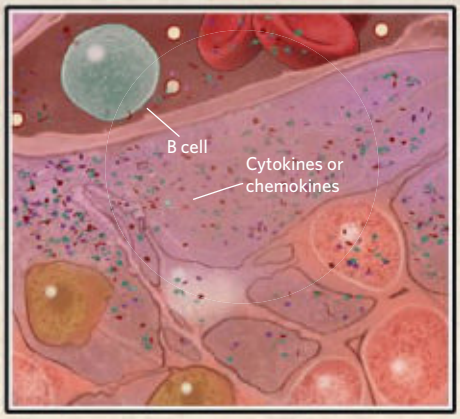
DIRECT CROSSING



TROJAN HORSE



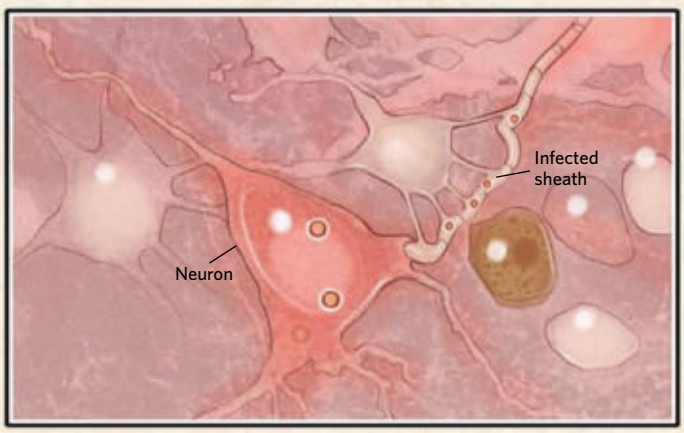
IMMUNE RESPONSE



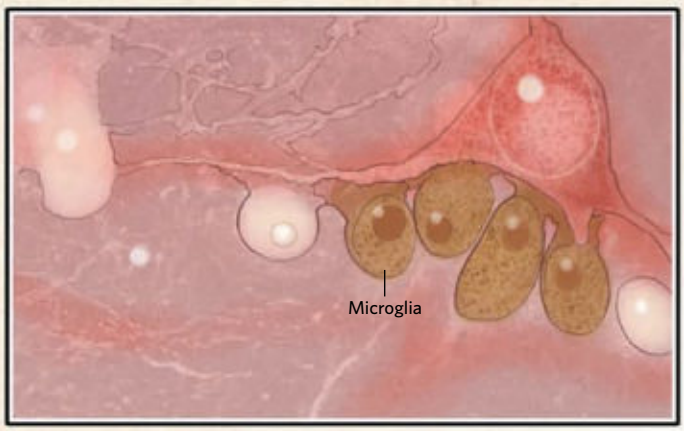
BRAIN DAMAGE

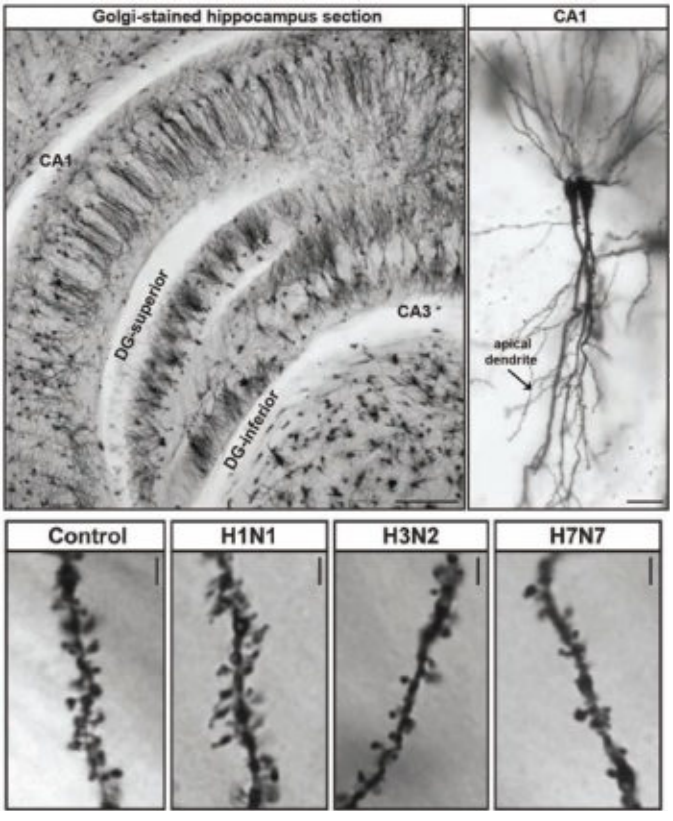
Once inside the brain, viruses can infect cells or their myelin sheaths and kill them (below, left). Viruses don't necessarily have to enter the brain to cause damage, though. They can also spark an immune response that activates microglia, which then consume otherwise healthy neurons (right).

DIRECT INFECTION OF NERVE CELLS



MICROGLIA CONSUMPTION





SMOOTHER NEURONS: Tiny bumps called dendritic spines are important structures for neuronal communication, receiving messages from other nerve cells in the brain. Mice infected with H3N2 and H7N7 experienced a drop in the number of these bumps, researchers recently showed. The number of bumps did not decrease following infection with H1N1.

tion in the brains of infected mice.[8] "More work is needed to look for a link between viral infection and neurodegenerative diseases," says microbiologist Terrence Tumpey, who coauthored that study.

Smeyne suspects the link between viruses and brain-centered diseases could be more subtle. To further explore the relationship between H1N1 and Parkinson's, he and his colleagues gave a toxin called MPTP to mice that had recovered from infection with the virus. The chemical was a byproduct of a bad batch of synthetic heroin cooked up in the 1970s that led users to develop Parkinson's disease. The MPTP-treated mice that had been infected with H1N1 developed signs of the disease and lost 25 percent more neurons in the substantia nigra than uninfected mice treated with the toxin or mice infected with the virus but not exposed to MPTP.[9]

"That suggested to us," Smeyne says, "that while the H1N1 infection alone did not cause Parkinson's, it primed the nervous system to be sensitive to other things that would."

A broader link between viruses and neurodegeneration

The flu-Parkinson's connection is not the only link researchers have made between viruses and neurological problems. In the late 1980s and early 1990s, researchers found that mice infected with viruses such as measles and herpes suffered the same kind of damage to their oligodendrocytes—cells in the central nervous system that produce myelin, the insulating fatty sheath wrapped around the axons of neurons—as patients with MS do. It's not clear whether the viruses invaded the oligodendrocytes directly, or simply provoked the mice's immune systems to attack the cells, but the end result was demyelination of neurons, van den Pol says, just like what is seen in MS patients.

One of the virus strains that induced MS symptoms in mice was herpesvirus 6, which has also been associated with the development of Alzheimer's disease. Tentative links between viral infections and Alzheimer's have been documented over the past few decades, but the possibility reemerged last year when Joel Dudley of the Icahn School of Medicine at Mount Sinai and colleagues, reviewing data from brain banks and published studies, found that patients with Alzheimer's disease had elevated levels of viruses, such as human herpesvirus 6 and human herpesvirus 7, in four key brain regions. Based on genetic and proteomic data, the researchers also found that human herpesvirus 6 may induce gene expression that spurs the development of the protein amyloid β, which forms plaques that are hallmarks of Alzheimer's disease.[4]

Such a correlation doesn't prove that viruses cause the disease, but it does suggest that pathogens may play a part in neurodegenerative diseases after all, Dudley says. "One thing that's different today compared to previous musings on the pathogen hypothesis is that we have much more powerful sequencing methods that can take a more unbiased look at the microbial DNA/RNA landscape of brain tissue," he says. "We are likely to get an even better look at this question as we apply long-read sequencing technology and single-cell sequencing technology to brain tissue samples." (See "Do Microbes Trigger Alzheimer's Disease?" *The Scientist*, September 2017.)

HIV is another virus researchers suspect could cause Alzheimer's-like or Parkinson's-like brain damage. In the 1990s, scientists showed that HIV could traverse the blood-brain barrier, and subsequent studies revealed that when the virus infiltrates the brain, it spurs neuronal death and a loss of synaptic connections.[10] More recently, physicians have started reporting on patients with HIV who develop dementia and a loss of brain matter that mirrors what's seen in Alzheimer's patients, Sara Salinas, a pathologist and virologist at the University of Montpellier in France, and colleagues explain in a 2018 review article in *Frontiers in Cellular Neuroscience*.[11] More-recent studies show that HIV patients develop plaques of amyloid β. And, Smeyne says, HIV patients can also develop slowness in movement and tremors.

A closer look at modes of neuronal communication may give some clues to the development of the neurodegenerative diseases. Earlier this year, two groups of scientists reported that, in addition to using electrical and chemical signals to talk to one another, neurons employ extracellular vesicles carrying messenger RNAs.[12,13] The structure of these vesicles is reminiscent of the way HIV and other retroviruses build protective shells called capsids that ferry the virus's genetic mat-

KORTE/HOSSEINI, DATA PUBLISHED IN HOSSEINI ET AL., *JNS*, 2018

erial from cell to cell, says Jason Shepherd, a neuroscientist at the University of Utah and coauthor of one of the studies. The genes encoding the vesicles could possibly be holdovers from past infections, he suggests, and these virus-mimicking capsids could be harboring toxic proteins, such as amyloid β, and spreading them throughout the brain.

"Clearly, viruses influence the brain," Shepherd says, but the nature of that relationship remains unclear.

> In science we often think of some cause and effect being often milliseconds. Here, you're talking about decades.
>
> —Anthony van den Pol, Yale University

Forgetfulness lingers

One challenge in understanding how the brain responds to viral infection is that the effects can linger long after our immune system has cleared the infection from our bodies. Earlier this year, for example, Martin Korte at the Technische Universität Braunschweig in Germany and colleagues reported that the brains of mice infected with certain strains of the flu virus suffered memory deficits even after they'd seemingly recovered. It turned out that their brains were full of microglia even 30 to 60 days after infection first took hold.[14] The microglia levels can start to return to the normal range around 60 days post infection, with the neurons in the young mice recovering completely, along with the animals' memory performance. Still, the microglia numbers can stay elevated for up to 120 days, Korte tells *The Scientist*; that's equivalent to more than 10 years in human time.

Van den Pol says such a lag is exactly why scientists have trouble accepting that viruses could cause neurodegenerative diseases. "In science we often think of some cause and effect being often milliseconds," he says. "Here, you're talking about decades. The virus goes in and then maybe decades later it can cause some potentially serious neurodegeneration"—such a long-term link is hard to demonstrate.

If the connection between viral infections and neurological problems can be more concretely established, researchers may be able to develop ways to mitigate the neurological effects, van den Pol says. Understanding how infections trigger the immune system, for example, could lead to ways to downregulate glia-driven inflammation in hopes of preventing long-term damage, he suggests.

In the meantime, Smeyne notes that vaccination for the flu—or at the very least, taking Tamiflu if a person gets infected—might help prevent neurological complications of influenza infection. He and his colleagues tested this approach in mice after their results revealed the link between flu, the MPTP toxin, and Parkinson's disease. The team gave a group of mice an H1N1 vaccine 30 days before infecting the animals with the virus. Another group of mice were treated with Tamiflu for the week after they were infected. Both groups of mice were allowed to recover before being given a low dose of MPTP. While control mice that did not receive either the vaccine or flu treatment developed Parkinson's-like symptoms, treated mice developed no neurodegenerative effects. "We had protected against [Parkinson's-like symptoms] just by early treatment or prophylactic treatment with the vaccine," Smeyne says.

It's further evidence to support the idea that viral infections can damage the brain, Smeyne says, but there's still no slam-dunk study that demonstrates a virus can cause Parkinson's, or Alzheimer's, or any number of other neurological disorders. "I do like the idea that viruses can cause a lot of different brain diseases as a hypothesis," van den Pol says. "But I also respect the fact that it really is a hypothesis." ■

References

1. H. Jang et al., "Highly pathogenic H5N1 influenza virus can enter the central nervous system and induce neuroinflammation and neurodegeneration," *PNAS*, 106:14063–68, 2009.
2. S. Sadasivan et al., "Induction of microglia activation after infection with the non-neurotropic A/CA/04/2009 H1N1 influenza virus," *PLOS ONE*, 10:e0124047, 2015.
3. U.G. Liebert, V. ter Meulen. "Virological aspects of measles virus-induced encephalomyelitis in Lewis and BN rats," *J Gen Virol*, 68:1715–22, 1987.
4. B. Readhead et al., "Multiscale analysis of independent Alzheimer's cohorts finds disruption of molecular, genetic, and clinical networks by human herpesvirus," *Neuron*, 99:64–82.e7, 2018.
5. E.T. Gamboa et al., "Influenza virus antigen in postencephalitic parkinsonism brain. Detection by immunofluorescence," *Arch Neurol*, 31:228–32, 1974.
6. A. Ogata et al., "A rat model of Parkinson's disease induced by Japanese encephalitis virus," *J Neurovirol*, 3:141–47, 1997.
7. S. McCall et al., "Influenza RNA not detected in archival brain tissues from acute encephalitis lethargica cases or in postencephalitic Parkinson cases," *J Neuropathol Exp Neurol*, 60:696–704, 2001.
8. J.C. Kash et al., "Genomic analysis of increased host immune and cell death responses induced by 1918 influenza virus," *Nature*, 443:578–81, 2006.
9. S. Sadasivan et al., "Synergistic effects of influenza and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) can be eliminated by the use of influenza therapeutics: Experimental evidence for the multi-hit hypothesis," *NPJ Parkinsons Dis*, 3:18, 2017.
10. S. Peudenier et al., "HIV receptors within the brain: a study of CD4 and MHC-II on human neurons, astrocytes and microglial cells," *Res Virol*, 142:145–49, 1991.
11. G. Canet et al., "HIV neuroinfection and Alzheimer's disease: Similarities and potential links?" *Front Cell Neurosci*, 12:307, 2018.
12. E.D. Pastuzyn et al., "The neuronal gene Arc encodes a repurposed retrotransposon gag protein that mediates intercellular RNA transfer," *Cell*, 172:P275–88.E18, 2018.
13. J. Ashley et al., "Retrovirus-like gag protein Arc1 binds RNA and traffics across synaptic boutons," *Cell*, 172:P262–74.E11, 2018.
14. S. Hosseini et al., "Long-term neuroinflammation induced by influenza A virus infection and the impact on hippocampal neuron morphology and function," *J Neurosci*, 38:3060–80, 2018.

