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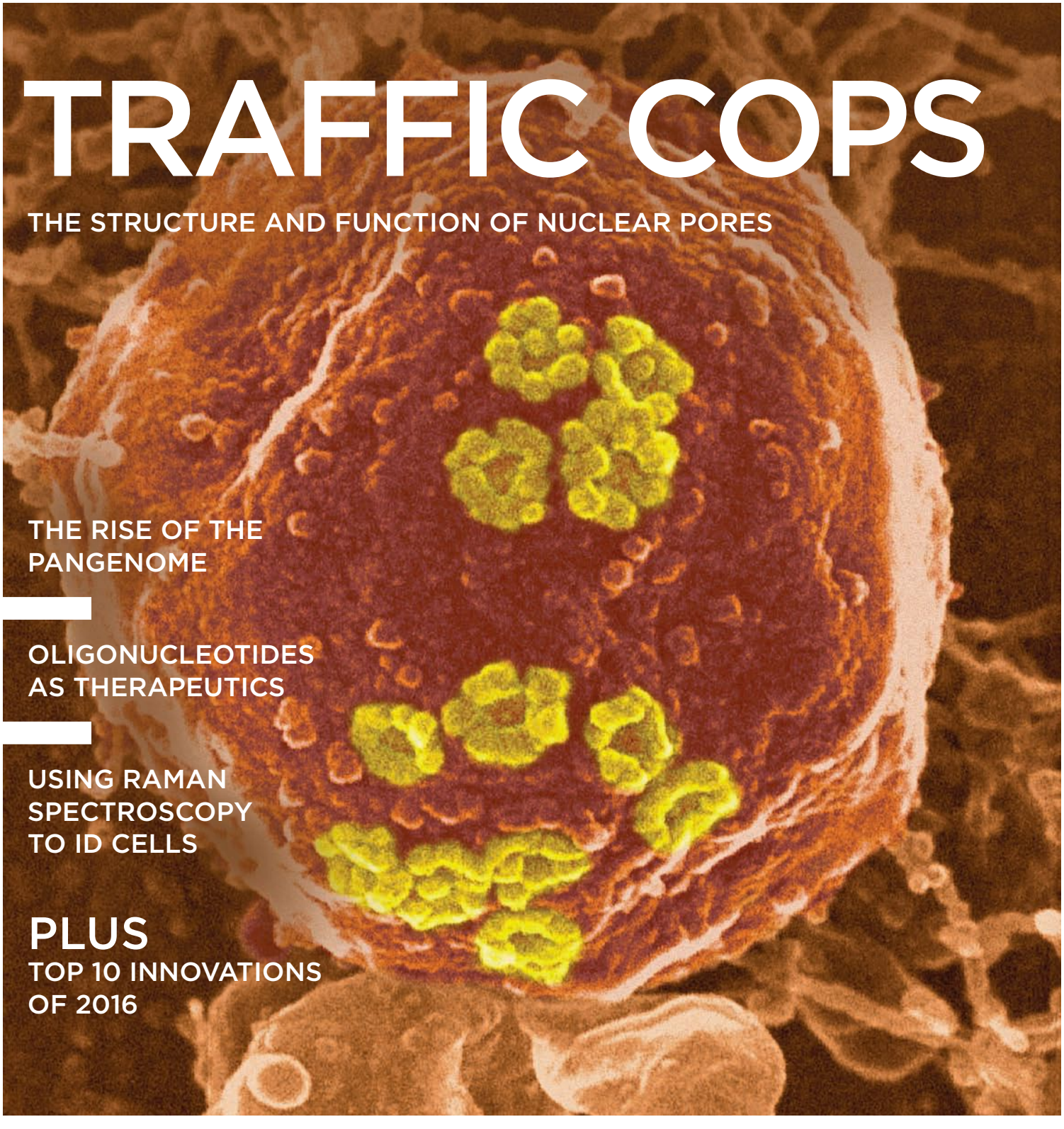

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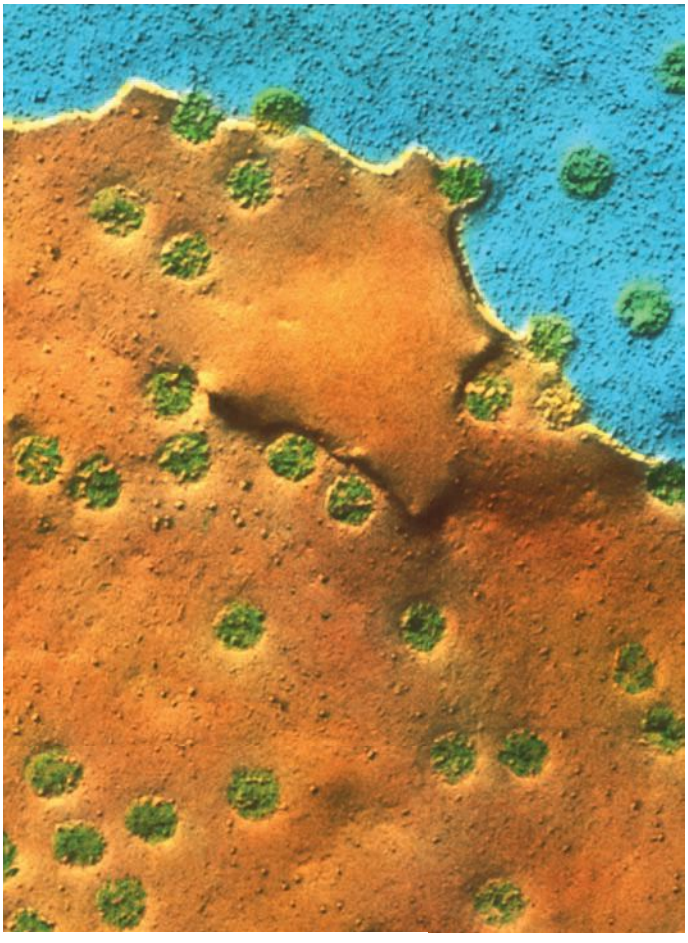

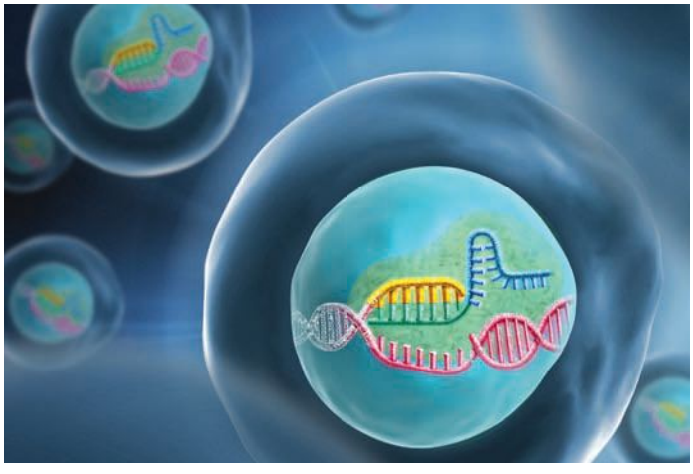

Features

ON THE COVER: © DR ELENA KISELEVA/SCIENCE SOURCE

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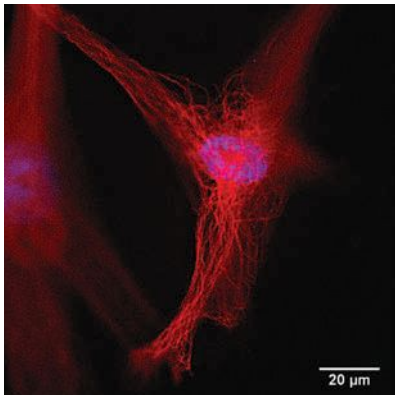

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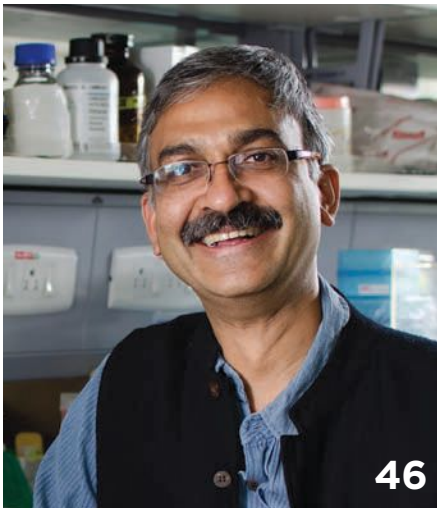
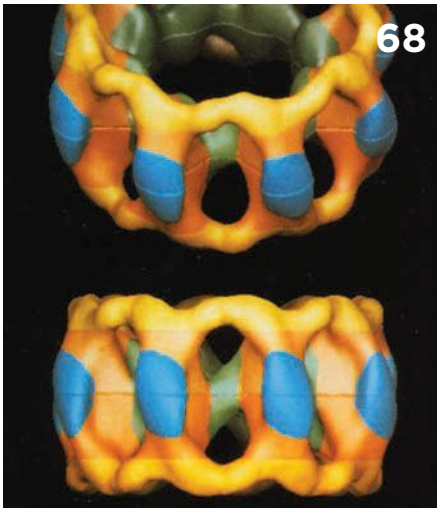


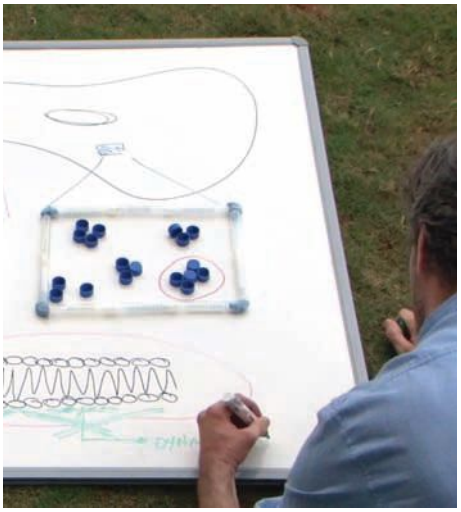


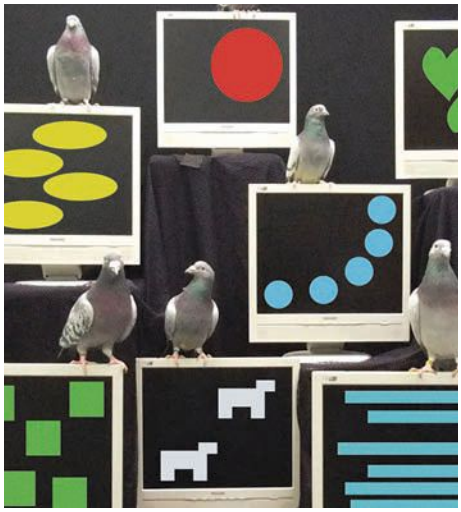
SALLY AITKEN; COURTESY OF SATYAJIT MAYOR; *CELL*, 69:1133-41, 1992, COURTESY RON MILLIGAN

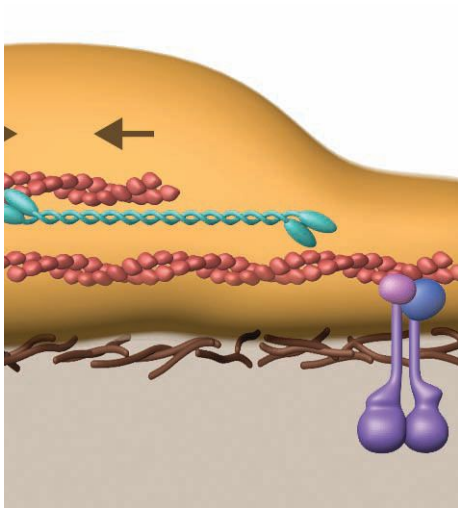
CORRECTION:
The print version of the 2016 Life Sciences Salary Survey (*The Scientist*, November 2016) failed to properly identify Paula Stephan. She is a labor economist at Georgia State University who studies the scientific workforce.