The Drugs that Put Us Under

Patterns of brain activity under general anesthesia reveal new ways to monitor and treat patients.

BY EMERY N. BROWN AND FRANCISCO J. FLORES

Before the advent of general anesthesia in the mid-19th century, surgery was a traumatic experience for everyone involved—the patient, of course, but also the medical staff and anyone who happened to walk by the surgery room and could hear the screams. The practice of putting patients in a reversible coma-like state changed surgery to a humane and often life-saving therapy. Because general anesthesia was such a game changer in medicine, these drugs were implemented in the operating room many decades before researchers understood how they worked.

Nowadays, researchers and anesthesiologists know much more about the mechanisms underlying the effects of anesthetic drugs and how they produce the profound change in behavioral state that implies a total lack of perception. Anesthetics primarily act on receptors located in the brain and produce oscillations in the brain's circuits, leading to a state of consciousness that it is much more similar to a coma than to sleep. Anesthesiologists typically used vital signs, such as heart rate and blood pressure, to assess the adequacy of the anesthetized state and the processing of pain signals. However, the effects of anesthetics in brain circuits result in conspicuous oscillations in the brain's electrical activity, which prompted the addition of electroencephalography (EEG) measurements to monitor the brain state of an anesthetized patient.

Starting in the 1990s, researchers developed algorithms to consolidate the signals recorded from several EEG electrodes into a single number that provided a simplified measurement of arousal level. More recently, direct observation of the raw EEG signals and their breakdown in time by frequencies, the spectrogram, is gaining traction for monitoring patients during general anesthesia. Learning to interpret the raw brain activity and its spectrogram, rather than relying on a single-number summary, has allowed anesthesiologists to assess how different anesthetics affect brain activity and produce the anesthetic state.[1]

By tracking brain activity during general anesthesia, researchers are also uncovering a wealth of new information that helps them understand the biological basics of how brain function is altered in an anesthetized state. In addition, general anesthesia has provided new options to treat a range of ailments, from sleep problems to depression.

Ether follies

Humans have practiced surgical procedures for thousands of years, and the search for ways to minimize pain and discomfort during invasive interventions is probably just as old. Wine and opium are among the first substances known to have been tried. Opium is a potent analgesic and mild sedative, and the ethyl alcohol in wine is a sedative, but neither of these drugs succeeds in making patients unaware of the trauma their bodies undergo during surgery.

In the first half of the 19th century, dentists stumbled upon two promising leads: nitrous oxide, which soon after its discovery became widely used in the US and Europe to perform tooth extractions, and chloroform, which was used for both veterinary and human surgeries for a few decades before it fell out of favor due to safety concerns. In the 1840s, Boston dentist William Morton was looking for ways to perform pain-free dental procedures and considered using nitrous oxide. But, Charles Jackson, a chemist at Harvard Medical School, advised him to try another option: ether.

At that time, it was common in academic and other social circles to hold parties, called "ether follies," where people would inhale ether for its exhilarating properties. Jackson had seen a man sustain a considerable leg injury during one such escapade. The man, who had been high on ether, showed no signs of pain. Morton took Jackson's advice and proceeded to experiment with ether on himself and his dog, and subsequently performed several dental procedures on his patients after administering the drug to them.

General anesthesia is a drug-induced reversible state defined by five end points:

- **Unconsciousness**, lack of awareness of sensory input
- **Analgesia**, lack of pain
- **Akinesia**, lack of movement
- **Amnesia**, lack of recall
- **Physiological stability**, the preservation of normal levels of all vital physiological functions, such as respiration, heart rate, blood pressure, and temperature

E.N. Brown et al., "General anesthesia, sleep and coma," *New Engl J Med*, 363:2638–50, 2010.

Morton contacted Harvard Medical School surgeon Henry Bigelow, and together they organized what would become known as the first public demonstration of surgery performed under general anesthesia. On October 16, 1846, in the operating theater now known as the Ether Dome at Massachusetts General Hospital, John Collins Warren, the founding dean of Harvard Medical School and the hospital's chief surgeon, removed a tumor from the neck of patient Edward Gilbert Abbott, while Morton held a glass flask containing an ether-soaked sponge that spouted ether vapor through a glass tube that was attached to Abbott's nose. Several prominent surgeons and physicians watched from the viewing area of the theater.

Warren performed the surgery with the patient showing minimal signs of pain;[2] at the end of the procedure, Warren famously declared: "Gentlemen, this is no humbug." Afterward Abbott did state that he had experienced sensations, though not pain, during surgery. The following day, flaws in ether administration were corrected, and a second tumor removal patient declared that she had felt and known nothing. Surgeries using ether-induced anesthesia were soon performed at nearby hospitals, and within a couple of months it began to change medical practice the world over.

The modern definition of general anesthesia requires that five endpoints are achieved. (See Box on page 39.) Ether provides all of these endpoints to some degree. Most modern inhaled anesthetics, such as isoflurane, desflurane, and sevoflurane, are chemical derivatives of ether but are more potent, less flammable, and are delivered using modern vaporizers and techniques. Improvements to the hypodermic needle achieved in the second half of the 19th century made possible the development of intravenous anesthesia, and physicians began combining anesthetic drugs with opioids to more effectively achieve analgesia. Later, muscle relaxants were added to ensure immobility.

The modern practice of general anesthesia, known as balanced anesthesia, uses combinations of drugs with the goal of distancing the patient from the trauma the body is undergoing while minimizing side effects. Recently, researchers have detailed the mechanism underlying the action of modern anesthetics, identifying links between the neural receptors on which the drugs act and patterns of overall brain activity that are linked to changes in neuronal firing. These connections allow anesthesiologists to track brain activity patterns during general anesthesia to improve patient experiences and outcomes, as well as to learn more about how the anesthetized brain functions.

How is general anesthesia achieved?

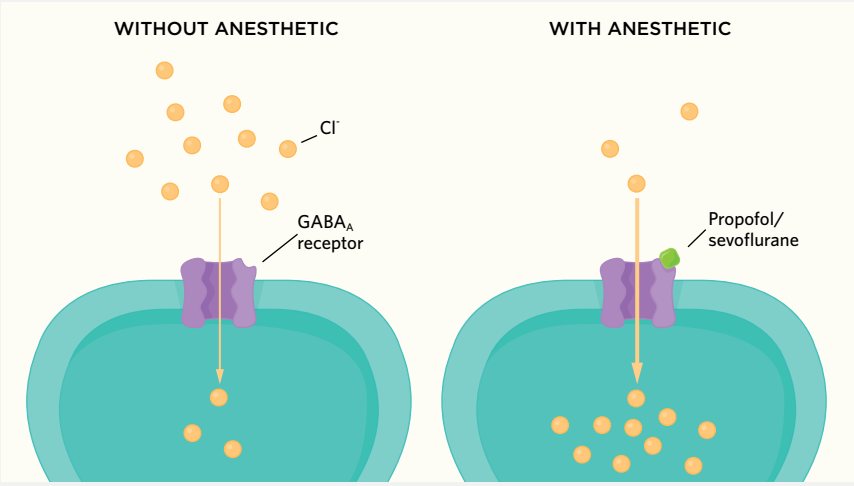
One of the most conspicuous features of general anesthesia is the profound state of unconsciousness that it produces. Up until the 1980s, the prevailing hypothesis on how the unconscious state was achieved was influenced by the observation that anesthetic potency directly correlated with solubility in olive oil, suggesting a hydrophobic site of action such as the lipid bilayer membranes of neurons. Researchers speculated that the drugs disrupted normal membranes function and prevented the conduction of action potentials. This idea was known as the lipid

ANESTHETICS AND NEURONAL RECEPTORS

General anesthetics work by altering the activity of specific neurons in the brain. One main class of these drugs, which includes propofol and the ether-derivative sevoflurane, work primarily by increasing the activity of inhibitory $GABA_A$ receptors, while a second class that includes ketamine primarily blocks excitatory NMDA receptors.

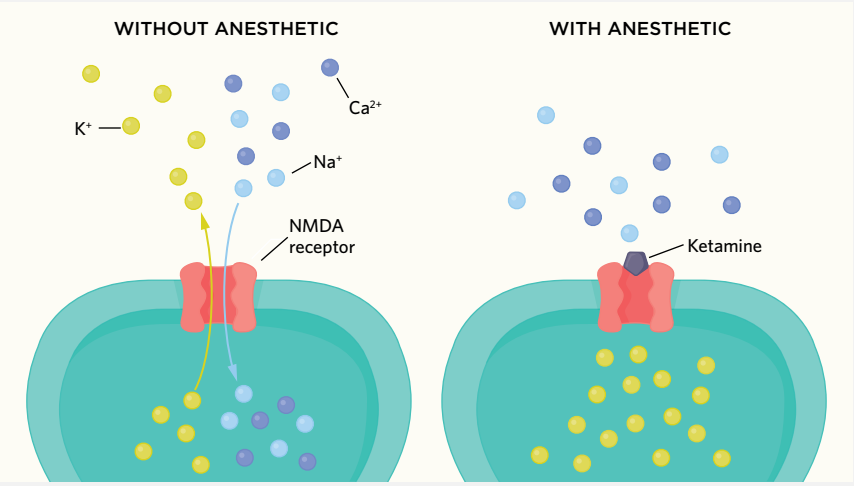
PROPOFOL AND SEVOFLURANE

The $GABA_A$ receptor is a channel that allows chloride ions to flow into the neuron, decreasing the voltage within the cell relative to the extracellular space. Such hyperpolarization decreases the probability that the neuron will fire. Propofol and sevoflurane increase the chloride current going into the cell, making the inhibition more potent.



KETAMINE

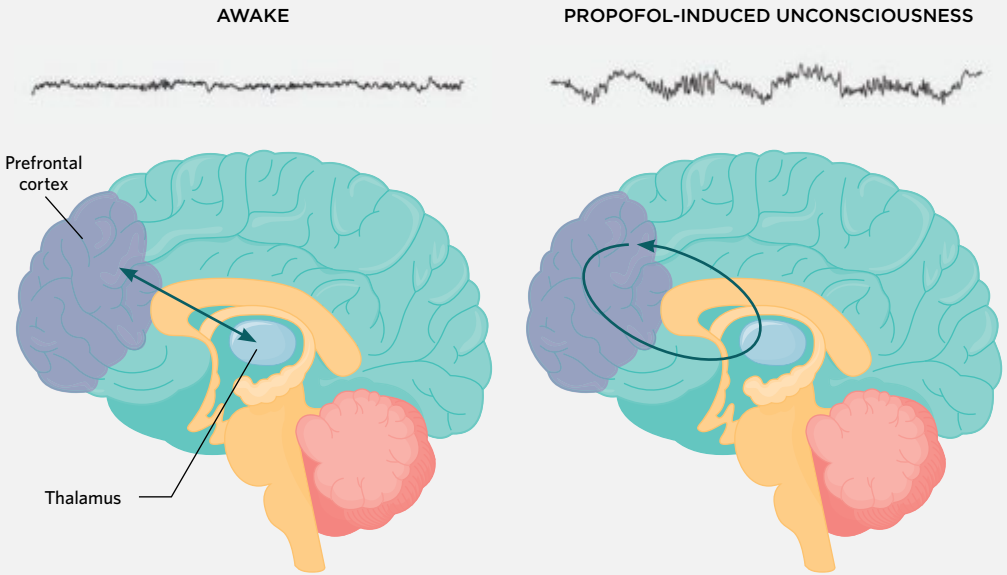
The NMDA receptor allows sodium and calcium ions to flow into the cell, while letting potassium ions out, increasing the voltage within the cell relative to the extracellular space and increasing the probability of neural firing. Ketamine blocks this receptor, decreasing its excitatory actions.

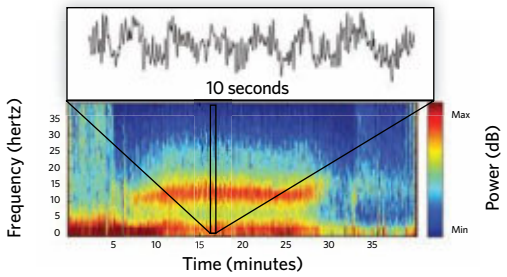
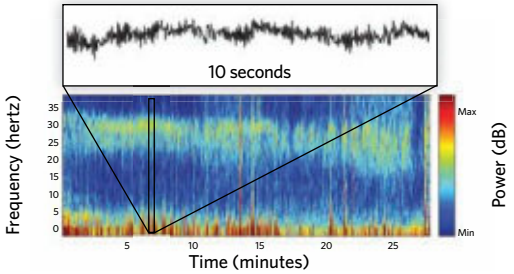


OSCILLATIONS IN THE ANESTHETIZED BRAIN

Anesthetics' interactions with neural receptors alter how neurons work, and as a consequence, how different brain regions communicate. These alterations manifest as highly structured oscillations in brain activity that are associated with the dramatic behavioral changes characteristic of general anesthesia.

The changes in brain activity can be readily observed using electroencephalogram (EEG) electrodes on the scalp. Slow oscillations of less than 1 Hz are seen in the brains of patients treated with any of the anesthetics in clinical practice. In addition, anesthetics elicit oscillations of other frequencies, such as the alpha oscillations observed following propofol administration (illustrated at right; see online for an animation of the activity patterns). These oscillations are directly related to the anesthetized state, and can be used to monitor level of unconsciousness.



DRUG	PRIMARY RECEPTOR	ANESTHETIC-SPECIFIC OSCILLATIONS	EEG READOUTS
Propofol	$GABA_A$	Alpha (8–12 Hz) oscillations result from synchronization of neural activity in the cortex and thalamus.	
Ketamine	NMDA	Beta/gamma (25–50 Hz) oscillations, perhaps due to an increased spiking rate of excitatory neurons in the cortex following ketamine-induced reduction of activity in nearby inhibitory neurons	

INTERPRETING THE EEG: The colored graphs, known as spectrograms, aid in visualizing the frequency and temporal dynamics of the oscillations by assigning hot colors to frequencies that are particularly prominent in the raw signal (black lines above spectrograms). Clinicians are beginning to use both types of readouts to monitor depth of anesthesia.

hypothesis. Then, Nicholas Franks and William Lieb of Imperial College London showed that the true targets of anesthetic drugs were neuronal receptors embedded in the membrane.[3]

Neuronal receptors regulate the probability that neurons will fire action potentials, often by acting to control channels for specific ions to go into and out of nerve cells. Activating excitatory receptors increases a neuron's firing potential, while activating inhibitory receptors decreases it. Hence, anesthetic drugs could, in principle, be grouped into two main classes: those that activate inhibitory receptors and those that inactivate excitatory receptors. (See illustration on page 40.)

Inhaled ether derivatives and intravenous propofol, the most widely used anesthetic drug, bind to the inhibitory $GABA_A$ receptor. Under normal, physiological conditions, the receptor is activated by gamma-aminobutyric acid (GABA) released from inhibitory neurons, and it allows the flow of chloride ions into the cell, dropping the relative voltage of the neuron's interior and thereby decreasing the probability of firing an action potential. Anesthetic drugs that target this receptor act as agonists to promote the influx of chloride ions, further suppressing the cell's ability to fire.

Other anesthetics such as ketamine, which was synthesized in 1962, and nitrous oxide block the channel of the N-methyl D-aspartate (NMDA) glutamate receptor. Normally activated by the neurotransmitter glutamate released from excitatory neurons, the NMDA receptor allows the flow of potassium ions out of the cell and calcium and sodium ions in, increasing the relative voltage of the neuron's interior and thereby increasing the probability of firing an action potential. Anesthetic drugs that target this receptor act as antagonists to block these ions fluxes, decreasing the ability of the cell to fire.

Knowing the actions of anesthetic drugs on the receptors still does not fully explain how unconsciousness occurs, however. Both GABA and NMDA receptors are found on the excitatory and inhibitory neurons that make up neuronal circuits. The function of these circuits and their relationship to behavior can be understood within the framework of systems neuroscience: the changes in ion fluxes produced by anesthetic binding to receptors dramatically alters neuronal activity across the brain, eliciting highly structured oscillations. In humans, these oscillations are readily visible in the EEG readouts. They are of high amplitude and lie within well-defined frequency bands lower than those of unstructured, low-amplitude oscillations seen in the brain of a conscious person.

Anesthetic-induced oscillations dramatically alter when neurons can spike, and impede communication between brain regions that play a role in consciousness.

The observed waves depend critically on which receptors are bound and how the targeted regions are connected to other areas of the brain. The oscillations change systematically with anesthetic drug class, drug dose, and patient age. For example, alpha oscillations (8–12 Hz) produced by GABAergic anesthetics depend critically on excitatory and inhibitory connections between the thalamus and the cortex.[4] The beta/gamma oscillations (15–50 Hz) produced by ketamine[5] might depend on blocking the NMDA receptors of inhibitory and excitatory neurons in the cortex, while the slow oscillations produced by both GABA agonists[6,7] and NMDA antagonists might depend on inhibition of the brainstem and its projections to the thalamus and cortex (See illustration on page 41.) In elderly patients the oscillations have lower amplitudes across all frequency bands. These oscillations also dramatically alter when neurons can spike, and impede communication between brain regions that play a role in consciousness.

The characteristics of the oscillations produced by anesthetics suggest that they are a significant part of the mechanism of anesthetic action, and explain how the brain state of a patient under general anesthesia can be reliably tracked using the EEG. However, monitoring brain activity has not been standard in anesthesiology practice. The early attempts to use EEG as an additional piece of information to monitor patients and inform the dose and rate of anesthetic delivery focused on developing an index that would provide a single readout of anesthetic state. However, EEG activity observed during general anesthesia is different across people of different ages, and these indices can be misleading when used in children or elderly people. Furthermore, EEGs captured under general anesthesia differ across drugs, and aggregated indices cannot take these differences into consideration.

For those reasons, in the last few years anesthesiologists have begun to monitor EEG readouts of brain signals during procedures involving general anesthesia. The oscillation patterns for common anesthetics are identifiable to the trained eye, and the assessment of their frequencies can be performed in real time with computer aid, providing a more nuanced picture of a patient's brain state. This has allowed clinicians to manage anesthetic drug dosing in a more nuanced way and to reduce the amount of anesthesia required to achieve the same anesthetic state.[8] As we understand more about how anesthetics work, and gain more experience directly observing EEGs generated during anesthesia, the practice will continue to be improved.

General anesthesia as treatment

In the last several decades, research on brain activity patterns and general anes-

thesia has yielded insights not only into the effects of anesthetics themselves, but also into neural processes related to conditions in which brain oscillations are altered, such as aging[9] and pathological conditions including autism.[10] In addition, the anesthetics methylhexital and alfentanil have proven useful in stimulating seizure activity in the brains of epilepsy patients, helping neurosurgeons to precisely locate the problematic tissue to resect. Advances in this field also point to the possibility of using anesthesia as a treatment for a handful of brain-related conditions.

This concept is not entirely new. For example, during deep general anesthesia and coma, an EEG pattern known as burst-suppression is observed. This pattern consists of bursts of electrical activity alternating with flat periods of inactivity. Neurologists frequently use anesthetics to induce a medical coma in patients with intractable seizures or raised intracranial pressure to arrest the seizure activity or decrease brain swelling. The comatose state is maintained by observing EEGs and titrating the anesthetic infusion rate to maintain a specific number of bursts per minute. This procedure ordinarily requires a human to assess the burst-suppression rate and manually adjust the anesthetic dose, but our research suggests that full automation of the process is possible.[11]

Another area where insights into the neural mechanisms of anesthesia might improve treatment options is sleep. Sleep has two main stages: rapid eye movement (REM) and non-REM sleep. Non-REM sleep is a state of profound unconsciousness that scientists consider to be most important for achieving properly restful sleep. Non-REM sleep is characterized by two main oscillatory patterns in brain activity: sleep spindles (10–15 Hz) and slow oscillations. Most sleep aid medications do not produce oscillatory brain activity that closely resembles the activity observed during natural sleep. However, the anesthetic dexmedetomidine, which affects circuits in the brainstem that are involved in control of wakefulness,[12] produces EEG patterns that are similar to those that occur during non-REM sleep.[13] Clinical trials are currently under way to test its efficacy as a sleep aid.

Anesthetics such as ketamine, xenon, and nitrous oxide have already been shown to have acute antidepressant effects. There is evidence that other anesthetics, such isoflurane and propofol, when dosed to the level of producing burst suppression indicative of a medical coma, have long-lasting antidepressant effects without the short-term cognitive impairment and amnesia associated with electroconvulsive therapy. Ketamine might exert its antidepressant effect by increasing the number of synaptic receptors and other synaptic signaling proteins, and even increasing the number of synapses in the brain.[14]

More than 170 years after its first public demonstration, general anesthesia allows millions of painless surgeries to be performed daily across the world and is still the bedrock on most surgical procedures are performed. At the same time, the study of the effects of anesthetics in brain function is opening many exciting opportunities for the development of novel anesthetic paradigms and for research on other questions in clinical neuroscience. ■

The brain state of a patient under general anesthesia can be reliably tracked using the EEG.

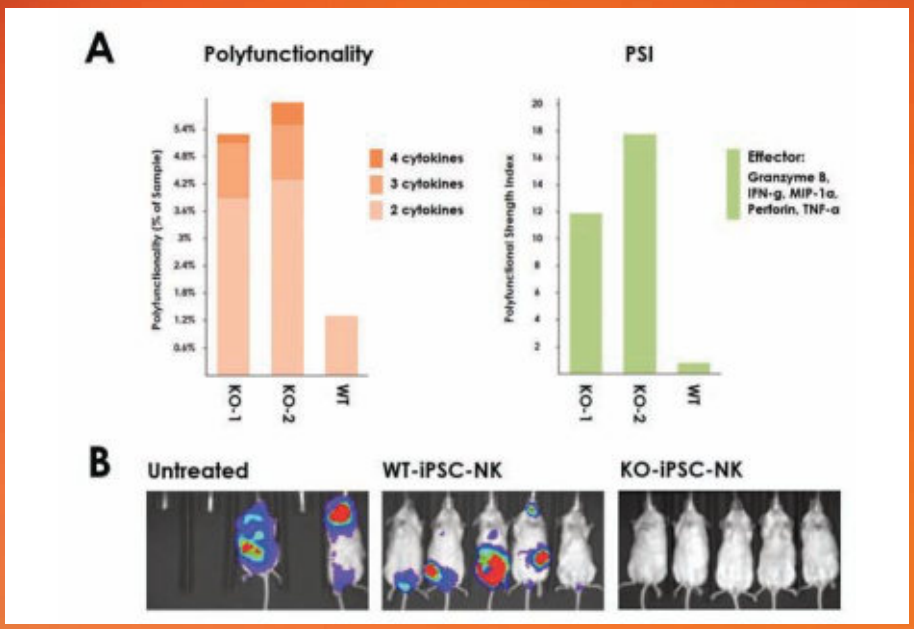
Emery N. Brown is the Warren M. Zapol Professor of Anaesthesia at Harvard Medical School, a practicing anesthesiologist at Massachusetts General Hospital, and the Edward Hood Professor of Medical Engineering and Computational Neuroscience at MIT. Francisco J. Flores is an instructor in Anaesthesia at Massachusetts General Hospital and Harvard Medical School.

References

1. P.L. Purdon et al., "Clinical electroencephalography for anesthesiologists: Part I: Background and basic signatures," *Anesthesiology*, 123:937–60, 2015.
2. H.J. Bigelow, "Insensibility during surgical operations produced by inhalation," *Boston Med Surg J*, 35:309–17, 1846.
3. N.P. Franks, W.R. Lieb, "Do general anaesthetics act by competitive binding to specific receptors?" *Nature*, 310:599–601, 1984.
4. F.J. Flores et al., "Thalamocortical synchronization during induction and emergence from propofol-induced unconsciousness," *PNAS*, 114:E6660–68, 2017.
5. O. Akeju et al., "Electroencephalogram signatures of ketamine anesthesia-induced unconsciousness," *Clin Neurophysiol*, 127:2414–22, 2016.
6. P.L. Purdon et al., "Electroencephalogram signatures of loss and recovery of consciousness from propofol," *PNAS*, 110:E1142–51, 2013.
7. K.J. Pavone et al., "Nitrous oxide-induced slow and delta oscillations," *Clin Neurophysiol*, 127:556–64, 2016.
8. E.N. Brown et al., "Multimodal general anesthesia: Theory and practice," *Anesth Analg*, 127:1246–58, 2018.
9. P.L. Purdon et al., "The ageing brain: Age-dependent changes in the electroencephalogram during propofol and sevoflurane general anaesthesia," *Br J Anaesth*, 115 (Suppl 1):i46–57, 2015.
10. E.C. Walsh et al., "Age-dependent changes in the propofol-induced electroencephalogram in children with autism spectrum disorder," *Front Syst Neurosci*, 12:23, 2018.
11. M.M. Shanechi et al., "A brain-machine interface for control of medically-induced coma," *PLOS Comput Biol*, 9:e1003284, 2013.
12. V. Breton-Provencher, M. Sur, "Active control of arousal by a locus coeruleus GABAergic circuit," *Nat Neurosci*, 22:218–28, 2019.
13. O. Akeju et al., "Dexmedetomidine promotes biomimetic non-rapid eye movement stage 3 sleep in humans: A pilot study," *Clin Neurophysiol*, 129:69–78, 2018.
14. N. Li et al., "mTOR-dependent synapse formation underlies the rapid antidepressant effects of NMDA antagonists," *Science*, 329:959–64, 2010.

Improving Preclinical Discovery of CRISPR-Engineered Immune Cell Therapies

Natural killer (NK) cells, known for their ability to kill tumor cells, are promising agents for cell-based cancer immunotherapies. CRISPR-Cas9 gene editing can be used to effectively modify the genetic makeup of inducible pluripotent stem cell (iPSC)-derived NK cells towards this end, but there is an urgent need to characterize their potency and efficacy as a preclinical cancer immunotherapy. IsoPlexis' single-cell proteomics system addresses this challenge by connecting each immune cell to the many cytokines it secretes, revealing correlations to in vivo outcome across a range of disease areas.



PSI reveals cell potency, post gene edits, which correlates with in vivo response. (A) CISH-/- knock-out cells showed increased polyfunctional cell subsets (left) and 10x higher PSI (right) compared to the wild type samples. These results indicate that CIS plays a key role in regulating NK cell activation-induced exhaustion and that Notch activation prevents this exhaustion and enables production of functionally hyperactive NK cells. **(B) CISH-/- iPSC-NK demonstrates better anti-tumor activity in vivo.** NSG mice were inoculated with the human leukemia cell line Molm13; mice were subsequently left untreated or were treated with either WT-iPSC-NK or KO-iPSC-NK cells. Thirty-five days after inoculation, two of four untreated mice had died and the other two showed significant tumor load, WT-iPSC-NK mice showed substantial tumor growth, while in CISH KO-iPSC-NK mice tumor growth was nearly absent, consistent with the 10x higher in vitro PSI of CISH KO-iPSC-NK cells. Reference: Zhu et al, ASH 2018.

The Polyfunctional Strength Index (PSI) delivers correlative potency data

The IsoPlexis system identifies which cells are polyfunctional (*i.e.*, those powerful cells that secrete multiple cytokines) and quantitates the cytokine concentrations within each cell. PSI combines these two single-cell metrics to effectively identify highly potent immunotherapies.