The Scientist regrets the oversight.

Online Contents







THIS MONTH AT THE-SCIENTIST.COM:

VIDEO
Cells, Skin Deep
Profilee Satyajit Mayor discusses his explorations of the plasma cell membrane, which are helping to update the classical fluid mosaic model of this dynamic cellular boundary.

SLIDE SHOW
Nerdy Birdies
Meet the pigeons that are redefining what it means to behave like a human by displaying remarkable pattern-recognition abilities.

VIDEO
Cells on the Move
Watch as physical forces guide the movement of a group of cells pulling themselves around specially designed petri dishes.

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

Coming in January

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

- Forensic anthropology, coming of age
- How gene drives could fight malaria
- Drug repurposing
- RNA G-quadruplexes
- PhDs and the biomedical job market problem

AND MUCH MORE

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Contributors

Born in Milwaukee, **Dan Lin** went to high school in a suburb of Chicago before he glimpsed his future scientific career at Washington University in St. Louis. A summer research project and a stint in a lab during his sophomore year oriented his interests toward research, which grew into a definitive focus on structural biology. Lin earned his degree in biomedical engineering, with extra study in biochemistry, graduating in 2011. As he was interviewing for graduate schools, André Hoelz was just setting up his lab at Caltech, and Lin felt it was the right fit. The nuclear pore complex is "just an amazing structural biology problem," Lin says, and a field steeped in questions that a young biologist could make his name answering. Progress in resolving the enormous structure has been staggeringly fast in Lin's few years, "beyond our wildest expectations," he says. Next year, he'll leave the lab to start a postdoc at the Whitehead Institute researching posttranscriptional gene regulation.

André Hoelz studied chemistry as an undergraduate at the University of Freiburg in Germany, and he wanted to apply this groundwork to biomedical research. "If you wanted to broaden your research interests . . . that was not easy to do in Germany," he says, but many more opportunities existed in the U.S. He came to Rockefeller University in 1997 to study the structure and function of protein kinases, working in the labs of John Kuriyan and Nobel Laureate Günter Blobel. Hoelz earned his PhD in 2004 and began to pursue an atomic model of the nuclear pore complex as a postdoc in Blobel's lab. He continued this ambitious work as a research associate and then as a research assistant professor, spending a total of 14 years at Rockefeller. Hoelz now runs his own lab at Caltech, where he continues to pursue not just the atomic structure of the nuclear pore, but the functional interactions of its many parts.

Lin and Hoelz offer a rundown of what's known and what remains to be solved about the nuclear pore complex in "Nuclear Comings and Goings" on page 24.

John Parrington first began writing about science during a media fellowship at *The Times*, where he leveraged his scientific background to pen stories about the latest advances in research. While there, he started researching the Human Genome Project and later wrote a book on the subject. His interests shifted then to a related topic: advances in genome editing, which became the subject of his second book, published this October. When he is not writing, Parrington studies the signaling pathways involved in egg cell activation and other key physiological events at the University of Oxford. Parrington arrived at the university in 2002 following a string of research fellowships that focused on the molecular mechanisms of fertilization. His next writing project will be a book exploring the science of human consciousness. "It's those big themes I seem to like," he reflects.

Read Parrington's essay based on *Redesigning Life: How Genome Editing Will Transform the World* on page 63.

Charles Strom is vice president of genetics and genomics at the Quest Diagnostics Nichols Institute in San Juan Capistrano, California, where he leads research into genetic tests for developmental disorders, including Down syndrome and Fragile X syndrome. He took his current position in 2002, before which he was both a practicing pediatrician and a researcher in the field of genetics. "My goal my whole life has been to apply state-of-the-art genetic techniques to help my patients," Strom says, with the particular goal of improving the accuracy of genetic testing while reducing its cost. He earned a PhD in biology and a medical degree from the University of Chicago, and has held faculty positions at Rush Medical College, University of Chicago, and University of California, San Diego, where he is now an assistant clinical professor. In recent years, he has begun research into cancer detection using next-generation sequencing and large-scale clinical databases.

Strom addresses the shortcomings and potential of clinical genetic databases in "Making the Grade" on page 20.

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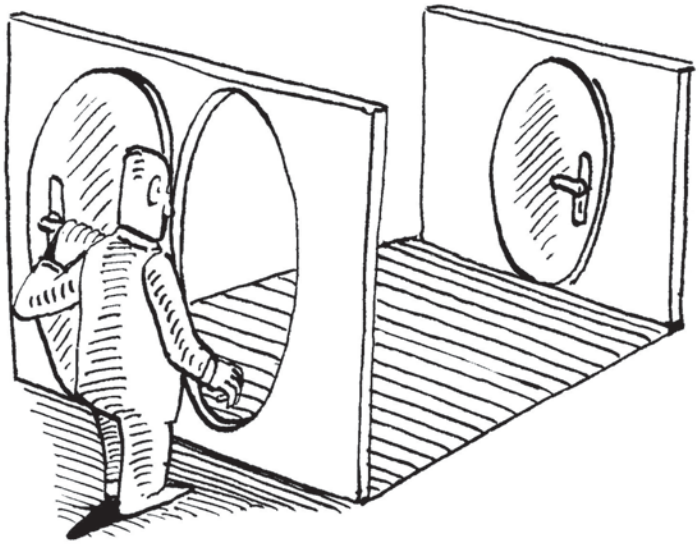
Doors and Pores

The awesome architecture of the gateways to the nucleus

BY MARY BETH ABERLIN



Hobbit houses, jigsaw puzzles, and nuclear pores have a lot more in common than you might think.

In 1937, J.R.R. Tolkien introduced his dearly beloved hobbits as "a little people, about half our height, and smaller than the bearded Dwarves." Their domiciles "had a perfectly round door like a porthole, painted green, with a shiny yellow brass knob in the exact middle. The door opened on to a tube-shaped hall like a tunnel." Not such a bad description of nuclear pore complexes either, those intricately constructed portals that pierce the double membrane enveloping the nucleus and control traffic into and out of the organelle. Of course, the aptness of the analogy has a lot to do with the level of magnification (not to mention imagination). But you can judge for yourself in this month's issue, with illustrations that go from a 3-D model pieced together from electron micrographs in 1992 (Foundations, page 68) to the zoomed-in photo on this month's cover and all the way down to the molecular twists and turns derived from X-ray crystallography in "Nuclear Comings and Goings" (page 24).

While Tolkien enthusiasts can YouTube how to construct a hobbit door (very tricky to hang, hinge-wise), structural biologists didn't design the object of their fascination—their task is to decode how it is built. Awe is the only appropriate response to the architectural masterpiece that is emerging.

Which brings me to jigsaw puzzles. I love doing them, the bigger the better. But the pieces I fit together are two-dimensional, and 3-D puzzles boggle my mind. Nuclear pore complexes raise jigsawing to a far more rarified level. In their feature, Daniel Lin and André Hoelz describe these "massive molecular machines" as put together from "more than 1,000 protein subunits with a total molecular mass of approximately 120 million daltons—the equivalent of more than 6.5 million water molecules." The subunits self-assemble to form more than 30 types of nucleoporin proteins, 17 of which constitute the beautifully symmetric three-ringed core of the nuclear pore complex. Through the pore's central channel smaller molecules enter and exit by diffusion while proteins or ribosomal units in their native states hop a ride on cargo ships known as karyopherins.

The study of another cellular envelope has been the research passion of Satyajit Mayor, this month's profilee ("Pushing Boundaries," page 46). His fascination with the organization and function of the plasma membrane began with his study of glycoproteins tethered to the outer surface of a parasite. He now investigates the nanoscale organization of such proteins and the role of the cytoskeleton in that organization.

All of these exciting discoveries owe much to technical innovations, which we take stock of in our ninth annual Top 10 Innovations competition. Assembled by Senior Editor Bob Grant, this year's panel of independent judges evaluated submissions of products or techniques new to the market from fall 2015 to fall 2016. Given the buzz surrounding CRISPR technology, it's no surprise that several products that facilitate the technique made the final list. Ditto for sequencing tools and methods that enhance the evaluation of new drugs. But the number one spot goes to a method for doing Western blots in single cells. (See "Top 10 Innovations of 2016," page 37.)

Whether it's zeroing in on single cells or individual nuclear pores, as more and more life science puzzles are fit together, we look forward to covering the story. ■

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

ANDRZEJ KRAUZE

Speaking of Science

We must keep funding scientific, technological, and medical research. And above all, we must embrace that quintessentially American compulsion to race for new frontiers and push the boundaries of what's possible. If we do, I'm hopeful that tomorrow's Americans will be able to look back at what we did—the diseases we conquered, the social problems we solved, the planet we protected for them—and when they see all that, they'll plainly see that theirs is the best time to be alive.

—President **Barack Obama** writing in the November issue of *Wired* about the importance of science innovation (October 12)

SCIENCE ADVOCATE IN CHIEF: President Barack Obama chats with entrants Evan Jackson, Alec Jackson, and Caleb Robinson from Flippen Elementary School in McDonough, Georgia, at the 2013 White House Science Fair.

Presidents have power over the direction of scientific research on a very large scale, which is an important factor for voters to consider when making their choices for elected officials.

—**Susannah Gal,** Penn State Harrisburg associate dean for research and outreach, on the impact a newly elected president can have on the direction of the US research enterprise (November 1)

So publishing more papers is like buying more tickets. And that's why you have a bigger impact during your more productive years.

—Statistical physicist **Roberta Sinatra** of Central European University in Budapest who coauthored a recently published *Science* paper that reported an analysis of more than 500,000 studies across multiple fields and suggested that dumb luck plays a big role in citation impact (November 3)

Our present analysis conclusively shows that females do have fewer distinct coauthors over their careers, but that this gap can be accounted for by differences in number of publications. We also find evidence for the hypothesis that female scientists are more open to novel collaborations than their male counterparts, a behavior that was shown to correlate with producing work of greater impact.

—**X.H.T. Zeng et al.,** authors of a recently published *PLOS Biology* paper that analyzed collaboration patterns by considering the publication records of nearly 4,000 faculty members from a variety of scientific disciplines (November 4)

I'm just trying to save people's lives, and now I'm being thrown into this abortion fight as a proxy. I have nothing to do with abortion, I don't encourage abortion—I just use tissue that would otherwise be discarded. And now I'm painted as this "baby killer" just for doing research as a medical student.

—**Eugene Gu**, a Vanderbilt University surgical resident who was the first to successfully transplant the (legally obtained) heart and kidney of a human fetus into a rat, on the Congressional investigation of his company, Ganogen (*The Huffington Post*, November 3)

Ralph Cicerone was a model for all of us of not only doing what counts, but doing it with honesty, integrity, and deep passion.

—**Marcia McNutt,** president of the National Academy of Sciences, remembering her predecessor, who died last month at the age of 73 (November 7)

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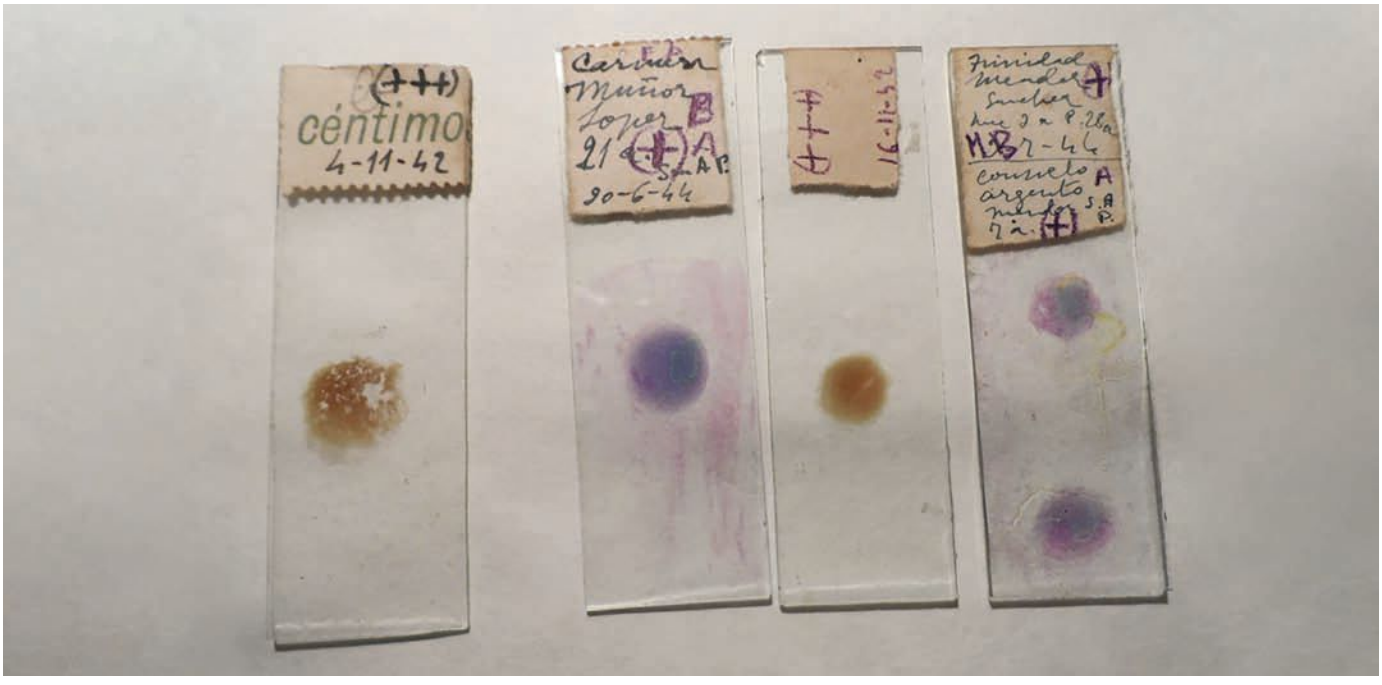
Notebook

DECEMBER 2016



BIOANTIQUES: These slides of stained blood droplets date to the 1940s and contain strains of the malaria parasite that are now extinct.

COURTESY OF CARLES ARANDA

Evolution, Cover-Slipped

The Ebro Delta is a low, marshy region in Catalonia, Spain, that borders the Mediterranean Sea—prime land for growing rice, but also perfect mosquito habitat. Like much of Europe, the area was long burdened with malaria until mosquito-control efforts following the Second World War drove down infection rates, eradicating the local strain of the parasite by 1964.

Decades later, scientists, like investigators at a crime scene, are trying to recover evidence of this lost European strain of malaria. The unicellular parasites responsible for the disease, *Plasmodium* spp., originated in Africa, scientists believe, and dispersed around the world by hitching rides in their human hosts. Just as globe-trotting humans fanned out from Europe in the colonial era, the now-extinct European strains of *Plasmodium* played a key role in malaria's spread.

Carles Aranda and Raul Escosa, government mosquito-control researchers based in Catalonia, devised a long-shot plan in the 1990s to recover this evolutionary missing link. During the eradication effort in the mid-20th century, the Spanish government conducted widespread malaria surveys to identify and treat affected communities. To confirm the pathogen's presence in an area, doctors inspected blood samples under microscopes. Aranda and Escosa wondered if anyone had saved a few of those old microscope slides.

The search for slides paid off two decades later when Escosa met the family of Ildefonso Canicio, a doctor who had led eradication efforts in the Ebro Delta. After Canicio's death in 1961, his daughter and son-in-law held on to his medical equipment, including some slides bearing droplets of blood from malaria patients. The family agreed to donate three of those slides, their yellowing labels dating them between 1942 and 1944.

Aranda and Escosa contacted geneticists and malaria experts to help them analyze the find. Carles Lalueza-Fox, a paleontologist for the Institute of Evolutionary Biology at Pompeu Fabra University, Barcelona, spearheaded the task of extracting readable DNA from the historical slides, drawing on his previous studies of genetic material in ancient human skeletons. "I am used to samples that are difficult, but these [malaria] samples, even though they are much more recent, were of a limited size—they are just blood drops," Lalueza-Fox says. In addition, *Plasmodium* cells were mixed in with human blood cells. "I was not sure if I would be able to extract DNA from the pathogen." So the team tried a few different approaches.

The researchers hit pay dirt when they used DNA "baits," molecules of biotin bound to short strings of RNA, to latch onto human DNA in the samples. They filtered the samples through beads coated in a substance that binds to biotin and sifted out the baits along with the DNA to which they had attached.

Lalueza-Fox and his colleagues were left with the first genetic material of extinct European *Plasmodium* species ever studied. They recovered DNA from both *P. falciparum*, the predominant species in Africa and the species responsible for the majority of today's malaria deaths, and *P. vivax*, a less virulent species found widely across the globe. Scientists have long debated how *P. vivax* arrived in the Americas, with one theory suggesting colonial Europeans brought the pathogen over, while another posits entry from the other direction, when early humans crossed the Bering Land Bridge from Asia. The two theories, notes Jane Carlton, a malaria researcher at New York University who was not involved in the study, are not necessarily mutually exclusive.

The extinct European *P. vivax* genome more closely resembled strains found today in South America than those found in East Asia, lending credibility to the theory of a more recent introduction by European colonists. The European *P. falciparum* genome, however, was starkly divergent from the modern South American subtype, supporting the theory that this more-deadly species came to South America directly from Africa during the slave trade. The team published its findings in *PNAS* this October (113:11495-500, 2016).

"You have to understand the pathogens and how they got there to understand the disease," Lalueza-Fox says. "It's crucial to understand the diversity and the history to try to get a global picture of the disease."

But while he and his colleagues reassembled most of the *Plasmodium* mitochondrial genomes, they could not capture the complete nuclear genomes due to the limited amount of blood on Canicio's old slides. So they're searching for other historical specimens. "I have discovered there is a whole market for antique microscope slides on the Internet," Lalueza-Fox remarks. A day after their paper was published, he saw a set of five malaria slides go up on eBay for a few dollars. They were dated to England, 1931. "I tried to buy them, and at the last moment of the auction, someone overpriced me and took them!" Until more slides surface on the Internet, he and his colleagues are exploring other sources, such as university medical collections and mass graves containing the skeletons of plague victims.

Piecing together the *Plasmodium* family tree arms researchers with information about the pathogen they are trying to erase, malaria researcher Richard Culleton of Nagasaki University wrote in an email to *The Scientist*. "If we can better understand the speed and capacity of the parasite to evolve and adapt to new populations, then we will be better placed to predict where it might go in the future, and how it will react to the implementation of new drugs and vaccines."

—Ben Andrew Henry

Bird Brains

After completing an undergraduate psychology course taught by Michael Colombo at the University of Otago in New Zealand, Damian Scarf was hooked on animal cognition. Colombo told Scarf how he and other behavioral researchers were demonstrating that nonhuman animals seemed to possess cognitive abilities that researchers had previously considered to be exclusively human. "Testing these human-unique abilities just seemed awesome, so I switched from zoology to psychology the following year," Scarf recalls.

Scarf completed his PhD work in Colombo's lab and is now a lecturer at the University of Otago, where he continues to test the ability of nonhuman animals to display traits supposedly unique to humans. Most of his work involves birds, which have repeatedly upended the concept of human uniqueness. For example, scientists have thoroughly documented the ability of Caledonian crows to use tools, a skill long believed to be employed

only by humans. Similarly, researchers have shown that scrub jays remember past events and act accordingly. "And when it comes to numerical discriminations or word discriminations, pigeons have taken them all," Scarf says.

Showing that pigeons have, to a remarkable degree, the ability to process relations between letters in allowable or not allowable sequences is in my view extremely interesting.

—Alex Kacelnik, University of Oxford

This year, Scarf, Colombo, and their colleagues tested pigeons' ability to recognize patterns of letters that appear in the English language. Nearly every day for two years, Scarf trained four pigeons. He would place the birds in a box with a touch screen, and then present the animals with either a real or fake four-letter word, along with a star below the letters. If the word was real, the birds were to touch it with their beaks; if it was fake, they were to touch the star. If the subjects answered correctly, they would get a bit of wheat. At the end of the training, the pigeons were able to recognize dozens of words—including ones they had never seen before—with about 70 percent accuracy (*PNAS*, 113:11272-76, 2016).

"It's quite a novel finding," says Alex Kacelnik, who studies comparative cognition at the University of Oxford. "Showing that pigeons have, to a remarkable degree, the ability to process relations between letters in allowable or not allowable sequences is in my view extremely interesting."

Pigeons, of course, do not use written language, but Scarf suspects that they are accustomed to picking up patterns of visual objects. "The plasticity that seems to be inherent in not only the visual cortex of primates but also the visual cortex of pigeons makes them code letter pairs maybe like they would have coded object combinations or object features in the environment," he says. Whether they are using the same part of the visual cortex that humans use to process words, however, remains to be seen, Scarf adds. "We do have plans to look, using electrophysiology, to see whether this is all localized to one part of the visual cortex."