PSI reveals cell potency of gene-edited, iPSC-derived NK cells

To study the role of cytokine-inducible SH2-containing protein (CIS) in human NK cell anti-tumor activity, IsoPlexis's systems analyzed the potency of iPSC-derived NK cells with the CISH gene deleted using CRISPR-Cas9 technology. A 10X higher PSI of the gene-edited NK cells was found relative to control wildtype NK cells, driven by increased antitumor cytokine production (*e.g.*, Granzyme B, IFN-γ, MIP-1α, Perforin, TNF-α). Researcher Dr. Dan Kaufman, PI of this study, said, "Our studies have used CRISPR-Cas9 gene editing in human induced pluripotent stem cells to produce natural killer cells with improved antitumor activity. Analysis of these cells using the IsoPlexis system was very valuable to characterize the improved polyfunctional cytokine response that plays a key role in improved activity of these iPSC-derived NK cells."

Increase in PSI of gene-edited NK cells correlates with improved in vivo response of the NK cell therapy

To test CISH$^{-/-}$ human NK cells in vivo, immunodeficient NSG mice were inoculated with the human leukemia cell line, MOLM-13, and split into three groups: untreated mice, mice treated with control wildtype NK cells, and mice treated with the *CISH*-knockout NK cells. The study revealed substantial tumor growth in untreated mice and wildtype NK treated mice, while tumor growth was nearly absent in mice receiving the *CISH*-KO NK treatment. This strongly suggests both a correlation between in vitro PSI and in vivo mouse antitumor activity, as well as therapeutic efficacy.

Conclusion

IsoPlexis' single-cell system improves engineered immune-cell therapy R&D, by providing metrics such as PSI, indicative of improved potency of gene-edited cells, and potentially correlating to in vivo efficacy of engineered cell therapies.

isoplexis

The Literature

GENETICS

Double Trouble

THE PAPER

Y. Yu et al., "Dna2 nuclease deficiency results in large and complex DNA insertions at chromosomal breaks," *Nature*, 564:287–90, 2018.

Few things are as dangerous for a cell as a DNA double-strand break. If both strands of the double helix are severed and left unrepaired, the cell could die at the next round of mitosis.

To protect against such a fate, a suite of DNA-repairing proteins is on standby for when breaks occur. One of them is the evolutionarily conserved enzyme Dna2, which helps prepare broken DNA strands for repair by other proteins and also degrades excess pieces of DNA produced during replication.

To better understand the enzyme's role, Grzegorz Ira, a geneticist at Baylor College of Medicine, and colleagues recently investigated the consequences of deleting *Dna2* in yeast. The deletion alone would be fatal to the cells, likely because the bits of DNA that Dna2 breaks down are so damaging, so the team engineered mutants that also carried a mutation in *pif1*, which codes for an enzyme that's believed to ready some of those substrates for processing by Dna2. In the double mutant, researchers think fewer toxic intermediates form, allowing the cells to survive.

The researchers also tweaked the yeast cells to conditionally express the enzyme HO endonuclease—which cleaves DNA at a specific locus known as *MAT*—when exposed to the sugar galactose. After letting the cells grow on plates of galactose for up to six days, the researchers examined the break sites by sequencing the DNA around the *MAT* locus. To their surprise, about 8 percent of the mutant cells carried a number of large insertions. By contrast, no insertions were found in control cells expressing HO endonuclease.

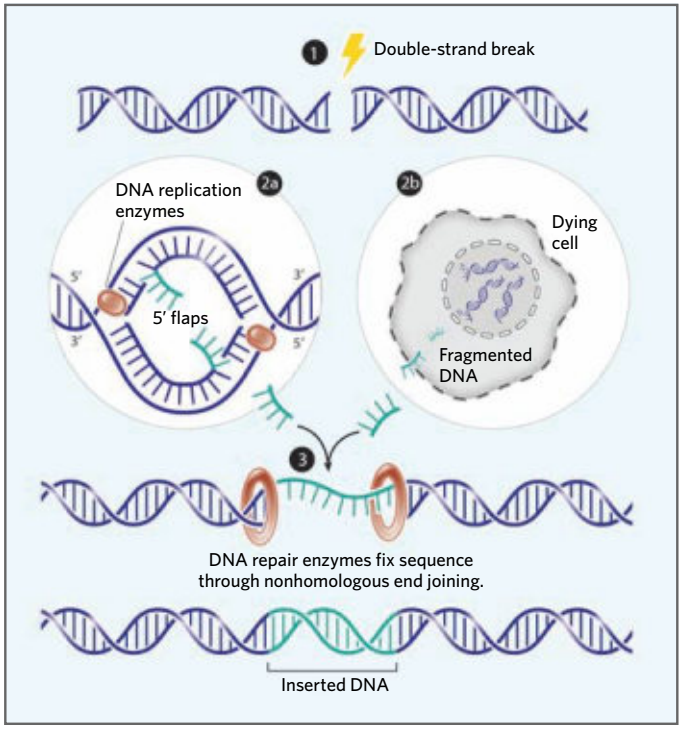
Sequencing revealed that DNA from just about anywhere in the genome—including retrotransposable elements, ribosomal DNA, genes, centromeres and telomeres, and sequences at which DNA replication is initiated—can land in these breaks.

The inserted sequences did not appear to be deleted from their original loci, suggesting that they arise through duplications. Ira explains this may have occurred due to the persistence of excess DNA that Dna2 would normally degrade.

Further experiments showed that once double-strand breaks occur in the yeast genome, the sites can collect any kind of DNA floating around inside cells, even without deletion of *Dna2*. Ira says the study demonstrates how easy it is for double-strand breaks to accumulate insertions, whether the stray DNA that's inserted results from Dna2 deficiency or from some other mechanism. "The entertaining part of the story is that any gene can jump from one locus to another using this mechanism," he says.

Robert Schiestl, a pathologist at the Fielding School of Public Health at the University of California, Los Angeles, who was not involved in the study, suggests that the vulnerability of double-strand breaks to insertions might be relevant for cancer, and says it would be interesting to know whether similar insertions occur in malignant cells with naturally occurring Dna2 deficiencies. However, the current study's results aren't a game-changer, he adds. "Knowing the function of the gene that they mutated, it's not that surprising. It's almost expected."

—Katarina Zimmer



MIND THE GAP: When a yeast cell is engineered to lack the enzyme Dna2, double-strand breaks in its DNA 1 collect stray sequences from all over the genome. Authors of a new paper suggest these insertions arise because Dna2 normally degrades excess DNA created during replication, such as so-called 5′ flaps 2a. Another possibility is that the rogue DNA is shed from dying cells 2b, although it is unclear whether Dna2 could be involved in that process. The excess bits can be integrated into breaks via nonhomologous end joining, in which repair enzymes weld the ends of severed DNA back together 3.

LET'S GET IT ON: Scanning electron micrographs show mating archaea fused together with cytoplasmic bridges.

IN THE BEGINNING: A primordial version of RNA might have incorporated inosine, a derivative of adenosine, in place of guanosine.

MICROBIOLOGY

Trading Spacers

THE PAPER

I. Turgeman-Grott et al., "Pervasive acquisition of CRISPR memory driven by inter-species mating of archaea can limit gene transfer and influence speciation," *Nat Microbiol*, 4:177–86, 2019.

Naturally occurring CRISPR-Cas systems in bacteria and archaea carry DNA memories of invasions by viruses or plasmids. These DNA sequences, called spacers, instruct Cas proteins to destroy the intruders should they enter the cell again. Curiously, several species of halophilic, or salt-loving, archaea isolated from water near Israel's Mediterranean coast possess spacers matching the DNA of closely related species, report Tel Aviv University's Uri Gophna and colleagues.

Archaea can mate by latching together with cytoplasmic bridges and exposing their genomes to each other to be recombined. To test whether archaea pick up spacers during mating, the researchers let two species of halophilic archaea mingle for 24 hours. Chromosomal markers allowed the team to identify archaeal spawn of two different parent species. Indeed, members of both species acquired spacers from each other during mating. "I was surprised to see that mating would really induce acquisition of spacers so broadly," says Gophna. In further experiments, the team found that mating efficiency fell when archaea had spacers matching their would-be partner of the other species, suggesting the spacers thwart such pairings.

The study shows that "CRISPR can target not only phages and plasmids, but also chromosomal DNA from other species," says Luciano Marraffini, a microbiologist at Rockefeller University who was not involved with the work. He thinks that in addition to warding off attacks, CRISPR influences evolution by interfering with mating between archaeal species.

Gophna's team observed another oddity—that in the genetic scramble of interspecies mating, archaea sometimes acquired spacers that instruct their CRISPR systems to pull out chunks of their own DNA, encoding mostly nonessential functions. Gophna sees this "semi-random process" as a way that archaea purge their genome of possibly unhelpful DNA integrated long ago.

—Carolyn Wilke

EVOLUTION

Strange Beginnings

THE PAPER

S.C. Kim et al., "Inosine, but none of the 8-oxo-purines, is a plausible component of a primordial version of RNA," *PNAS,* 115:13318–23, 2018.

Proponents of the RNA world theory argue that life on Earth originated from a mixture of self-replicating, information-storing molecules. But while researchers have discovered ways that RNA's pyrimidine nucleosides, uridine and cytidine, could have formed in primordial conditions, they've had less success with the purine nucleosides adenosine and guanosine, casting the theory into doubt.

Biologist Jack Szostak's lab at Harvard Medical School recently set out to test a new hypothesis: that compounds called 8-oxo-purines could have acted as substitutes for modern purines in primordial RNA. His team used an adenosine derivative, inosine, as a control.

Under early-Earth conditions, 8-oxo-purines turned out to perform poorly—RNA molecules containing them copied slowly and with low accuracy. But inosine, unexpectedly, served as an excellent guanosine substitute. "We were really surprised to see that actually inosine works almost as well as guanosine, and in some cases, slightly better," says Szostak. While it's impossible to confirm that inosine really was a component of primordial RNA, "we're pretty convinced that it could have happened this way."

By removing the need for a plausible chemical pathway to generate guanosine under early-Earth conditions, the paper "goes a long way to suggesting a solution to a long-standing problem," says John Sutherland, a chemist at the MRC Laboratory of Molecular Biology at the University of Cambridge who was not involved in the work but, like Szostak, is part of the Simons Collaboration on the Origins of Life.

Researchers now need only to find out how adenosine could have formed in order to complete the story of how primordial RNA might have come together. "The value of this work is not just in what [Szostak's group] does next," says Sutherland, "but what it suggests other people should do next as well."

—Catherine Offord

Master Decoder

After a career as a neurologist in the United States, Kári Stefánsson founded Iceland-based deCODE Genetics to explore what the human genome can tell us about disease and our species' evolution.

BY ANNA AZVOLINSKY

Kári Stefánsson remembers exactly where he was when he formulated the idea for his company, deCODE Genetics. It was 1995, and he had recently moved from the University of Chicago to Harvard University. "I was sitting in Starbucks at the Beth Israel Hospital, and I put together this narrative for identifying genetic variants of a large number of diseases using data from a large number of people and also figuring out the structure of a population," he says. "I proposed to do this in Iceland because the Icelandic population has the advantage of having started from a small number of colonizing individuals," leading to what's called a "founder effect." He reasoned that because of this founder effect, the number of disease-associated genetic variants would be relatively small for each individual disease, so they'd be easier to identify among people in Iceland.

You free yourself from the necessity of beginning with a hypothesis, which I thought was very liberating.

Stefánsson was a seasoned molecular biology and protein biochemistry researcher with a focus on neuroscience, but he had never done human population genetics studies. Still, he realized that such genetic analyses were the only way to probe the nature of human diseases in a model-independent way. "You can look for [genetic] variants without prior ideas of what causes the disease, so you free yourself from the necessity of beginning with a hypothesis, which I thought was very liberating."

After that day, there was no turning back, Stefánsson says. He was determined to form this company. "I realized that to make a significant contribution to the field of genetics, the scope was too large to fit into academic research and to obtain the proper grant funding." So Stefánsson went to venture capitalists in New York City, San Francisco, and Los Angeles to convince them to fund his company.

While his goal was to find genetic variants at the root of common diseases, he sold his idea to investors by proposing that deCODE would turn a profit by finding novel drug targets for pharmaceutical companies and by using human genetics data to identify subpopulations that would be more likely to respond to certain treatments—what is today called precision medicine.

Pitching this idea, he convinced seven companies to give him a total of $12 million in the span of six weeks. "I didn't know much about how to raise funding," he says. "I was just this eccentric man from Iceland who was telling potential investors his story." Stefánsson moved back to Iceland in 1996 and began to build deCODE from scratch. "I thought that would take four years," he says, "but instead it took almost 20."

STARTING WITH BOOKS

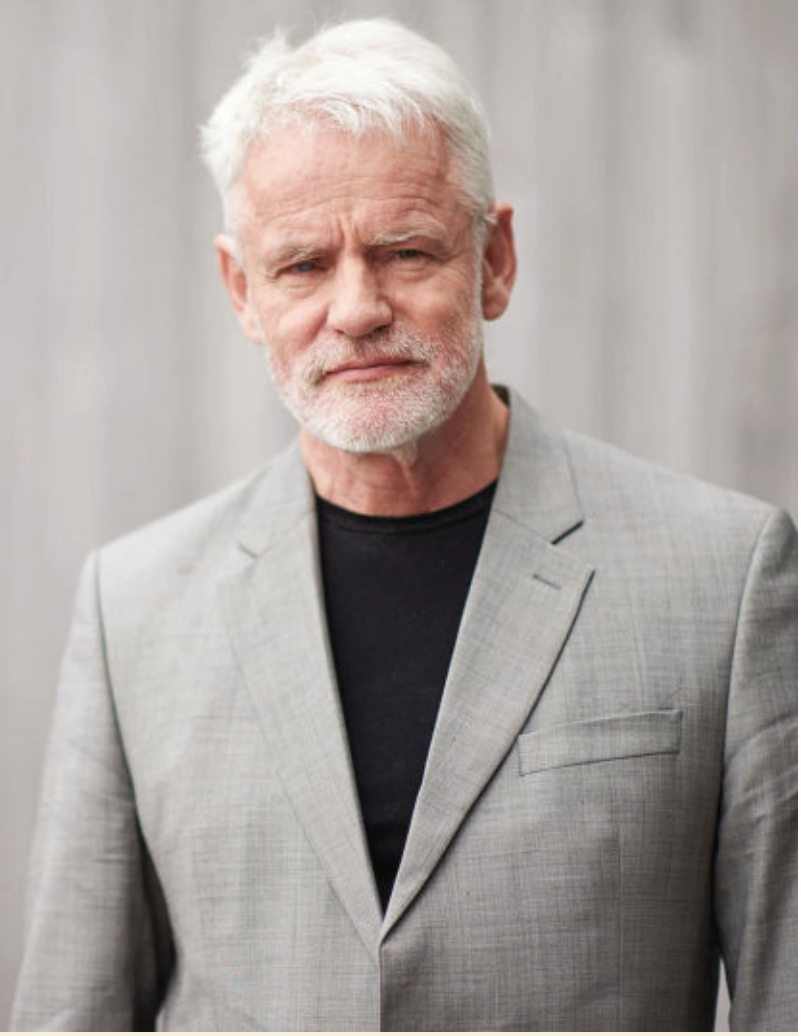
Stefánsson was born and grew up in Reykjavik. He was the second youngest of five children. "I was in the worst position in the order of children that you can think of, the child that tends to get the least attention," he says. He doesn't recall being starved of his parents' attention, although that is how his eldest sister remembers it, he says. Stefánsson's father was a radio journalist, writer, and then a member of Iceland's parliament, while his mother stayed home with the kids.

At a young age, Stefánsson was more interested in books and writing than in science. His father wrote biographies and published an autobiography of his early life, growing up in an Icelandic fishing village, that "straddled fiction and nonfiction," Stefánsson says. Stefánsson was a prolific reader and aspired to become a writer too. "When I was growing up, popular writers in Iceland had the status pop stars have elsewhere," he says.

Iceland was a relatively poor country then. "Our family was economically not at all privileged, but we had cultural privileges. . . . We were encouraged to read good literature, to write, and to be creative." Stefánsson also enjoyed the outdoors, fishing with his father in the summer and riding horses.

Stefánsson excelled academically. He entered the College of Reykjavik in 1966 and majored in math. After graduating, Stefánsson went to medical school—following the lead of his best friend because he couldn't decide what to do next. At the University of Iceland Medical School in Reykjavik, Stefánsson initially wanted to pursue psychiatry but then switched to neurology. "I was interested in the brain for the same reason as I am interested in it still today—the brain is the last frontier of biology, it is the organ we don't understand, the organ of consciousness and emotion, which define us as a species," he says. "I think it requires an extraordinarily dull mind not to be fascinated by the brain."

After completing medical school in 1976, Stefánsson was accepted into the neurology residency program at the University of Chicago. He had missed the deadline to apply for that academic year, but the neurology department chair, Barry Arnason, was a



KÁRI STEFÁNSSON

President, CEO, and director of deCODE genetics, Reykjavik, Iceland
Professor of Faculty of Medicine, University of Iceland, Reykjavik, Iceland
One of Time Magazine's 100 most influential people of the year (2007)
One of 10 most important biologists of the 21st century, Newsweek Magazine (2007)
Jakobus Award (2007)
Anders Jahre Award (2009)
One of the world´s 10 most cited scientists, Thomson Reuters (2010)

Greatest Hits

- Using extensive genealogy data on the Icelandic population going back hundreds of years, provided evidence of a genetic component to human longevity.
- Demonstrated that the most common late-onset form of Parkinson's disease has a genetic component.
- Identified 10 percent of the human genome that likely has evolved faster as a result of meiotic recombination by analyzing the relationship between recombination rate and reproductive success.
- Identified common gene variants that raise schizophrenia risk, and a link between creativity and predisposition to schizophrenia.
- Isolated genetic variants linked to educational attainment and found that there is negative selection against these genetic elements, as individuals with these variants tend to have fewer children.

Canadian of Icelandic origin and was impressed that an Icelander had applied. Arnason offered Stefánsson a position in his lab for a year before Stefánsson could join the clinical program. In Arnason's lab, Stefánsson initially conducted laboratory research, isolating and culturing glial cells called oligodendrocytes from sheep. Oligodendrocytes produce myelin—a substance composed of fat and protein that wraps around the axons of nerve cells. The goal of the research was to better understand the pathology of multiple sclerosis, a myelin-related disease. This was Stefánsson's first lab experience, and he became fascinated with research, publishing on the work in 1980. "Looking back, I am pleased to have done hands-on experiments where 95 percent of the time you are generating data and 5 percent of the time you are analyzing it," he says. "In comparison, at deCODE, we spend 95 percent of our time on the data analysis."

Stefánsson completed his clinical residency in neurology, trained as a neuropathologist, and then spent another year in a lab at the University of Chicago, where he found that Müller cells, the glial cells in the retina, had similar structural properties to oligodendrocytes, including the presence of myelin-associated glycoprotein.

In 1983, Stefánsson became an assistant professor in neurology at the university, running a lab to study the role of myelin in multiple sclerosis and other autoimmune diseases and specializing in multiple sclerosis as a clinician. In 1991, he became a full professor, and in 1993, he moved his laboratory to Harvard University to become the chief of the neuropathology division at what was then called Beth Israel Hospital, now known as Beth Israel Deaconess Medical Center.

CHANGING COURSE

Having secured funding for deCODE in 1996, Stefánsson moved back to Iceland, originally turning an old building that had housed a photography store into a laboratory. He hired a few colleagues from the US, and within a year, the company grew to about 100 employees.

The deCODE team started constructing a database of the existing genealogy of almost the entire Icelandic population, going back hundreds of years—and making that information accessible to the people of Iceland. The researchers initially asked, with just birth and death data, whether individuals who lived until at least the age of 90 were likely to have relatives who also lived past 90. In fact, those who did live to 90 were more likely to be related to each other, indicating that there is a genetic component to long life. Analyzing the pattern of longevity among families, the researchers also showed that the people who live longer are more likely to inherit a positive factor—one or several genes—that can stave off multiple diseases. Stefánsson's team then used a similar approach, adding medical record data, to show that

there is a genetic component to the most common late-onset form of Parkinson's disease.

DeCODE also explored how the human genome has evolved. In 2004, the team analyzed how reproductive success is affected by the way that the maternal and paternal genomes are scrambled during the formation of egg and sperm. They used genome-wide microsatellite data on 23,066 individuals and came to the conclusion that a small portion of the genome, just 10 percent, has a higher rate of recombination, suggesting that characteristics driven by genes in these regions evolved faster than phenotypes coded by genes in other parts of the genome.

It's still not clear what these characteristics are, according to Stefánsson, but the team is investigating this further. And while new mutations across the genome are more likely to come from the father (because spermatogenesis is a continuous process throughout a man's life while oocytes do not divide postnatally), in this 10 percent of the genetic code both the father and mother equally contribute to the de novo mutation rate. The analysis also showed that a high recombination rate was linked to increased viability of a fetus, particularly in older mothers. In addition, mothers who had a high oocyte recombination rate were more likely to have more children. Again, the results raised more questions than they answered, so Stefánsson and his colleagues are continuing to dig deeper.

As Stefánsson had envisioned on that fateful day in Starbucks, human disease risk is another focus of the company. In 2009, the deCODE team identified common genetic variants that result in an increased risk of developing schizophrenia. "We showed that those who carry the variant but don't develop schizophrenia have a departure from the norm in terms of cognition, in a similar way as schizophrenics but not as severe," says Stefánsson. "This suggests that what comes first is the abnormality in thought and then the schizophrenia, and not that the schizophrenia is a prerequisite for thinking differently."

For Stefánsson, this is among the first clues to figuring out how thoughts develop in the brain. Another clue is that there's an overrepresentation of individuals with bipolar disorder and of healthy siblings of people with schizophrenia and bipolar disorder among creative professions, but, according to Stefánsson, it hasn't been clear whether creativity and these mental illnesses had shared biology. "We showed that Icelandic writers, painters, and others in creative professions have a higher predisposition for schizophrenia, suggesting that at least in part, creativity and schizophrenia share biology."

In another study, deCODE researchers uncovered a genetic link underlying the phenomenon that individuals who obtain a higher education typically have fewer children, showing that genetic predisposition to obtain education may also predispose an individual to have fewer children. Analyzing about 130,000 people in Iceland, the team identified genes associated with completing higher education (educational attainment) and found that these genes decreased in frequency in the population between 1910 and 1990. In other words, the genes associated with educational attainment were under negative genetic selection. If an individual harbored these "education" genetic variants, they were also more likely to have later and fewer children. It was not simply a matter of investing more time in education and thus less in childrearing: those who had these gene variants but did not attain higher education also had fewer children than individuals who did not harbor the "education genes," suggesting a link between these education-associated variants and fertility.

A GENOMIC FRONTIER

In 2015, Amgen bought deCODE, but Stefánsson says the biotechnology company has left him and his team alone to continue to make new discoveries and publish their basic human genetics research. DeCODE now has genealogy data on all Icelanders, blood samples from about 160,000 of those individuals, and whole genomes on about 60,000 volunteers who have given deCODE informed consent to use their data for research. "We have sequence information on most of the nation, which puts us in a position to do fairly powerful genetics but also places a high responsibility on our shoulders on privacy and data protection," Stefánsson says.

I think it requires an extraordinarily dull mind not to be fascinated by the brain.

Iceland's governmental bioethics committee oversees the data. The deCODE database includes individuals' disease-causing genes, such as a mutation in *BRCA2* that's common among Icelanders and confers an 86 percent probability of developing cancer on the women who carry it, as well as increasing the risk of prostate cancer in men. The Icelandic government, however, does not allow itself or any private institution to provide people with their genetic information without explicit consent. "It's a complicated issue," says Stefánsson. "It seems self-evident that anyone should have access to their data." But, because deCODE is sequencing data for bulk population studies, and not in a clinical-grade laboratory, the company cannot guarantee that each individual's sequence is completely accurate. Therefore, because the national law prohibits both deCODE and the healthcare system from contacting those with disease-related genes, the company's solution is a website where the team has deposited better-quality sequencing information on *BRCA2* and other genes, allowing individuals to find their data if they wish.

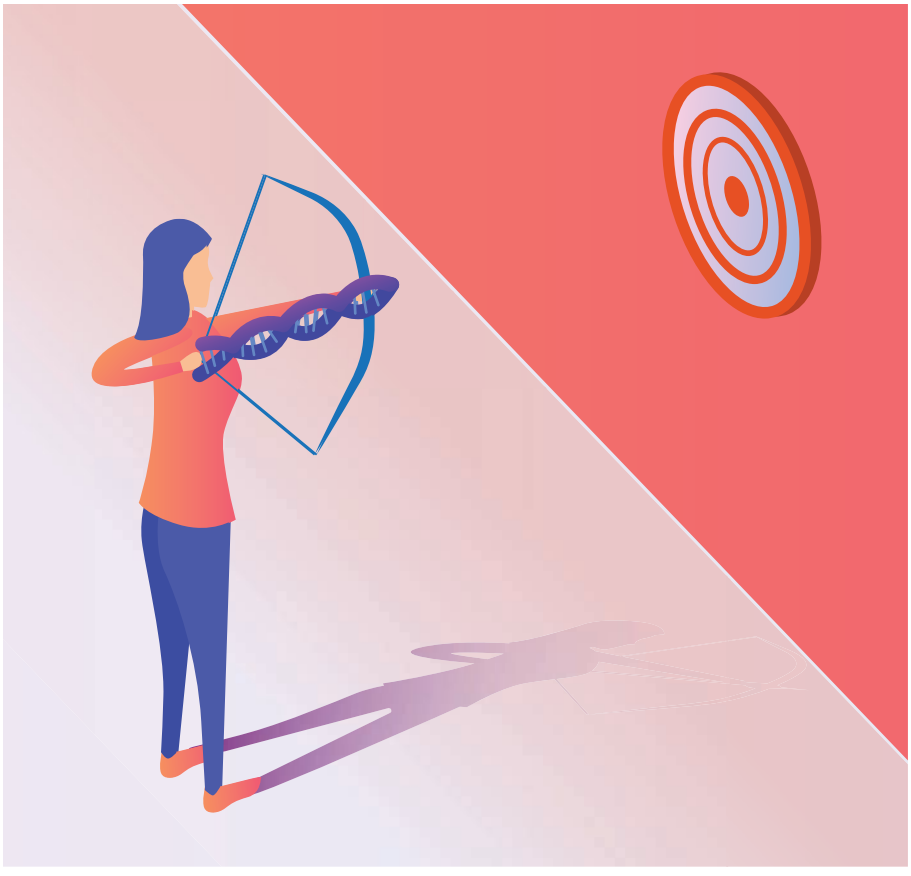
After 23 years at deCODE, Stefánsson says he has achieved more than what he expected when he hatched his plan for the company in Starbucks, and he has no plans of slowing down now. "I am extraordinarily pleased with the contributions we've made to human genetics and evolution."

While staying engaged with his research, Stefánsson still manages to read about 40 novels a year. "Language is the equipment with which you think, and the best way to train yourself on the use of language is to read good literature," he says. "Reading is as important for me as eating. I cannot survive without it." ■

Knock It Into the Park

Techniques for upping the efficiency of knocking in genes

BY ANNA NOWOGRODZKI



Almost always, building something is harder than tearing it down. Similarly, knocking in genes poses a greater challenge than knocking them out. It's a reality that researchers will have to overcome in order to get the most out of gene editing. Knocking in genes allows scientists to study the effects of specific gene variants, to use reporter genes like green fluorescent protein to track gene products in time and space, to probe genome regulation, and ultimately, to repair disease-causing genes. "It's a really effective way to interrogate every base of a gene," says Greg Findlay, an MD/PhD candidate at the University of Washington.