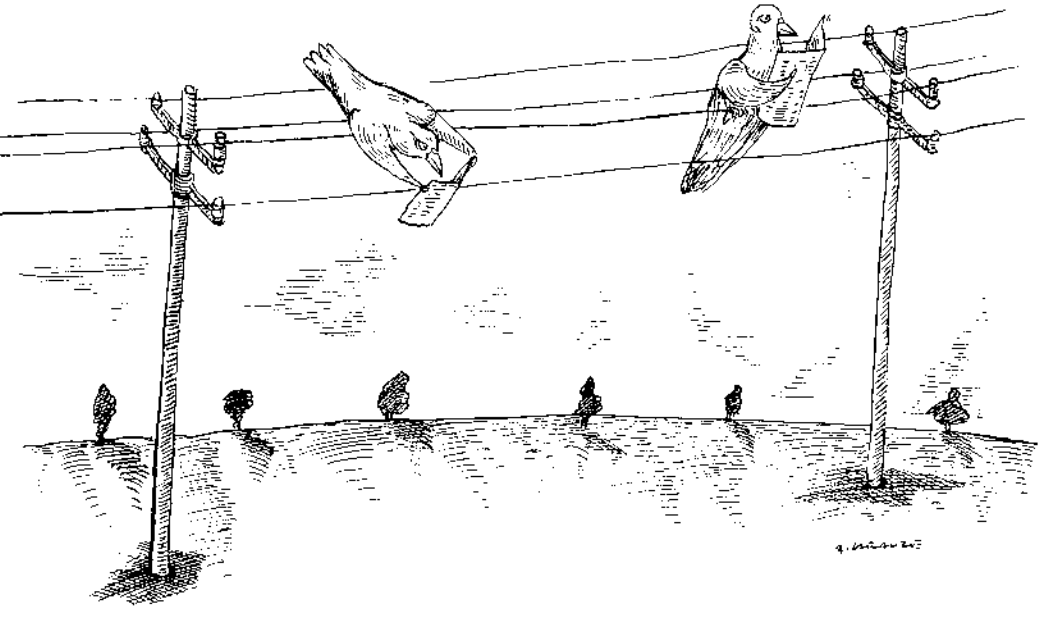
Understanding how birds are capable of performing such advanced mental feats has proved tricky. For instance, in 1998, Nicky Clayton of the University of Cambridge and her colleagues found that scrub jays only searched for cached perishables soon after they had stored them, suggesting the birds think about the future and plan accordingly, but exactly how they do it remains a mystery. "We know they are thinking about it, but we don't know how they are," says Clayton. "The way humans do it, we subjectively travel back in the mind's eye to remember those events and subjectively travel forward in the mind's eye to think about the future. And in doing so we're aware of the passage of time, and we're aware of the subjective nature of self. We don't know if that's how animals are doing it."

In some cases, it's obvious that the birds are processing stimuli differently than humans are. Just this year, for example, Kacelnik and his University of Oxford colleague, zoologist Antone Martinho, showed that newborn ducklings imprinted the relative sizes and colors of two different objects: if they saw two equal-size objects when they were born, they will follow equal-size objects, even different ones, later in life (*Science*, 353:286-88, 2016). "This is a quite striking ability," Kacelnik says. Such relational concepts are thought "to be only available to highly intelligent animals with a high level of training, and these animals do it in 15 minutes after they come out of the egg."

Indeed, no two species are the same, Kacelnik says. "All species have a common ancestor and share many common processes, but they have also evolved special abilities that are definitely not the same between species," he says. "Humans cannot navigate home as a pigeon does, and a pigeon cannot play chess as humans do. Claiming that there are no differences is not helpful."

But Scarf plans to keep his eyes open for more claims of such differences, and in particular, of uniquely human abilities. "There'll be something else that comes out that people promote as [human- or] primate-unique," he says, "and we'll do the same thing we always do, which is try and test it in pigeons."

—Jef Akst



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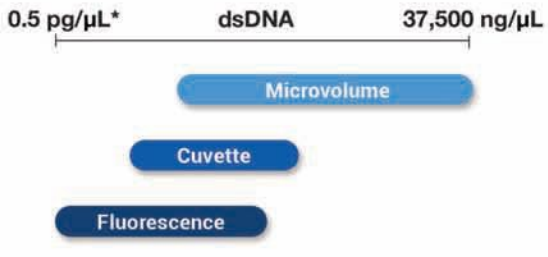

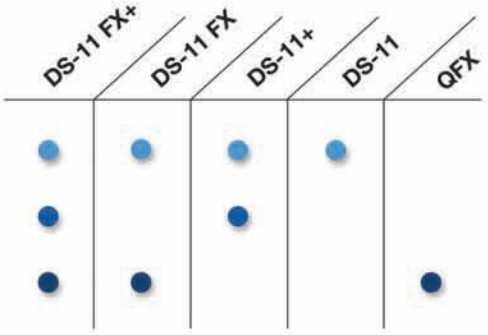

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Living in Giant Footsteps

Wolfram Remmers, a graduate student at the University of Koblenz and Landau in Germany, had always wanted to visit a rainforest. So when the chance to take a summer field course with a nongovernmental organization, the Tropical Biology Association in Kibale National Park, cropped up in 2014, he leapt at the opportunity. "It was my first time in Africa," Remmers says. "I was always fascinated in the biology of the tropics—and I was not disappointed!"

Spread over nearly 300 square miles in southern Uganda, Kibale National Park is famous for its diverse primate communities, as well as sizeable resident populations of big cats, birds, and the world's largest land animal, the African bush elephant. The park has been a study site for more than four decades, providing data on everything from lion conservation to wild-grown coffee harvesting. But while exploring the swampy regions of Kibale's tropical rainforest with his peers between classes, Remmers stumbled upon something that had never before been documented in Uganda, nor in any other African environment.

After five days, we could already take more than 400 individual insects out of the 18 buckets. It was amazing.

—Wolfram Remmers
University of Koblenz and Landau

It all began with a puddle. "We came across these elephant footprints while we were walking through the forest," he explains. "I was surprised to see that, even in the dry season, these footprints were filled with water."

Remmers knew from having studied aquatic ecology that small animals—particularly invertebrates—use pools of stagnant water as habitats and breeding grounds. And a study in 2010 had found that the water-filled footprints of elephants are used for breeding by mosquitoes in India (*EcoHealth*, 7:498-506, 2010). But most research on the environmental impact of elephants has focused on more direct effects, from the animals' dispersal of seeds through their guts or invertebrates on their skin, to their trampling of vegetation and erosion of landscapes. Remmers says he wondered whether elephants may be playing a larger, indirect role in invertebrate ecology if their footprints are actually inhabited by these little creatures.

Remmers teamed up with two other students and set out into the forest. They used a sieve to sample 30 elephant footprints over the course of three days. Their work turned up more than 2,700 specimens, including water beetles, mosquitoes, and dragonfly larvae, representing 61 distinct taxa, including 27 families or

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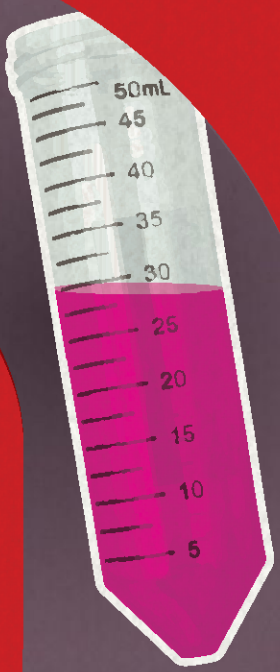

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7. SPRAY C
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5. REMOVE CELLS FROM LIQUID NITROGEN AND PLACE IMMEDIATELY ON DRY ICE

6

6. THAW CELLS IN A 37 °C WATER BATH FOR 2 MINUTE WHILE GENTLY AGITATING

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8

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ROLL AGAIN

8. PIPET CELLS FROM THE VIAL AND PUT INTO A CENTRIFUGE TUBE DROPWISE

Warning! Never pour your cells directly from the cryovial into the medium.

11

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A POWER OUTAGE!

YOUR INCUBATOR SHUT OFF LAST NIGHT, LEAVING YOUR CELLS IN THE COLD. THAW A NEW VIAL.

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9. RINSE CELL VIAL WITH MEDIUM TO COLLECT REMAINING CELLS

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15. PLACE IN INCUBATOR AND CHECK CELLS 24-48 HOURS LATER

15

13

13. QUANTIFY CELLS USING A HEMOCYTOMETER OR CELL COUNTER

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14

14. PLATE CELLS AT THE RECOMMENDED DENSITY

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BIG SHOES TO FILL: University of Graz undergraduate researcher Isabella Schaberl passes a water-filled elephant footprint, home to rich communities of tiny invertebrates, in Kibale National Park, Uganda.

COURTESY WOLFRAM REMMERS

orders (*Afr J Ecol*, doi:10.1111/aje.12358, 2016). "It's a preliminary study," notes elephant researcher Gary Haynes of the University of Nevada, Reno. "But when you look at the fact that they identified 61 taxa—and there's probably a lot more they haven't yet identified—that's a lot."

Remmers and his team didn't stop there. "We wanted to know how quickly—and in general how—[invertebrates] were colonizing the footprints," he says. Because existing footprints were already colonized, he and his fellow researchers decided to make their own. Armed with buckets and digging equipment, the three students laid out six transects perpendicular to a stream; then, they sank buckets into the earth—three per transect at various distances from the stream—and filled them with water to create a set of footprint-size puddles. "It was a lot of work," Remmers acknowledges. "We had a pickaxe and a shovel, and we dug 18 of those holes and carried the water from the stream to fill [them]."

The backbreaking effort paid off. By taking samples from the artificial footprints, the team observed the colonization of these pools in real time and found that it took place surprisingly quickly—particularly in footprints close to the stream, notes Remmers. Within hours, the team found water beetles paddling around in several of the recently constructed footprints, and spiders spinning webs over the pool's surface. After five days, "we could already take more than 400 individual insects out of the 18 buckets," Remmers says. "It was amazing."

The findings both are and are not a surprise, notes Western Kentucky University's Bruce Schulte, a biologist who studies herbivorous mammals and had previously found that vegetation damage by elephants could create habitat for amphibians and reptiles in Northern Tanzania (*Afr J Ecol*, 49:133-40, 2010). "It makes sense that these organisms would take advantage of these types of pools that the elephants create," he says. "But what struck me was the scale. Elephant footprints are big relative to other species' footprints, but you don't really think of them as being a microcosm of aquatic macroinvertebrates."

Although data were collected over only a few days, Remmers says that community composition differences between old and recently made natural footprint pools suggests a sort of succession—similar to how plant communities change with age or time from last disturbance. But researchers will need to collect longer-term data to confirm this hypothesis. For now, Schulte notes, the work provides only a snapshot. Remmers says he hopes to collect such data after completing his PhD, both on the long-term dynamics of footprint communities and on the importance of these pools in invertebrate ecology.

In the meantime, the team's research provides food for thought, not to mention further incentive for elephant conservation, notes Haynes. "Elephants are ecosystem engineers," he says. "But there's a whole lot of things about elephants we don't know much about." The current study reflects that observation, Haynes says, and adds yet another facet of elephant-driven ecosystem engineering to the list.

"Anyone driving past the forest would notice the large-scale changes [elephants make], like the clearing out of vegetation and so on," Haynes says. "But the little things in the footprint, that's a whole other dimension. It's one more thing that you can think of elephants having a hand in—or I guess a foot in, in this case."

—Catherine Offord

Bugs in the Methane Paradox

Until 2008, a simple molecule—one carbon flanked by four hydrogens, aka methane—had oceanographers scratching their heads. Methane levels are supersaturated on ocean surfaces, meaning they are higher than expected given atmospheric concentrations. Yet, scientists only knew of methanogenesis in anaerobic archaea deep down on the oxygen-depleted ocean floor. The abundance of methane in oxygen-rich surface waters became known as the "methane paradox," a curiosity with potential implications for global warming given that the greenhouse gas moves easily from supersaturated water into the atmosphere.

But the source of methane underlying the paradox remained unsolved until David Karl at the University of Hawaii and colleagues discovered that laboratory-grown

aerobic marine microbes can break down methylphosphonate and produce methane (*Nat Geosci*, 1:473-78, 2008). Four years later, researchers found an archaeon that produces the starting material for this decomposition, methylphosphonic acid, and, in 2014 researchers identified marine bacteria that use the molecule to make methane. While this series of discoveries offered an explanation for the marine methane paradox, it did not address a similar phenomenon seen in lakes.

Numerous freshwater systems also have methane in their upper, oxygen-rich layers, and, like oceanographers, limnologists didn't know how it got there. Even out in the middle of the Great Lakes, methane is supersaturated, says Paul del Giorgio of the University of Quebec in Montreal. "Very far from anoxic sediments, very far from shore, it's not obvious where the methane comes from," he says. Researchers raised a variety of possibilities: perhaps it blows in from anoxic waters near the edges of lakes; maybe there are anoxic micropockets in the upper layers where methanogens can do their thing; or perhaps aerobic microbes are releasing methane like those in the ocean.

Several years ago, Hans-Peter Grossart of the Leibniz-Institute for Freshwater Ecology and Inland Fisheries in Germany and colleagues produced evidence supporting the third idea: that methane production can occur in oxic lake water. In the laboratory, they incubated water samples from the upper levels of nearby Lake Stechlin, a clear body of water in northwestern Germany that reaches 70 meters deep, and observed methane concentrations increase (*PNAS*, 108:19657-61, 2011).

"We really had problems to get [the paper] accepted because we were acting against the current paradigm that methane is only produced in anoxic environments," says Grossart. "We showed there is internal production, but we couldn't well define the processes. That was the problem."

Earlier this year, Julia Maresca of the University of Delaware filled in some of the missing pieces of the methane puzzle. She had been studying how microbes obtain phosphorus in an unusual lake in Indonesia. At nearly 600 meters deep, Lake Matano is one of the deepest lakes in the world. It is also rich in iron and poor in phosphorus. Maresca thought it might be possible that, to get the phosphorus they need, bacteria there break down methylphosphonate just like microbes do in oxic ocean waters.

ROLLIN' IN THE DEEP: A view of the Indonesian village of Sorowako from Julia Maresca's research vessel floating on iron-rich Lake Matano

To find out, she and her colleagues motored for more than an hour to the middle of the clear, blue lake and collected water samples from 10 meters below the surface. They froze the samples in a kitchen-turned-laboratory at a house in a nearby mining town and then brought them back to the U.S. for metagenomic analyses. Genes coding for enzymes responsible for producing phosphate and methane from methylphosphonate popped up in the results (*Appl Environ Microbiol*, doi:10.1128/AEM.02399-16, 2016). "What we saw was that pathways for phosphonate degradation seemed to be pretty enriched," says Maresca.

Her team took a closer look at one gene in particular, *phnJ*, which is responsible for cutting the carbon-phosphorus bond and finally releasing methane. The Alphaproteobacteria, Gammaproteobacteria, and Actinobacteria Maresca's group isolated from Lake Matano express *phnJ* when methylphosphonate is present and produce methane as long as phosphate isn't around. Presumably, the microbes do not need this pathway if phosphate is abundant.

Maresca's group also found homologs of *phnJ* present in numerous other metagenomic data sets from freshwater systems, including Lake Stechlin. "This might be a pretty common mechanism," she says.

One outstanding question is how much this mechanism contributes to the methane levels observed in lakes' oxic waters. "It's interesting that they identified this process," says David Bastviken of Linköping University in Sweden. "It may be important, it may not be. We do not know."

Bastviken says he suspects that horizontal transport of methane from shallow, anoxic zones toward surface waters above deeper zones may be the main source of methane at freshwater surfaces. Frank Peeters, head of the environmental physics group at the University of Konstanz's Limnological Institute in Germany, also points out that the mechanism Maresca identified operates in low-phosphorous conditions, and "most lakes have substantially larger phosphorus concentrations than the limiting value." It is possible, he added in an email, that the depth at which temperature and oxygen concentration change drastically—the thermocline and oxicline, respectively—change seasonally. "The high methane concentrations typically found in the deep water might extend to depths shallower than 100 meters during some years or seasons," he speculates.

Maresca says it will be important to figure out how much these various methods contribute to methane levels at the surfaces of freshwater systems to better model their contributions to global methane cycling. "Globally, freshwater produces a lot of methane and we don't know how much and how [these bodies of water] do it."

—Kerry Grens

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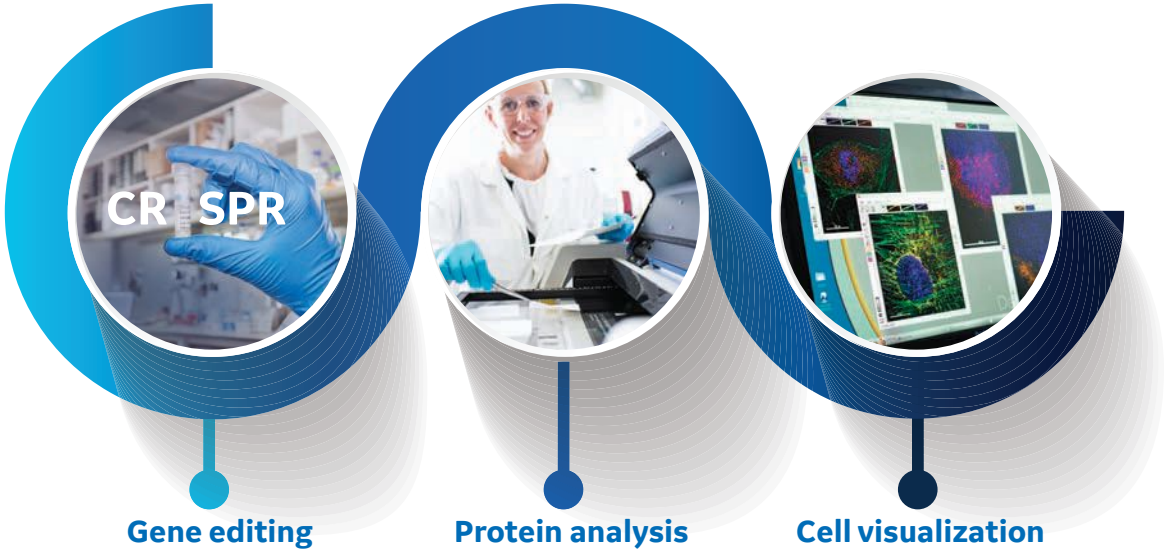

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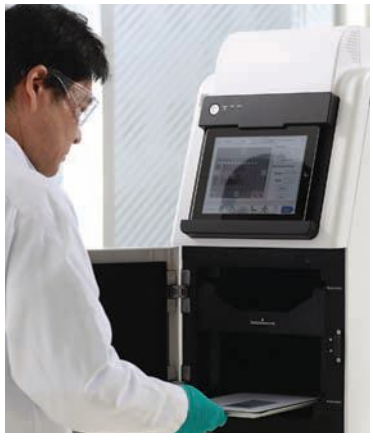

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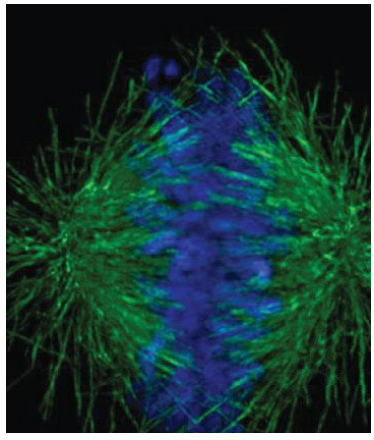

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Making the Grade

Sorting out which data sets are clinical-grade is key to helping patients.

BY CHARLES M. STROM

In the face of unprecedented amounts of sequencing data generated by the genetic and genomic revolution, researchers have established a dizzying number of databases aiming to harness this new information. Sharing and pooling of such data have accelerated scientific progress, but questions remain about how the results will impact medical practice. Each of these databases has a different purpose and different structure—and different data. It is vital that oncologists and other health-care providers, who increasingly rely on genetic databases for information to predict patients' inherited cancer risk, understand that these databases are not equivalent, and that only some contain clinical-grade data.

To detect rare variants, robust databases must include information beyond prevalence, such as family segregation studies, coinheritance patterns, in silico prediction, and functional studies.

To understand the differences in databases, it is first necessary to know how gene variants correlate with pathogenicity. In general, variants are classified into one of five categories: known benign, likely benign, variant of uncertain clinical significance, likely pathogenic, or known pathogenic.

The most rudimentary variant databases allow for pathogenicity assessment based on prevalence in a given patient population (e.g., women with breast cancer) compared with prevalence in an unaffected control population. This approach requires that disease-linked variants occur with sufficient frequency to allow meaningful statistical comparisons. While this is suitable for common variants, it is less useful for rare variants, such as the many missense mutations where one amino acid is substituted for another in the highly variant genes *BRCA1* and *BRCA2*.

To detect rare variants, robust databases must include information beyond prevalence, such as family segregation studies, coinheritance patterns, in silico prediction, and functional studies. And, the databases must be regularly updated, such as when research reveals that a variant of uncertain significance is more likely to be benign or pathogenic. They should also account for differences in how laboratories classify a variant.

Curation differences are another concern. Curation is the process of monitoring data quality, completeness, and consistency. Curation can be prospective or retrospective, automated or manual. With prospective curation, contributors upload data into a quarantined area where curation occurs prior to allowing entry to the database. Retrospective curation allows open data uploads, after which attempts are made to identify duplicate or incorrect entries and other necessary changes. The word curation is perhaps used too loosely at times; employ caution when considering databases to ascertain the degree of curation performed.

One final hurdle to taking advantage of clinical-grade variant databases is ease of use. Identifying relevant information on a variant can require considerable expertise when a database consists of thousands or millions of variant entries.

A concern about open databases is that curation is minimal or nonexistent prior to database entry. In these cases, curation must be done retrospectively.