CRISPR-Cas9, a gene editing technology known for its user-friendliness, can knock genes in or out. Knocking out a gene involves inserting CRISPR-Cas9 into a cell using a guide RNA that targets the tool to the gene of interest. There, Cas9 cuts the gene, snipping through both strands of DNA, and the cell's regular DNA repair mechanism fixes the cut using a process called non-homologous end joining (NHEJ). NHEJ is highly efficient but inaccurate. The process tends to introduce errors in the form of small insertions or deletions that are usually enough to knock out the gene.

To knock a gene in, however, the cuts must be repaired very precisely, with no extra insertions or deletions. This requires harnessing a second DNA repair mechanism called homology-directed repair (HDR), which—in mammalian cells, at least—occurs less efficiently, so its frequency is dwarfed by that of NHEJ. Complicating the process further is the fact that some gene loci and cell types are inherently less hospitable to CRISPR-Cas9 editing.

In the past few years, researchers have developed many new strategies to boost the efficiency of knocking in genes both large and small using CRISPR-Cas9, and along the way they've proposed and tested new applications for this type of gene editing. Here, *The Scientist* explores a few of the most promising approaches.

SELECT IT

RESEARCHER: Jon Chesnut, senior director of synthetic biology R&D, Thermo Fisher Scientific

PROJECT: In developing a gene tagging kit called Truetag that Thermo Fisher will put on the market later this year, Chesnut used selectable markers to improve efficiency. A selectable marker—in this case, an antibiotic resistance gene—is stuck to a fluorescent protein tag and knocked into mammalian cells. Those cells are then grown in culture with the associated antibiotic. The resistance gene confers a selective advantage to the cells that carry it; they alone are able to grow, and thus those that grow contain the gene tag of interest. Even if the efficiency of gene insertion is low, researchers can use antibiotic selection for a week or more to end up with a high percentage of cells with successful insertions.

Using the antibiotic puromycin or blasticidin with the kit, Chesnut's team managed to boost the gene insertion rate from 10–30 percent to 90 percent or more in some cell populations. A few especially difficult genes went from an insertion rate of less than 1 percent to greater than 90 percent. It's important to test multiple doses of antibiotics on the cell line you plan to

use to find the correct dose, Chesnut says: you want to kill cells without insertions but not cells with successful insertions.

TRY IT: Selectable markers work best when the gene of interest is highly expressed, Chesnut says. "If it's not, you may still get selection but you may not get enough expression of your fluorescent protein tag to be able to detect it." Also, the general limitations of CRISPR-Cas9 apply. "There are regions of the genome that don't cut very well with CRISPR, and we're still not sure why," he adds. And some cell types don't easily accept foreign DNA, RNA, or RNA-protein complexes—the three methods of CRISPR-Cas9 delivery.

For better luck inserting selectable markers, make sure there is a so-called PAM [Protospacer Adjacent Motif] sequence, a short tag in the target DNA that CRISPR-Cas9 must recognize before it cuts, within 10 base pairs of the desired gene insertion site, says Chesnut. Farther away from the cut site than that, and the insertion efficiency may be too low to be functional. Without a PAM site, you can try TALENs or zinc finger nucleases, although those older gene editing techniques are trickier than CRISPR.

TIMED INHIBITION

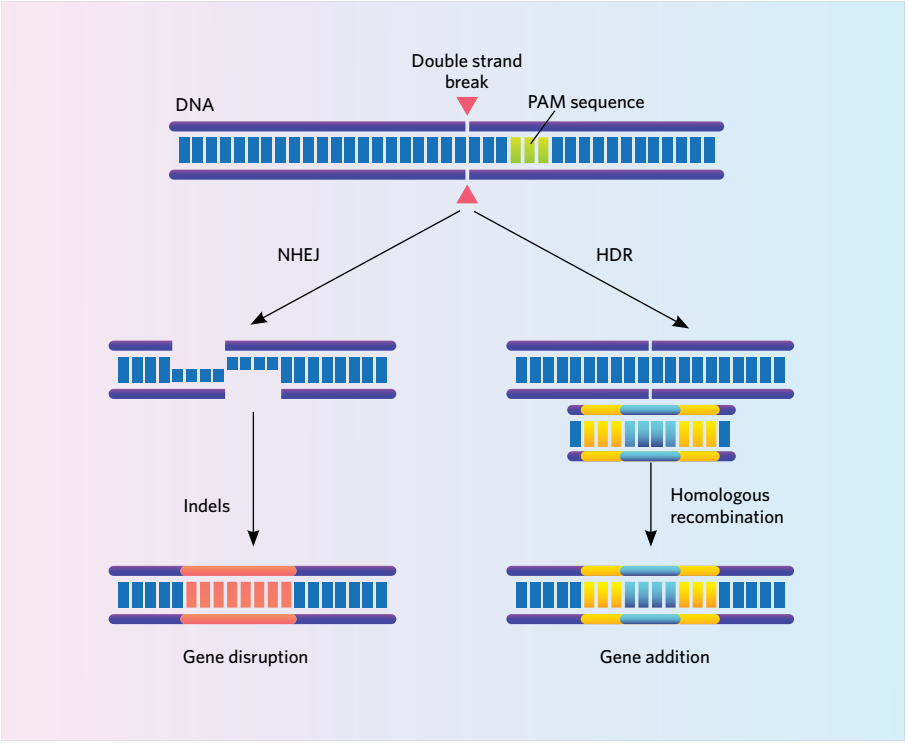
RESEARCHER: Jacob Corn, genome biologist, Swiss Federal Institute of Technology, Zurich

PROJECT: Researchers don't understand why the NHEJ pathway vastly outcompetes the HDR pathway in mammalian cells. "Yeast do HDR like crazy," Corn says. In an effort to rev up this DNA repair process in human cells and improve gene knock-in control, he and his team are trying to pinpoint how HDR is regulated. They screened human cells for genes whose knockdown led to increased HDR in the cell, and then searched for small molecule inhibitors of those genes. One of the genes that popped up codes for CDC7, a kinase that regulates the cell cycle transition to S phase; its inhibitor, XL413, boosted gene knock-in efficiency two- or threefold (*BioRXiv*, DOI: 10.1101/500462, 2018). That's because HDR occurs in only some parts of the cell cycle, including S phase, Corn says. If you add the inhibitor XL413 at the same time as you use CRISPR-Cas9 to edit your target gene, the cells pile up in the phase immediately before S phase. Then you remove XL413, and all the cells go into S phase and increase knock-in efficiency.

Corn has used this technique in many immortalized human cell lines and in human T cells. It can knock in short stretches of DNA, such as SNPs, as well as large genes. There is no reason it shouldn't work in mice, he says, although he hasn't tested it.

TRY IT: "Timing is absolutely key," Corn says. The Cas9 must cut the DNA at the same time that XL413 is added. If you inhibit first and then release while editing with CRISPR-Cas9, homologous recombination efficiency drops threefold instead of increasing, because the cells are released into the wrong phase of the cell cycle.

And as with any HDR effort, Corn says, always run a no-nuclease control to make sure you aren't accidentally amplifying contaminant DNA that's floating around your lab. After introducing the knock-in, "sequence, sequence, sequence, sequence," he says. Just using a reporter system such as a fluorescent protein tag to demonstrate successful gene insertion can backfire. Sequencing verifies that the insertions were made at the correct site.



FIX IT: Non-homologous end joining (NHEJ), the main DNA repair mechanism in mammalian cells, simply joins the double-stranded DNA break back up, often inserting or deleting small stretches of DNA (indels) in the process. Homology-directed repair (HDR), on the other hand, inserts a new DNA segment that is surrounded by sequences that match the DNA on either side of the break site.

PLAYING THE LONG GAME

RESEARCHER: Channabasavaiah Gurumurthy, director of the mouse genome engineering core facility, University of Nebraska Medical Center

PROJECT: A few years ago, musing over the difficulty of knocking in genes while trying to do so into mouse zygotes, Gurumurthy and his colleagues had a revelation.

THE SCIENTIST STAFF

Researchers were successfully inserting short, single-stranded DNA, so why not try making a knock-in by inserting long, single-stranded DNA? Indeed, the approach, which Gurumurthy calls Easi-CRISPR (efficient additions with ssDNA inserts -CRISPR), boosts efficiency by 2.5 times, and using single-stranded DNA slashes the rate of off-target insertions 100-fold in cell culture (*Nat Protoc* 13:195–215, 2018; *Nature* 559:405–09, 2018). "It is quite huge," he says. In Gurumurthy's lab, Easi-CRISPR has generated a knock-in mouse line for 9 out of every 10 genes they have tried. A collaborator has also used it in human T cells to create CAR-T cells, patient-specific immune cells for fighting cancer.

TRY IT: Easi-CRISPR is far from foolproof, Gurumurthy cautions. Sometimes the technique inserts only part of the gene. Also, he adds, it can scramble the homology arms—the short sequences on either side of the gene that home it to its correct target in the genome. And some loci are inexplicably more difficult to insert than others.

Few commercial vendors design and synthesize custom long, single-stranded DNA. You can make your own, but the stability of single-stranded DNA varies; less-stable sequences will have lower yields, so you may need to synthesize more of them, says Gurumurthy.

Researchers unable to insert CRISPR into single-cell mouse embryos can pay a core facility to make the mice with their DNA sequence, says Gurumurthy. Core facilities such as his charge from $5,000–$15,000 to generate one or two breeding pairs; commercial facilities charge $20,000–$50,000, he says.

KNOCK-IN BY NUMBERS

RESEARCHER: Greg Findlay, MD/PhD candidate in the lab of Jay Shendure, University of Washington

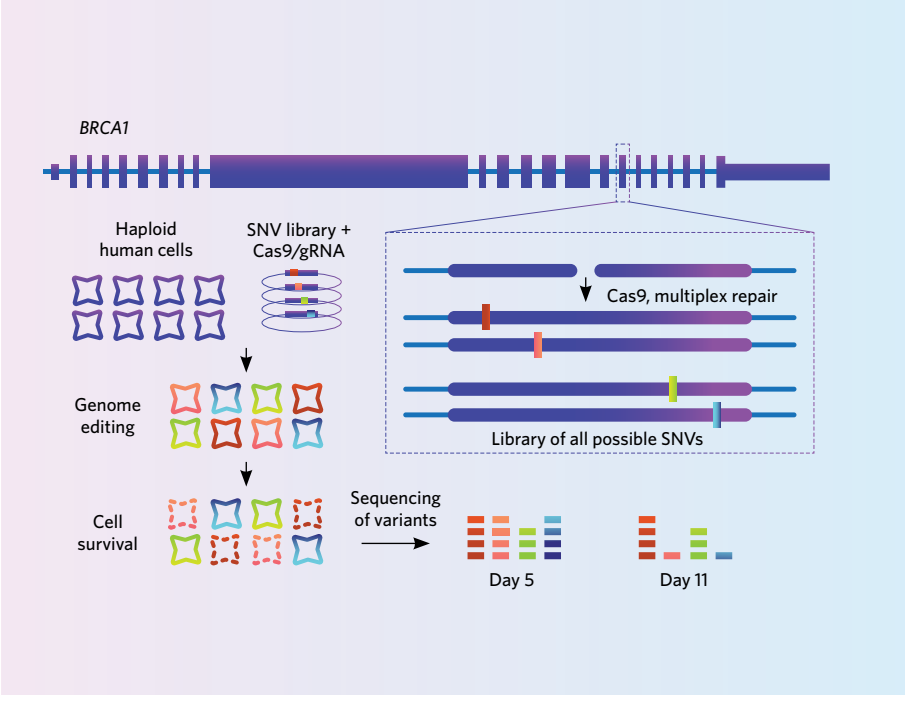
PROJECT: Findlay and his colleagues were aiming to improve how clinicians interpret mutations in the breast and ovarian cancer gene *BRCA1*. That gene has thousands of variants, but researchers don't know how most of them affect its function. To study the impact of these variants, they used a knock-in technique they developed called saturation genome editing (*Nature*, 562, 217–22, 2018).

In an immortalized haploid human cell line, they used CRISPR-Cas9 to knock in 4,000 tiny variants in millions of cells at once in vitro. The genome is cut at the same spot in each cell, but each cell's genome receives a different variant. To promote HDR, they also knocked out the *ligase4* gene, disabling the NHEJ repair pathway—a step that yielded a threefold gain in efficiency, Findlay says. Finally, since all the cells' knock-ins are different, they sequenced the cells deeply, covering the same genomic region millions of times, to make sure they actually knocked in the 4,000 variants they wanted to study. They sequenced at two time points, and deduced that the knock-ins that didn't come up in the sequencing at the second time point were ones that interfered with the gene's function, because the cells carrying them must have died.

TRY IT: Findlay's team had the DNA oligos for the 4,000 variants manufactured for them on a microarray. You can buy arrays of 6,000 to 250,000 oligos, so consider getting more bang for your buck by combining multiple experiments on the same array, says Findlay. Their lab pays about $5,000 for 100,000 oligos.

This strategy comes with limitations: it has so far only been used to knock in single-nucleotide variants, and all the edits need to be in the same gene. The method works best when editing a fairly narrow region of DNA, about 110–120 base pairs, because longer DNA oligos would have too many errors, Findlay says. It's also important to sequence very deeply to make sure that you account for the full number of variants you intended to knock in. ■

SEARCH FOR SURVIVORS: Researchers knocked in all 4,000 known single-nucleotide variants (SNVs) of the *BRCA1* gene in millions of cultured cells. Then, they sequenced all the variants -- once five days after knocking them in, and again six days after that. They deduced that variants that disappeared on the second round of sequencing had interfered with the gene's function, causing the cells that carried them to die.



ADAPTED FROM AN ILLUSTRATION BY GREG FINDLAY

The Aging Workforce

Researchers, institutions, and funding agencies are struggling to come up with ways to make academic science sustainable as more people opt to stay in their positions longer.