Two archetypical variant databases are ClinVar, sponsored and maintained by the National Center of Biotechnology Information, and the Leiden Open Variant Database (LOVD). ClinVar and LOVD are open databases that allow an individual to enter a variant. The submission may be as simple as a representation of an allele and its interpretation. It may also include the pathogenicity assessment assigned to that variant by the contributor, along with literature references and other information used for the assessment. (See "The Genetic Components of Rare Disease," *The Scientist*, July 2016.) Other recent *BRCA* databases, such as that championed by the American Society of Human Genetics and the University of Washington, may also help contribute understanding about *BRCA* science, if appropriate curation is in place.

A concern about open databases is that curation is minimal or nonexistent prior to database entry. In these cases, curation must be done retrospectively. If a mistake is identified, it is the contributor's responsibility to correct the entry. For instance, ClinVar staff do not review all submissions, and the interpretation of the relationship of variation to health is provided only by the submitter. ClinVar and LOVD are laudable efforts to advance genetic research, but their utility as references, particularly for clinical use, is currently limited.

BRCA Share, which I helped launch in 2014, is designed to overcome some traditional limitations to variant databases. The database focuses on *BRCA1* and *BRCA2*, genes that contain tens of thousands of unique variants. New and rare variants continue to be discovered, and the link between each and a woman's risk of breast and ovarian cancers is still being resolved.

BRCA Share is based on the Universal Mutation Database (UMD) developed by INSERM, the French National Institute of Health and Medical Research. For more than a decade—while *BRCA* testing in the United States was limited to a single commercial laboratory thanks to a patent on the gene variants and testing technology—more than a dozen laboratories in France were performing *BRCA* testing and contributing their data to the UMD. Manual prospective curation is performed by INSERM's curation team prior to uploading data to the UMD. Supporting data, such as family studies, prevalence in international databases, and occurrence in UMD (curated to eliminate duplicate entries from the same family), are also available.

BRCA Share is free to scientists, physicians, and others with a research focus; commercial laboratories must pay a fee on a sliding scale. After little more than a year of existence, BRCA Share has led to the identification of 334 pathogenic or likely pathogenic variants and classifications of 375 variants whose role in cancer risk was previously uncertain (The BRCA Share Consortium, 6th Biennial Meeting of The Human Variome Project, June 2016).

Beyond BRCA Share, other genetic databases show promise for clinical use, including BRCA Challenge and ClinGen. How well these initiatives evolve will shape the course of genetics research—and the quality of genetic patient testing—for years to come. ■

Charles M. Strom was one of the principal architects of BRCA Share. He is a full-time employee of Quest Diagnostics, and he owns stock in Quest Diagnostics. Quest Diagnostics is a founding member of BRCA Share.

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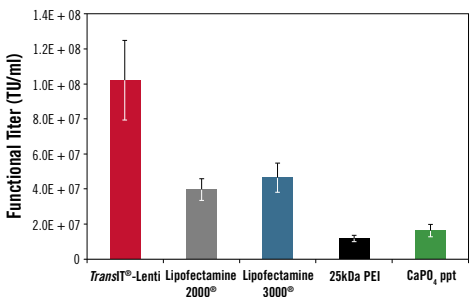

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Synthetic Sensors

Engineered circuits detect endogenous transcription factors to drive cellular outputs.

BY RUTH WILLIAMS

Some synthetic biology applications use cells as mere hosts for engineered genetic circuits—for example, when cells act as factories for desired molecules. For other applications, however, researchers would like to integrate the synthetic circuits with the cell's own pathways.

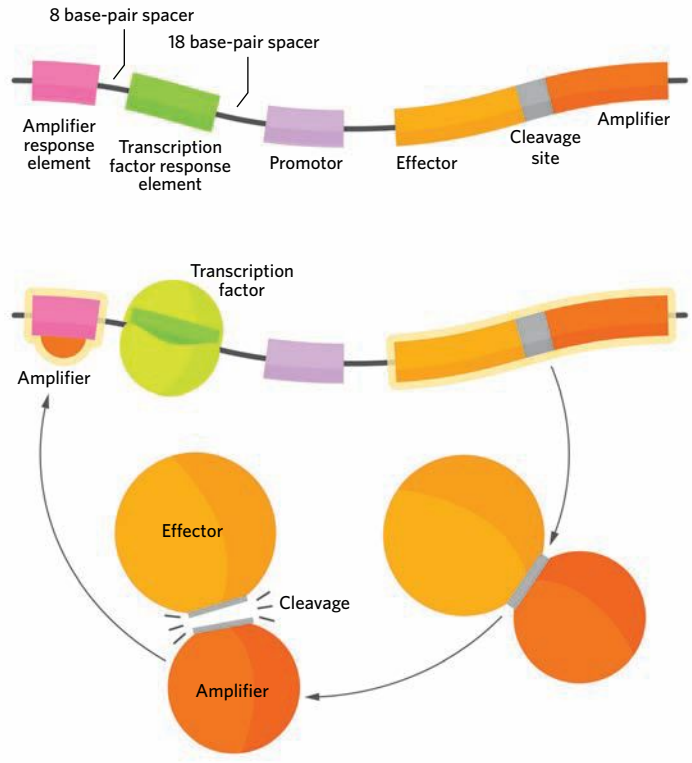
Such integrated systems could be used to sense particular molecules and induce appropriate responses. For instance, detection of a metastasis-inducing protein in a cancer cell might be used to trigger that cell's suicide. "The concept is that these synthetic circuits will be able to read out bits and pieces of information from the cell and interpret them to make decisions and drive the cell in different directions," says Yaakov "Kobi" Benenson of the Federal Institute of Technology, or ETH, in Zurich.

Benenson and colleagues have recently generated genetic constructs—or "transducers"—that can detect the presence of specific transcription factors in human cells and, in response, activate synthetic processes—such as the expression of particular proteins, the silencing of mRNAs (via microRNA production), or the induction of targeted genetic recombination.

The constructs are similar to tissue-specific expression vectors, in which promoters with cell type–specific activity are used to drive transcription of a desired gene or genes. The difference, however, is in the constructs' precision design and ability to detect cell states, not just cell types, says Benenson.

Having established key design principles, the team was able to create transducers that detect a variety of different transcription factors. In one scenario, for example, detection of the developmental transcription factor Sox10 drove Cre recombinase activity and, in turn, the recombination-dependent expression of a fluorescent reporter.

The nice thing, says biological engineer Ron Weiss of MIT, is that "in principle, [the constructs] are relatively modular so you can plug-and-play" with the particular factor you want to detect. (*Cell Rep*, 16:2525-37, 2016) ■



SYNTHETIC CIRCUIT: In this example of a typical transducer, the genetic construct (top) includes an amplifier response element and a carefully positioned transcription factor response element upstream of a promoter that drives expression of a fusion gene (the combined effector of choice and an amplifier). In the presence of a specific endogenous transcription factor, which binds to the transcription factor response element, the fusion gene is expressed. Cleavage of the fusion protein releases the amplifier, which together with the transcription factor drives much stronger expression. The system is like a positive feedback loop, but neither the transcription factor nor the amplifier alone can drive strong expression—they need each other.

AT A GLANCE

INTRODUCED ELEMENT	DESIGN	SPECIFICITY	UTILITY
Cell-specific expression vectors	DNA sequence contains a cell-type specific promoter and the gene, or genes, of interest.	Low. Promoter region may bind many transcription factors.	Expressing a gene of interest in a desired cell type, e.g., production of a fluorescent protein for observation of a specific tissue under a microscope
Transcription factor–specific transducers	DNA sequence contains one or more carefully designed transcription factor response elements, a minimal promoter, an amplifier system, and the gene, or genes, of interest.	High. These constructs are designed to detect one, or a combination of, specific transcription factors.	Inducing a particular designed activity in response to the presence of one or more specific transcription factors. Unlike cell-specific promoters, these constructs can be turned on during discrete cell states, including transient conditions.

DOTTED WITH PORES: This colored freeze-fracture transmission electron micrograph shows part of the nuclear envelope of a mouse liver cell. Nuclear pore complexes (green) penetrate both the inner (blue) and outer (brown) membranes, regulating transport into and out of the nucleus.

Nuclear Comings and Goings

Solving a long-standing structural puzzle will open the door to understanding one of the cell's most enigmatic machines.

BY DANIEL H. LIN AND ANDRÉ HOELZ

Eukaryotic cells store their DNA in the nucleus, cordoned off from the cytoplasm by the nuclear envelope. Made up of two lipid bilayers called the inner and outer nuclear membrane, the nuclear envelope protects DNA from damage by reactive by-products and intermediates of cellular metabolism. It also serves as a critical regulator of gene expression, restricting access to the genome and dictating which transcripts can exit the nucleus. This regulatory responsibility ultimately belongs to the thousands of massive molecular machines that penetrate both nuclear membranes to form gateways between the nucleus and cytoplasm.

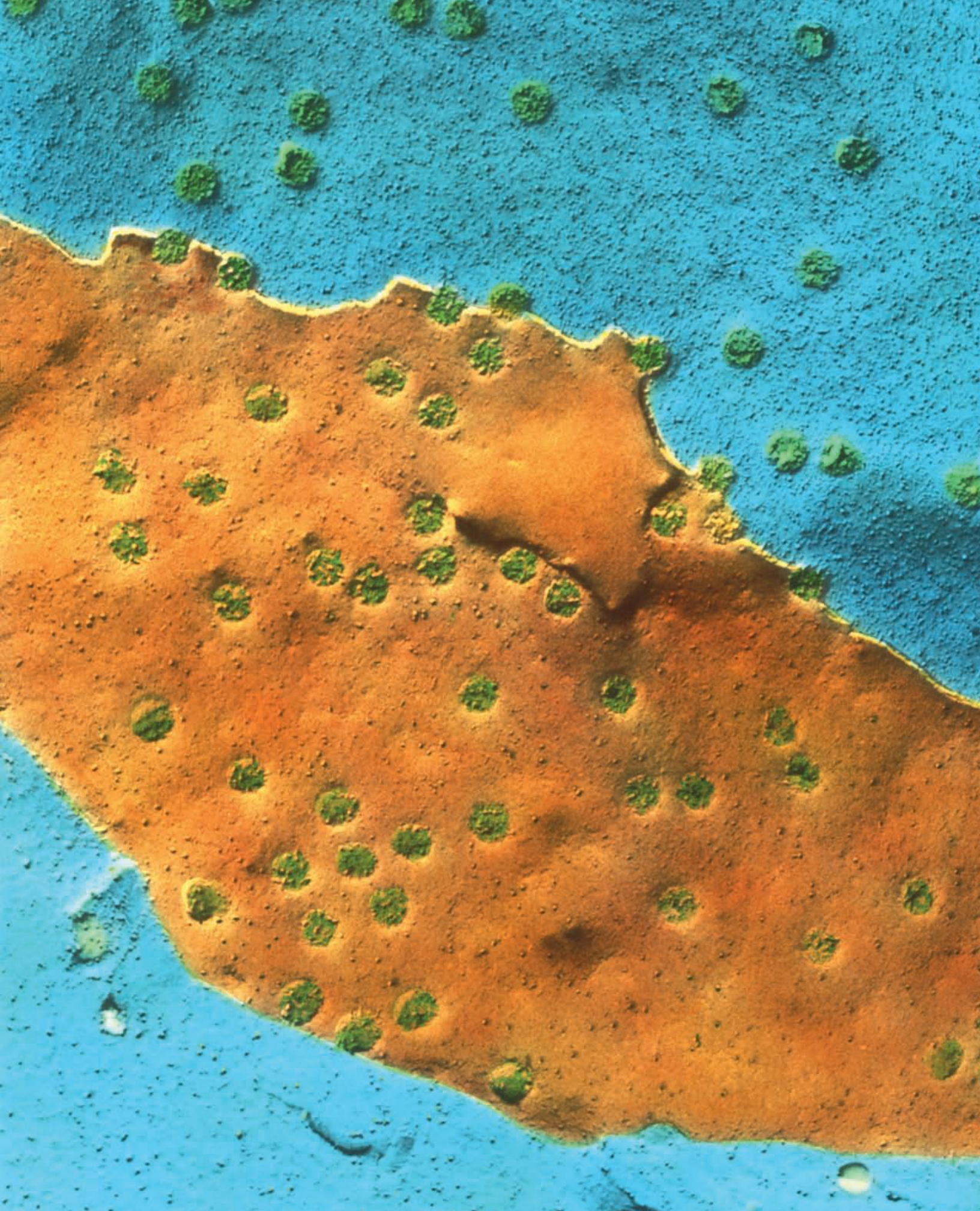
Each nuclear pore complex (NPC) consists of more than 1,000 individual protein subunits with a total molecular mass of approximately 120 million daltons—the equivalent of more than 6.5 million water molecules. The size and complexity of the NPC has prevented any single technique or experiment from revealing its entire structure in detail. Over the last decade, however, technological advances have spurred an explosion in data on the structure of the NPC. Improvements in electron microscopy have led to snapshots of NPCs in their native environment at moderate, but improving resolution. Simultaneously, X-ray crystallographic analyses of the individual protein subunits and their interactions with one another at the atomic level have given us high-resolution details of how the pieces fit together. And this year, the powerful combination of these two approaches finally revealed the structure of the NPC's symmetric core.[1,2] Continued interrogation of the structure and function of the NPC will open the door to a deeper understanding of one of the cell's most important machines.

NPC structure

In 1950, Harold Callan of the University of Edinburgh and S.G. Tomlin of King's College London used electron microscopy to observe tiny pores in the nuclear envelope.[3] Nine years later, Michael Watson of the University of Rochester described the protein complexes embedded in those pores.[4] In 1982, the first NPC protein, called a nucleoporin, was identified using a monoclonal antibody raised against purified rat nuclei. The 414 antibody, which is still in use today, was subsequently found to be reactive to an entire family of nucleoporins found in a variety of organisms, from yeast to humans. Over the next two decades, researchers used genetic screens to identify many more nucleoporins in yeast. (See "The Pattern of a Pore, 1992" on page 68.) By 2000, scientists had verified approximately 30 different constituents of the yeast NPC, and improvements in mass spectrometry brought the total number of unique nucleoporins to around 34, depending on the species.[5] The vast majority of nucleoporins are conserved in eukaryotes, suggesting that NPCs emerged in a proto-eukaryotic ancestor as it first developed nuclei.

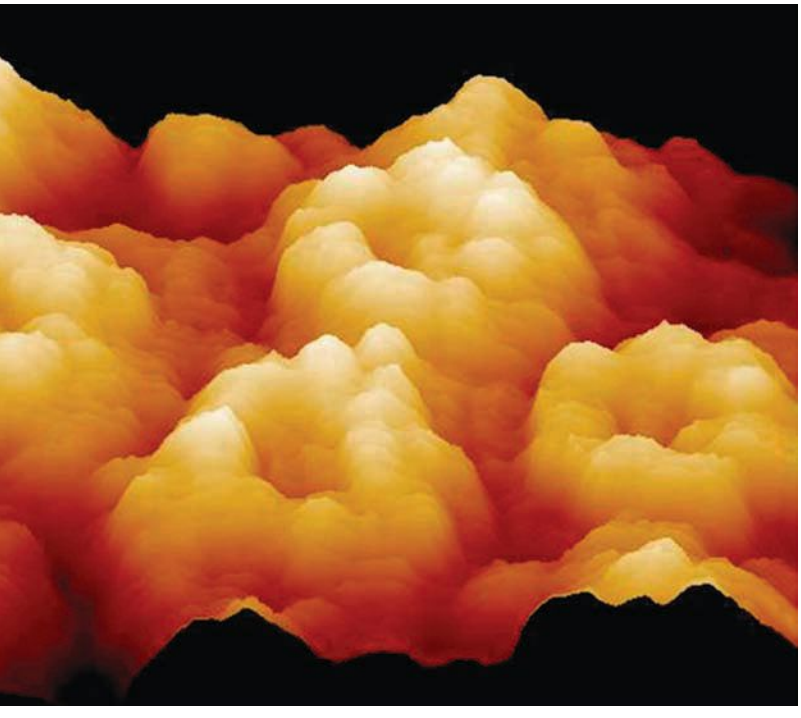
The impressive size of NPCs arises as a consequence of extensive symmetry. Early electron micrographs showed that NPCs possessed eightfold rotational symmetry around the central transport channel. In addition, the cytoplasmic and nuclear halves of the interior core of the NPC are identical but rotated 180 degrees relative to each other. (See illustration on page 27.) The interior core is therefore known as the symmetric core.

The symmetric core is composed of three major ring structures: an inner ring that surrounds the central transport channel and two outer rings that sit on either side of the nuclear envelope.



Inside the nucleus, nucleoporins specific to the nuclear side of the NPC attach to the symmetric core and form a nuclear basket, which interfaces with chromatin and the transcription machinery. On the cytoplasmic side, a different set of nucleoporins form flexible structures known as the cytoplasmic filaments, which are involved in facilitating transport through the NPC.

This year, we and our colleagues at the California Institute of Technology published our work detailing the molecular architecture of the symmetric core.[1] In the same issue of *Science*, an international team led by Martin Beck and colleagues at the European Molecular Biology Laboratory (EMBL) in Heidelberg, Germany, described its own efforts to elucidate the NPC's structure.[2] Electron microscopy reconstructions done by Beck's group and high-resolution crystal structures determined by our group and others provided the frame and jigsaw pieces, respectively. But to confidently piece together the structural puzzle, we also needed a detailed understanding of the biochemistry of nucleoporins and the network of protein-protein interactions that link them. Beck and colleagues used crosslinking reagents to create covalent bonds between protein atoms that sit directly adjacent to each other in the NPC, and they performed mass spectrometry to identify the cross-linked nucleoporins. Our group tackled the problem by purifying the individual nucleoporins and building up the components of the NPC piece by piece to understand the rules of how they assembled. As a result of these complementary approaches, we now know the relative orientation and position of essentially all 17 nucleoporins that constitute the symmetric core in the human NPC.



RUGGED TERRAIN: This false-colored atomic force micrograph of the surface of a nucleus shows several nuclear pore complexes rising from the nuclear envelope like cavernous mountains.

One critical role of this architecture is stabilizing the extreme curvature of the nuclear membranes in the nuclear pores. In order to form a pore through the nuclear envelope, the nuclear envelope's inner and outer lipid bilayers must fuse together in a U-shape. Because lipid bilayers prefer to be flat at microscopic scales, the membrane curvature needs to be stabilized by protein complexes, the most well-studied of which exist in the membrane coats that encapsulate small vesicles involved in protein trafficking between intracellular membranous organelles. Atomic-resolution crystal structures of nucleoporins have revealed similarities to complexes found in vesicle coats, including proteins such as Sec13 that are present in both. And, just like proteins in vesicle coats, the majority of nucleoporins are not transmembrane proteins and do not make contact with the nuclear envelope.

Each nuclear pore complex consists of more than 1,000 individual protein subunits with a total molecular mass of approximately 120 million daltons—the equivalent of more than 6.5 million water molecules.

Taken together, the structural details of the NPC provide us with a much better understanding of the molecules and forces that hold such a crucial machine together and how it is able to operate a portal between the nucleus and cytoplasm. Importantly, we are now able to design targeted studies to probe how the NPC interacts with other cellular machineries and processes.

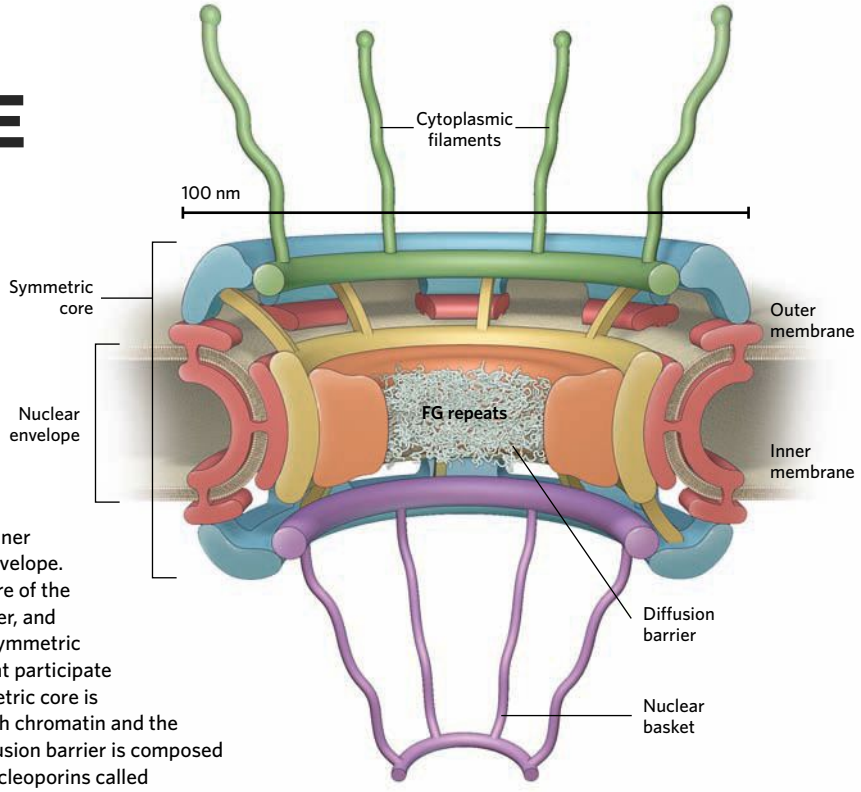
Molecular transport through the NPC

Unlike many channels and transporters, the 100-nanometer-wide NPC does not open and close to regulate transport. Instead, it generates a passive barrier that prevents diffusion of molecules larger than about 3 nm in diameter, while allowing selected cargoes many times larger (up to 40 nm) to move through. The diffusion barrier is generated by several nucleoporins that contain stretches of amino-acid sequences called phenylalanine-glycine (FG) repeats. Such FG repeat regions are intrinsically disordered and show a strong propensity for self-assembly. Projection of the repeats into the central transport channel creates a mesh-like protein barrier that prevents passive diffusion of macromolecules larger than approximately 40 kilodaltons, while still facilitating the rapid transport of large cargoes.