BY KATARINA ZIMMER

Had he been at almost any other institution in the UK, Hagan Bayley could have studied membrane proteins for as long as he wanted. But at the University of Oxford, the chemical biologist was asked to retire from his professorship of 16 years this coming September, and to give up his lab—along with the 20 graduate students and postdocs who work there—at what he considers the relatively young age of 68.

Fortunately for Bayley, he is able to stay three additional years, but that's only because he applied to university administrators for an extension—a process he says dragged on for seven years, after he was denied twice and had to go through an arduous appeals process.

It's a challenge that several Oxford academics have taken on since the university introduced a so-called "employer justified retirement age" in 2011, which effectively forces staff to leave their positions at a fixed age—originally 67 years old, but recently raised to 68. In August 2018, an Oxford literature professor went as far as suing the university for lost earnings, claiming age discrimination after being asked to retire at 67 in 2016. "You have to be very tenacious, because you can easily give up," says Bayley.

The UK abolished its nationwide retirement age in 2011, 25 years after the US made the same move. But institutions can implement their own retirement rules if they can make a legitimate business argument. Oxford, along with the Universities of Cambridge and St. Andrews, decided to exercise that option in recent years. The reason, according to Oxford's policy: to promote "inter-generational fairness," equality, and diversity.

AN OLD INSTITUTION: The University of Oxford is one of several UK universities that have introduced mandatory retirement ages for faculty.

Mandatory retirement is just one approach that university administrators on both sides of the Atlantic are considering in order to curb what many view as a troubling trend in academic research, and particularly in the sciences—that senior researchers are retiring later and later, while siphoning away limited resources such as faculty positions and funding from younger researchers.

For Bayley, however, dismissing experienced researchers at the height of their careers isn't just unfair—it would do more harm than good for science. "I don't think that firing faculty members at 68 is going to give you the best science," he says. "And it's also not good for young people," as lab members will have to find alternative posts after their PI leaves. "You're not firing one person, you're firing an entire research group."

A senior cohort

Since the US abolished mandatory retirement in 1986, followed by Australia in 2004, and later the UK, the numbers of professors pushing 70 in those countries have soared. In the US, the mean age of life scientists employed in academic faculties has climbed from

45 in 1993 to 48 in 2010, according to a 2017 *PNAS* study (114:3879–84). The study's authors note in their paper that they expect the trend to continue.

For some academics, the reasons for sticking around may be partly financial: the 2008 global recession has made early retirement a less attractive option than before. But many university researchers simply want to keep on working for as long as they're healthy. Ecologist David Goodall of Edith Cowan University in Australia, for instance, kept his unpaid faculty position until the age of 104.

The effects of an aging academic cohort on younger researchers are hard to quantify, notes Bruce Weinberg, a labor economist at Ohio State University and one of the *PNAS* study's authors. But a few statistics are telling: the percentage of National Institutes of Health (NIH) grant recipients who are 36 years of age or younger dropped from 16 percent in 1980 to only 3 percent in 2014. And while in 1980 new investigators had to wait just one year on average to get federal funding, these days they wait up to five years.

In addition to delayed retirements that have diminished the number of available tenure-track positions, the number of new PhD candidates is dramatically rising. As a result, younger researchers spend years trapped in a cycle of temporary roles such as postdocs, traineeships, or adjunct positions—and may opt to leave academia altogether. "I am concerned that if we're increasingly supporting an aging scientific workforce, that we may risk losing part of a generation of younger researchers and innovators," says Weinberg. The imbalance is particularly troubling because the older researcher cohort tends to be less diverse in terms of gender and ethnicity, he adds.

Some institutions are addressing this situation by trying to gently ease out older professors. For instance, MIT's engineering school announced a post-tenure, "semiretirement" position for senior researchers in 2016. Senior workers are paid less, according to the program, but can still teach and mentor, which frees up tenure-track positions for postdocs. Several other universities are trying to coax senior faculty into shutting down their labs by offering them lucrative retirement packages. The hardline route that Oxford and some other UK universities have taken, however, has been heavily criticized—even by many junior researchers.

Aside from being discriminatory, and possibly hampering scientific progress by pushing out the most experienced investigators, the policy only addresses part of the problem. "Even if everyone were to retire who's 65 and over, right now, it probably wouldn't help my generation," notes Gary McDowell, who left postdoctoral research in developmental biology at Tufts University in 2016 to direct Future of Research, a nonprofit that advocates for young scientists. "It's not like an 80-year-old professor retires and that job goes to someone who's in their 30s. It's going to be someone in their 50s or 60s."

You're going to find yourself in ten, fifteen, twenty years . . . where you have a huge hole in the middle of your workforce.

—Shirley Tilghman, Princeton University

Some academics think that the biomedical community needs to take a more holistic view of the situation. "I do worry that we have done nothing effectively to ensure that we are continuing to bring in a robust number of young people who are going to be the leaders of their field in another 25 years, nor have we frankly done enough to give our most senior colleagues ways in which they can retire with dignity," says Shirley Tilghman, a professor of molecular biology and public policy and president emerita of Princeton University.

For her, the issue speaks to a wider crisis in academia. "At its core, this is an issue about how we structure the workforce," Tilghman says. As she and colleagues outlined in an influential 2014 paper in *PNAS* (111:5773–77), shoring up the academic pipeline will require individual institutions, as well as funding bodies, to come up with solutions to distribute precious resources more fairly.

Correcting the funding landscape

Recently minted principal investigator Prachee Avasthi recalls well the struggle she experienced launching her own lab at the University of Kansas Medical Center. In 2015, she was given a position as assistant professor, along with an empty room and some small start-up funds—everything she needed, save a federal grant to provide long-term support for her research on ciliary function in the green alga *Chlamydomonas*. (See "Prachee Avasthi: Cell Cosmetologist," *The Scientist*, December 2018.)

Like other early-career scientists, she had to compete with senior researchers for a finite pool of grant money, putting her at a disadvantage. "People are more likely to believe the [older] investigator can pull this off because they've already pulled it off before," she says. "Early-career people don't have that track record."

Avasthi recalls the period as particularly stressful because she had to secure funding before her start-up funds expired—a hurdle faced by many researchers in the process of setting up their own lab. Eventually, after around eight large grant proposals were unsuccessful, she secured an R35 award—a five-year grant that supports both early-career and established investigators—from the National Institute of General Medical Sciences.

How to distribute money more fairly across age groups is a question that several funding bodies have grappled with over the years. According to McDowell, the European Research Council has set a good example by creating separate funding schemes for early-, mid-, and late-career investigators. That way, you're comparing "peer with peer, rather than comparing a 70-year-old professor at Harvard Med who has 40 years of papers and track records to a 35-year-old new investigator," he says.

The NIH has gone back and forth on the issue for years. In 2013, the agency floated the idea of an "emeritus award" to help senior investigators in the process of winding down their research, But the proposal drew criticism from researchers who perceived it as a way to channel even more money to older investigators. In 2017, the newly proposed Grant Support Index (GSI), which would have capped the number of grants an individual can receive, was scrapped after one month, again in response to concerns voiced by the research community. Instead, the NIH decided to put about 3 percent of its budget towards grants for early- and mid-career investigators, as part of the so-called Next Generation Researchers Initiative.

Tilghman says that one of her main concerns is the potential impact of this hypercompetition on innovation in science. "What I often hear from my junior colleagues is, you won't get funded if you're proposing something that looks at all risky," she explains. The system favors proposals that can guarantee results but will only move a field forward incrementally, rather than potentially propelling it into new, uncharted territory. The NIH has tried to address this issue by creating the New Innovator Awards (DP2), which reward early-career researchers with innovative proposals. But these need to be scaled up, Tilghman says. "There are simply not enough of them."

For now, the main beneficiaries of such initiatives are younger researchers. Often, this leaves mid-career

I am concerned that if we're increasingly supporting an aging scientific workforce, that we may risk losing part of a generation of younger researchers and innovators.

—Bruce Weinberg, Ohio State University

investigators out of the picture, notes Christopher Pickett, director of Rescuing Biomedical Research, a non-profit cofounded by Tilghman that researches solutions to systemic issues in life science. "I think there's an assumption that once you've established your lab, then you're out on your own."

How to distribute money more fairly across age groups is a question that several funding bodies have grappled with over the years.

Everyone for themselves?

Rather than compete directly with senior investigators, many early-career scientists are stepping outside the research community in search of financial support.

Geneticist Melissa Wilson Sayres of Arizona State University, for instance, recently raised $9,000 to sequence the genome of the Gila monster (*Heloderma suspectum*) through a crowdfunding campaign on experiment.com, a platform where many other scientists have begun to crowdfund their projects. Similar digital fundraising platforms, such as SciFundChallenge, have sprung up in recent years.

Other young researchers are experimenting with new ideas for obtaining funding. Last May, social scientist Irena Schneider of King's College London co-launched Lyrical Science, an online platform where biomedical researchers can "pitch" their research in a presentation similar to a TED talk and directly engage with an online community of laypeople, corporations, and foundations interested in sponsoring their research. Ultimately this would "add express lanes into the funding ecosystem," explains Schneider. So far only two scientists have signed up for the platform, which is still in beta mode, she says.

Some philanthropic organizations offer specific grants to relieve the pressure for early-career researchers to secure money straight off the bat. For instance, the Chan Zuckerberg Initiative's Ben Barres Early Career Acceleration Awards support young investigators studying Alzheimer's, ALS, and other neurodegenerative diseases at academic institutions. And many disease foundations, such as the Children's Tumor Foundation, the Foundation Fighting Blindness, and the Leukemia Research Foundation, all have funding mechanisms geared towards younger researchers.

A handful of academic institutions have also been stepping up to create fellowships for early-career scientists who want to lead their own lab and conduct research after completing a PhD. These include New York's Cold Spring Harbor Laboratory, Harvard University, MIT, and Rockefeller University.

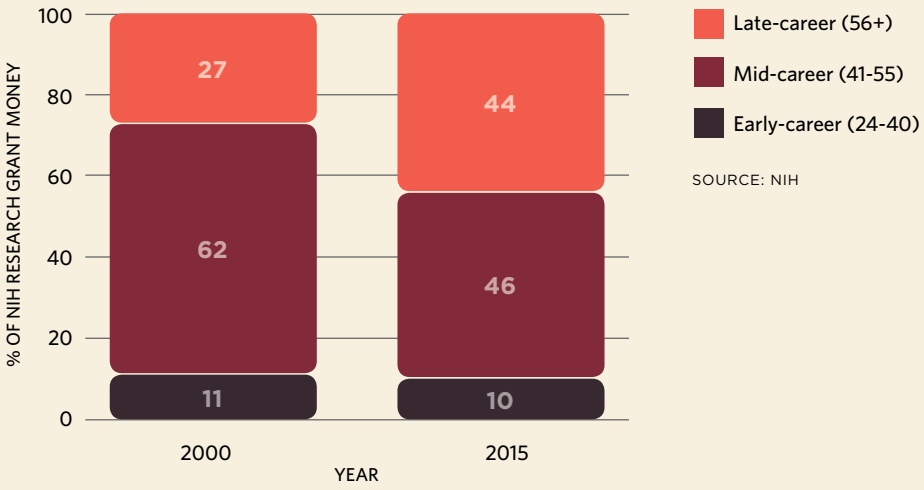
"Such opportunities may be able to help early-career researchers get in the door," writes McDowell in an email—although for the scientific enterprise to be sustainable, researchers need to be sure of financial support throughout their entire research careers, he adds.

While individual initiatives can help offset the effects of an aging academic workforce and a lopsided funding landscape, the scientific community must consider the risk that too many researchers will leave academia in search of a more-secure career, notes Tilghman. "If you're not doing that, you're going to find yourself in ten, fifteen, twenty years . . . where you have a huge hole in the middle of your workforce," she says. "You're missing a generation, basically, and that has pretty significant implications." ■

Katarina Zimmer is a freelance science writer living in New York City.

MORE OF THE PIE

Senior investigators' share of the total NIH funding available for researchers has been growing in recent years. From 2000 to 2015, the portion of money going to researchers over 56 years old rose from 27 percent to 44 percent. This trend is largely driven by the increase in the proportion of grants going to senior researchers: between 2000 and 2015, the number of awardees over 56 years old nearly doubled, while the number of mid-career awardees only increased slightly and the number of early-career awardees moderately.



SOURCE: NIH

THE SCIENTIST STAFF

In Praise of Crazy Ideas

Many of the truly transformative innovations in science were initially met with scorn.

BY SAFI BAHCALL

In 1971, Judah Folkman, then a 38-year-old surgeon at Boston Children's Hospital, proposed a radical idea: tumors in the body send out signals that trick surrounding tissues into helping them grow. The signals instruct these tissues to sprout new blood vessels, which can deliver oxygen and other nutrients to cancer cells. He suggested designing a new kind of drug, one that would block those signals, preventing or destroying the new blood vessel growth and starving tumors.

Cancer at the time was treated mainly by flooding the body with poison (chemotherapy) or searing it with radiation. Folkman's idea of subtly targeting some mysterious communication channel between tumor and host was met with scorn. When he spoke about his idea at scientific meetings, Folkman said, the room would empty out: "Everybody had to go to the bathroom at once." One year, the criticism was so intense that Boston Children's Hospital convened an external committee to review his research. The committee judged his work to be of little to no value. He was asked to resign as chief of surgery should he choose to continue the studies. He resigned and, fortunately for all of us, continued his research.

Today, Folkman's thesis underlies nearly all modern approaches to treating cancer, from anti-angiogenesis therapy to cancer immunotherapy. The work has resulted in dozens of FDA-approved drugs that have helped hundreds of thousands of cancer patients, and has inspired the development of a broad new category of drugs, VEGF inhibitors, that can reverse a certain form of blindness (that caused by macular degeneration).

Tasked with writing this essay about pursuing "ideas that are outside the boundaries of what some would call rational thinking," adapted from my forthcoming book, *Loonshots*, I hardly knew where to start. So many great breakthroughs in drug discovery were initially considered crazy.

In the 1970s, when Japanese biochemist Akira Endo sought to develop a compound to lower blood cholesterol, the research community wrote off the concept, reasoning that every cell contains cholesterol, so any such drug must harm every cell. Fortunately, he persisted. The statins he developed have prevented millions of heart attacks and contributed significantly to a three-decade decline in deaths from heart disease.

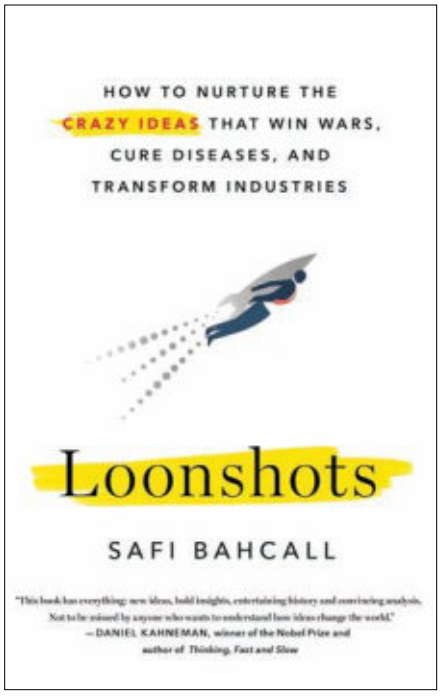
In the 1990s, chemist Julian Adams insisted on developing a cancer drug known as PS-341, which blocked proteasomes—the waste receptacles of cells. Every cell, like every home, needs waste disposal. Blocking that system is a crazy, irrational idea. Except that it worked. The drug became a revolutionary new treatment for multiple myeloma.

Folkman liked to include a slide in some of his presentations with a clip from a 1903 *New York Times* article. The article described yet another failed attempt at building a flying machine and explained exactly why any such machine could never work. Nine weeks later, two brothers took off from Kitty Hawk, North Carolina, in the world's first successful airplane.

Overconfidence in accepted wisdoms discourages true innovation. The recent proliferation of spectacular scientific tools—from full-genome sequencing to CRISPR—increases this danger, by encouraging the pursuit only of rigorously validated targets. But complex systems such as human cells and tissues often produce results that can't be predicted from simple laboratory analyses.

If we want great breakthroughs, we need to encourage crazy—even irrational—ideas, whose logic might defy our best current models. Doing so requires humility: the willingness to question our beliefs.

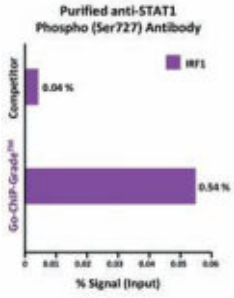
Former US Vice President Joe Biden declared a moonshot against cancer in 2016. Similar goals may soon be set against other diseases. It's important to keep in mind that a moonshot is just a destination. Nurturing loonshots—the neglected ideas whose champions are often dismissed as crazy—is how we get there. ■



St. Martin's Press, March 2019

Safi Bahcall is a physicist, a former biotech CEO, and author of Loonshots: How to Nurture the Crazy Ideas that Win Wars, Cure Diseases, and Transform Industries.

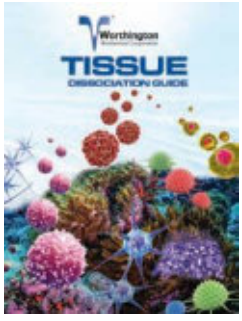
Go-ChIP-Grade™ Antibodies and Kits



BioLegend offers high-quality antibodies and kits validated for use in ChIP assays directed toward diverse targets including histone modifications, chromatin-associated proteins, and transcription factors. Many of our Go-ChIP-Grade™ antibodies are suitable for use in other applications including western blotting, immunofluorescence, and flow cytometry for added functionality within the research workflow.

BioLegend
Phone: Toll-Free (US & Canada): 1-877-246-5343
Fax: 1-877-455-9587
E-mail: cs@biolegend.com
Web address: www.biolegend.com/chip

Technical. Guide. Free. Update.

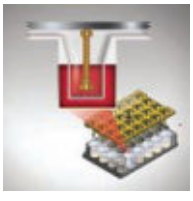


Worthington educational materials have guided generations of life science research for decades.
And thanks to you, we are cited in respected scientific journals more than any other primary enzyme producer across the globe.

Order your NEW Tissue Dissociation Guide today. Call: 1.800.445.9603, Fax: 1.800.368.3108,
or go to: worthington-biochem.com
Expertise for a new generation of researchers.

WORTHINGTON BIOCHEMICAL CORPORATION
1.800.445.9603
worthington-biochem.com

Continuous Measurements of Cell Monolayer Barrier Function (TEER) in Multiple Wells.



The TEER 24 System electrically monitors the barrier function of cells grown in culture upon permeable membrane substrates. The new **TEER 24** system accommodates standard 24 well membrane inserts in a disposable 24 well microplate. The instrument is placed in a standard CO2, high humidity incubator and connected to a PC. Dedicated software presents real-time continuous measurement of TEER in **ohm-cm²**.

For more information visit
APPLIED BIOPHYSICS
www.biophysics.com/products-ecisTeer24.php

Flow Cytometer ZE5

- New automation features enable high-throughput flow cytometry in biomarker discovery and phenotypic screening
- Processes 96- or 384-well plates in under 60 minutes with no drop-off in data quality
- Helps researchers glean more results from rare cell populations in less time using their most precious samples

Bio-Rad
www.bio-rad.com

Single-Use Bioreactor BIOSTAT® RM TX

- Developed specifically for closed, automated expansion of consistent quality cell products such as ex vivo cellular immunotherapies
- Combines the company's established single-use Flexsafe® bag technology with its expertise in biopharmaceutical automation
- Equipped with a special port for hands-free gravity harvesting

Sartorius Stedim Biotech
www.sartorius.com

Flow Cytometer ZE5

- Offers a unique option for histology laboratories
- Can be placed on any benchtop as a plug-and-play product
- Combines a quiet fan and a Peltier design optimized for robust performance

TECA
www.thermoelectric.com

How Chromosomes X & Y Got Their Names, 1891

BY JOSEPH KEIERLEBER

Why are the human sex chromosomes called "X" and "Y," while the other 22 chromosomes are identified only by numbers?

The answer begins in the late 1800s, when insect gonad cells, whose large chromosomes are easy to view through a microscope, were the specimen of choice for investigating the cellular basis of heredity. In 1891, German biologist Hermann Henking counted 11 chromosomes in firebug (*Pyrrhocoris apterus*) sperm nuclei. Some nuclei, he discovered, contained an additional large chromatin element, which he identified in his drawings with *"x."*

A few years later at the University of Kansas, Clarence McClung observed that half of grasshopper sperm contained a chromosome similar to Henking's *x* element. Like Henking, McClung used *"x"* to label this chromosome in the figures of his 1899 paper, but he coined the term "accessory chromosome" to identify it from then on. McClung soon hypothesized that the accessory chromosome determined male sex.

In the early 1900s, Nettie Stevens at Bryn Mawr College and Edmund Beecher Wilson at Columbia University tackled the puzzle of how chromosomes relate to sex differences in insects. Although they worked independently, they followed each other's research. In 1905, Wilson, the more established scientist, described a pair of unequally sized chromosomes, which segregated in a 50:50 ratio among insect sperm. A month later, Stevens reported a similar discovery in beetle gonads. Half of beetle sperm carried a small chromosome, which Stevens labeled *"s,"* and half carried its larger companion, *"l."* Female somatic cells contained two copies of the large chromosome, while male cells contained one small and one large. "This seems to be a clear case of sex determination," Stevens wrote, concluding that sperm carrying the small chromosome, not McClung's large accessory chromosome, determined male sex.

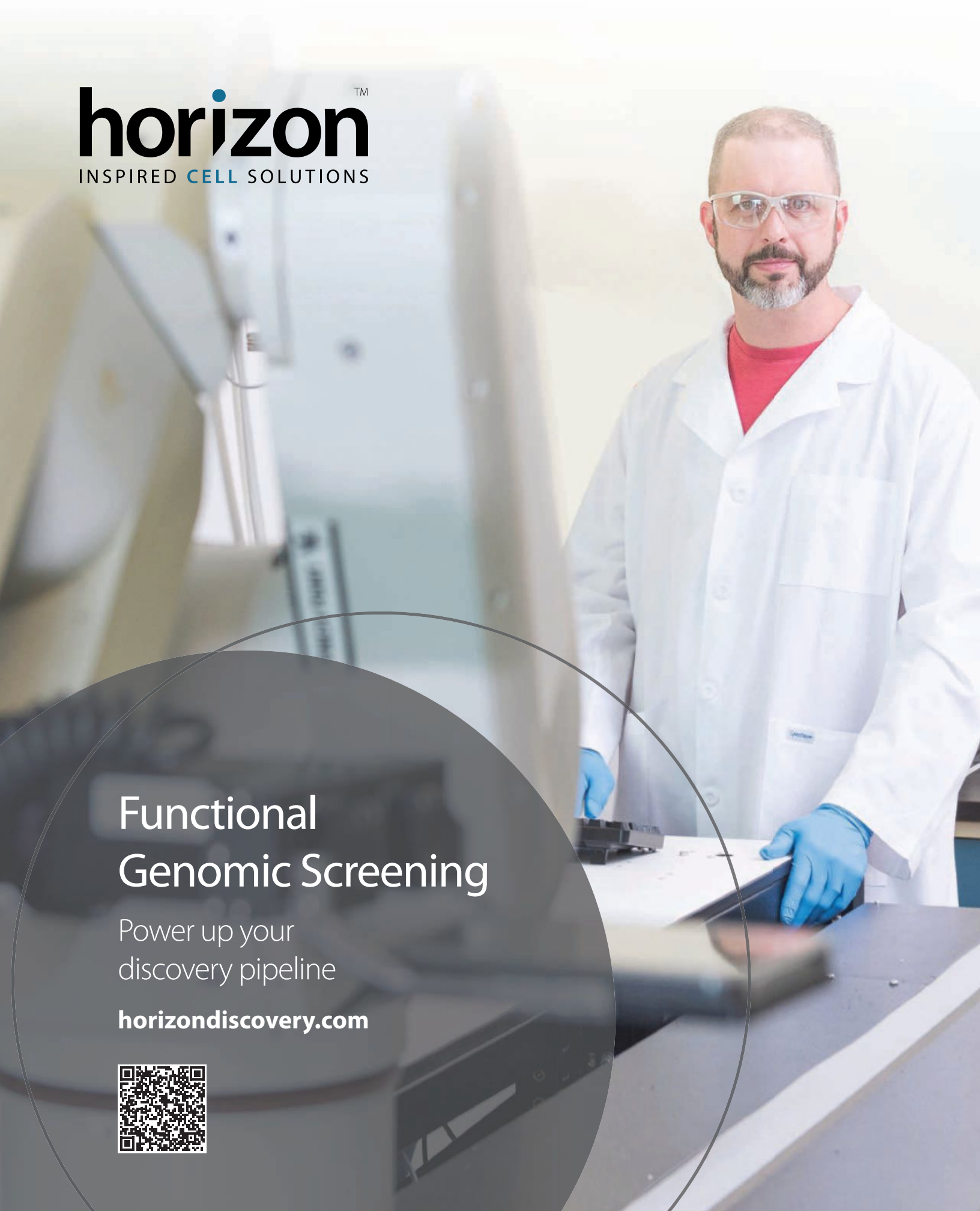
Wilson cited Stevens's "very interesting discoveries" in a footnote, but he was skeptical that these chromosomes determined sex. By the time of Stevens's death in 1912 at age 50, Wilson's scientific stature had overshadowed Stevens's groundbreaking theory. In 1909, Wilson concluded that the unequal chromosomes were indeed sex determinants. Following Henking's precedent, he called the large chromosome "X." For the small chromosome he chose "Y."

Improved microscopy techniques in the 1910s revealed sex-correlated chromosomes in other animals, including humans. By 1917, scientists were using X and Y to identify human sex chromosomes. But for decades, they couldn't agree on how to name the other human chromosomes; some used numbers, while others used letters. Nor did they agree on the number of human chromosomes, which are small, numerous, and difficult to count. In 1960, an international panel concluded there are 22 human autosomes, and they should be numbered in an ascending fashion, from shortest to longest. The two sex chromosomes, the panel advised, "should continue to be referred to as X and Y, rather than by a number, which would be an additional and ultimately, a superfluous appellation."

BRYN MAWR COLLEGE LIBRARY, SPECIAL COLLECTIONS; BIODIVERSITY HERITAGE LIBRARY, N.M. STEVENS, *STUDIES IN SPERMATOGENESIS PART II*

WHAT'S IN A NAME?: Cytogeneticist Nettie Stevens (top left) drew these images of beetle chromosomes in 1906, labeling one chromosome pair *"l"* and *"s"* in figures 102 and 107. These chromosomes would come to be known as the sex chromosomes, X and Y.

While using both letters and numbers to identify chromosomes may be confusing, this convention can be helpful. Robert Resta, a genetic counselor at Swedish Medical Center in Seattle, says the XY system helps his patients understand the inheritance patterns of sex-linked traits. "Patients remember X and Y from their high school or college biology courses, even if their understanding of it is imperfect," Resta says. "They are at least familiar terms that help make them a little more comfortable with technical genetic discussions." ■


horizon™
INSPIRED CELL SOLUTIONS
Functional
Genomic Screening
Power up your
discovery pipeline
horizondiscovery.com

MilliporeSigma

EXPLORE ANTIBODIES WITH CONFIDENCE

The path to discovery starts with an antibody that you trust.

Rely on the expertise and experience of our family of highly cited research antibodies. Our antibodies now encompass the products you know and trust from Millipore, Chemicon, Upstate, CalBioChem and Sigma-Aldrich.

Search with Confidence
SigmaAldrich.com/antibodies

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