In contrast to transport into the endoplasmic reticulum or the mitochondrion, which requires that proteins be unfolded to pass through the organelle's membrane translocation machinery, macromolecules are transported through the NPC in their native state. Proteins called karyopherins recognize specific molecules and also bind to the FG repeats, allowing them to shuffle the over-

THE NUCLEAR PORE

Nuclear pore complexes (NPCs) are huge molecular structures that penetrate the nucleus's two lipid bilayer membranes and mediate the transport of macromolecules into and out of the cell's command center. The structure of the NPC, which consists of more than 1,000 individual protein subunits, is coming into sharper focus, and biologists now have a better understanding of the function of this massive molecular machine.

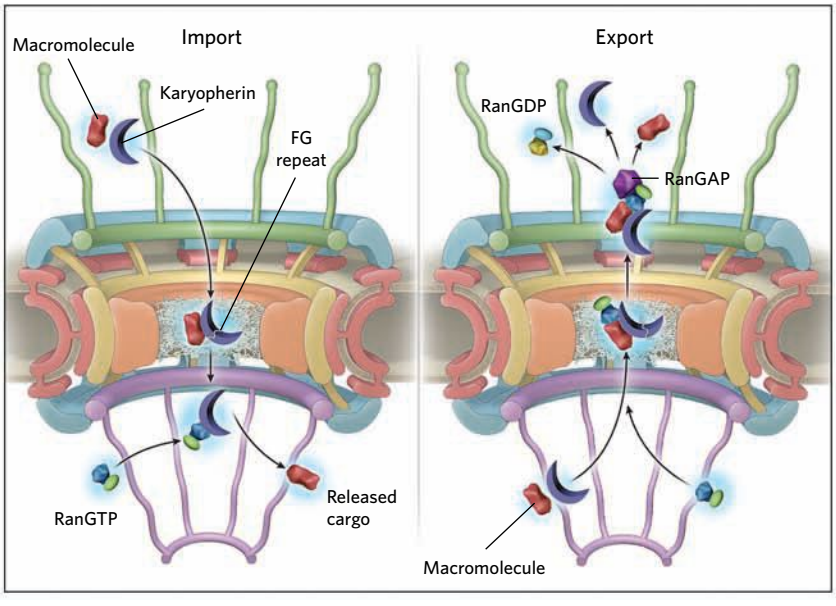


STRUCTURE

The human NPC is composed of approximately 34 nucleoporin proteins. The interior, or "symmetric," core is composed of an inner ring, and two outer rings that sit on either side of the nuclear envelope. The NPC symmetric core helps to stabilize the extreme curvature of the nuclear membranes abutting the pore, forms the diffusion barrier, and provides a central transport channel. On the outside, the NPC symmetric core is decorated with proteins called cytoplasmic filaments that participate in protein transport and mRNA export; on the inside, the symmetric core is associated with nuclear basket nucleoporins, which interact with chromatin and the transcription machinery. In the central transport channel, a diffusion barrier is composed of extensive amino-acid regions found in about a third of the nucleoporins called phenylalanine-glycine (FG) repeats, which are intrinsically disordered and self-assemble into a mesh-like network that prevents passive diffusion of macromolecules.

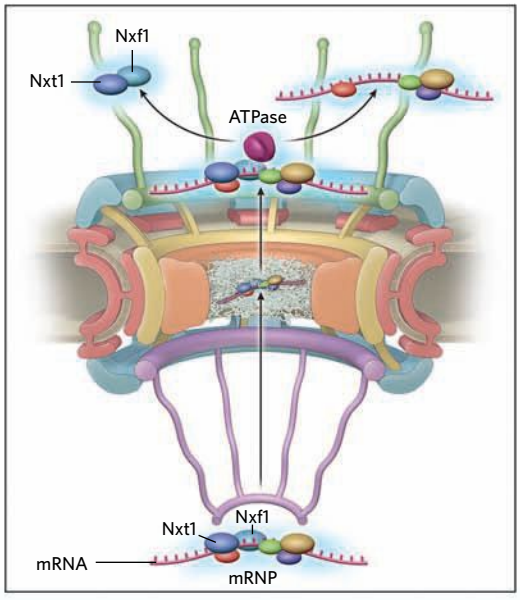
FUNCTION

MACROMOLECULE TRANSPORT



Macromolecules—including proteins, tRNAs, and even fully assembled pre-ribosomal subunits—are transported through the NPC in their native states with the help of proteins called karyopherins that bind to both target molecules and to FG repeats. Upon entering the nucleus, incoming karyopherins release their cargo when they are bound by RanGTP, the GTP-bound conformation of a small GTPase protein called Ran. For cargoes leaving the nucleus, RanGTP is often incorporated into karyopherin transport complexes inside the nucleus, but encounters the Ran-activating protein RanGAP after exiting. RanGAP triggers Ran to hydrolyze GTP into GDP, causing a conformational change and the release of the cargo into the cytoplasm.

mRNA EXPORT



Messenger RNAs (mRNAs) transcribed in the nucleus are loaded with diverse proteins to form messenger ribonucleoproteins (mRNPs) that are exported through the NPC. These proteins include Nxf1 and Nxt1, which bind to FG repeats in the central channel. Nucleoporins on the cytoplasmic side of the pore recruit and activate an ATPase to remove Nxf1/Nxt1, freeing the mRNA to be translated by the ribosome.

sized molecules across the nuclear envelope.[6] The karyopherins are a large protein family, including at least 19 known members in humans and 14 in budding yeast. Each family member recognizes its own set of cargoes, which can include proteins, tRNAs, and even pre-ribosomal subunits. Most karyopherins specialize in either import or export, though some karyopherins can mediate both.

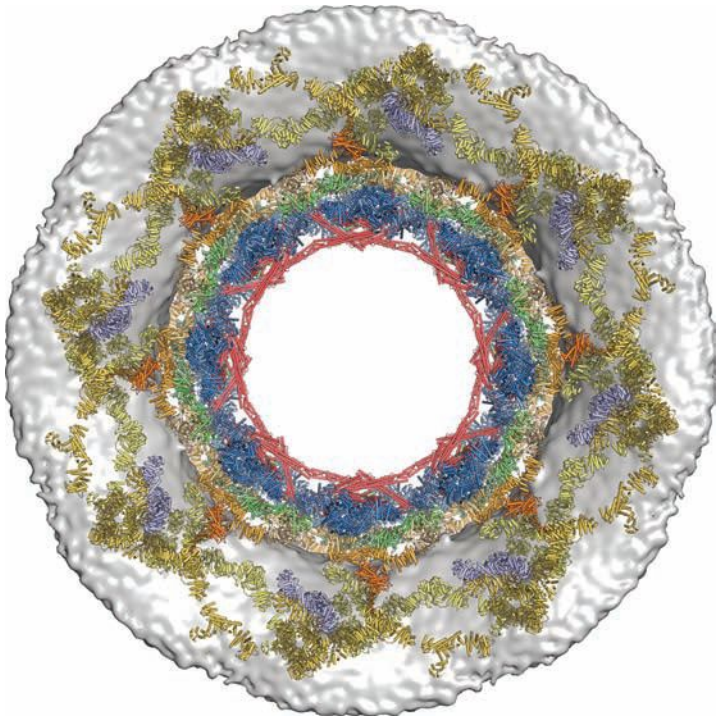
The best-characterized method of cargo recognition involves short amino acid motifs known as nuclear localization signals (NLSs). First identified in viral proteins, NLSs are also used by cellular proteins. The presence of an NLS in a protein is a strong predictor for nuclear localization, and synthetic sequences are now commonly used to ensure the nuclear localization of engineered proteins. Cargoes can also be linked to karyopherins through adaptor proteins, and many macromolecules are recognized by their three-dimensional folds. Thus, while several canonical pathways for regulating nuclear import or export are now well understood, the specific mechanisms that control the localization of many proteins remain unknown.

Scientists do understand what dictates the directionality of transport, however. For most proteins and some small RNAs that depend on karyopherin-mediated transport, their release on the appropriate side of the NPC is determined by what is known as the Ran gradient. Ran is a small GTPase protein that binds and hydrolyzes GTP to GDP and adopts different conformations in its GTP- and GDP-bound states. The Ran-activating protein Ran-

Unlike many channels and transporters, the 100-nanometer-wide NPC does not open and close to regulate transport.

GAP is localized to the cytoplasmic filaments on the outside of the NPC. In contrast, the guanine exchange factor called RCC1, which facilitates the exchange of GDP for GTP in Ran, localizes to chromatin in the nucleus. As a result, Ran is activated almost exclusively in the cytoplasm, and thus exists there in the GDP-bound form (RanGDP), while it remains in its GTP-bound conformation (RanGTP) in the nucleus. These two conformations interact with karyopherins to regulate the uptake and release of nuclear pore cargo.

When a karyopherin shuttles cargo from the cytoplasm into the nucleus, it encounters RanGTP, which binds to the karyopherins in a manner that is mutually exclusive with cargo binding. Because of the high concentrations of RanGTP, imported cargoes are released into the nucleus. In contrast, export complexes require RanGTP for their assembly. But, the complex of karyopherin, cargo, and RanGTP is in a strained conformation, as if spring-loaded. After exiting the nucleus, export karyopherins encounter RanGAP, which stimulates Ran's GTPase activity. The bound GTP is hydrolyzed into GDP, causing Ran to adopt a conformation incompatible with karyopherin binding, which in turn triggers the karyopherin to release its cargo into the cytoplasm.



In contrast to molecules that are transported by karyopherins, the export of mRNAs occurs independently of the Ran gradient. Prior to export, mRNAs undergo several processing steps and are loaded with many proteins to form export-competent messenger ribonucleoproteins (mRNPs). One set of added proteins is a transport factor composed of the proteins Nxf1 and Nxt1. Similar to karyopherins, Nxf1/Nxt1 bind to FG repeats and pass through the diffusion barrier, shuttling the mRNP through the NPC. Nucleoporins on the cytoplasmic side of the pore recruit and activate an ATPase to remove Nxf1/Nxt1, freeing the mRNA to be translated by the ribosome.

Many viruses have developed ways to subvert regulated nucleocytoplasmic transport, either by interfering with bulk mRNA export or by developing novel ways to hijack existing transport pathways. HIV-1 genomes, for example, hijack karyopherin-mediated transport by encoding a specific RNA sequence that is recognized by the HIV-1–encoded protein Rev, which serves as an adaptor to karyopherins. Together, the genomic RNA and Rev are exported to the cytoplasm like any other karyopherin-dependent cargo. An alternative strategy is exploited by some simian retroviruses, which encode a specific RNA element that is recognized by Nxf1/Nxt1, so that their RNA genomes are exported through the canonical mRNA export pathway. Both approaches help the virus bypass the regulatory steps in the nucleus that normally ensure only the correct mRNAs are exported from the nucleus.

Other roles and links to disease

While a number of facets of transport through the NPC are now well understood at a mechanistic level, much remains to be decoded about the NPC's many other cellular functions. For

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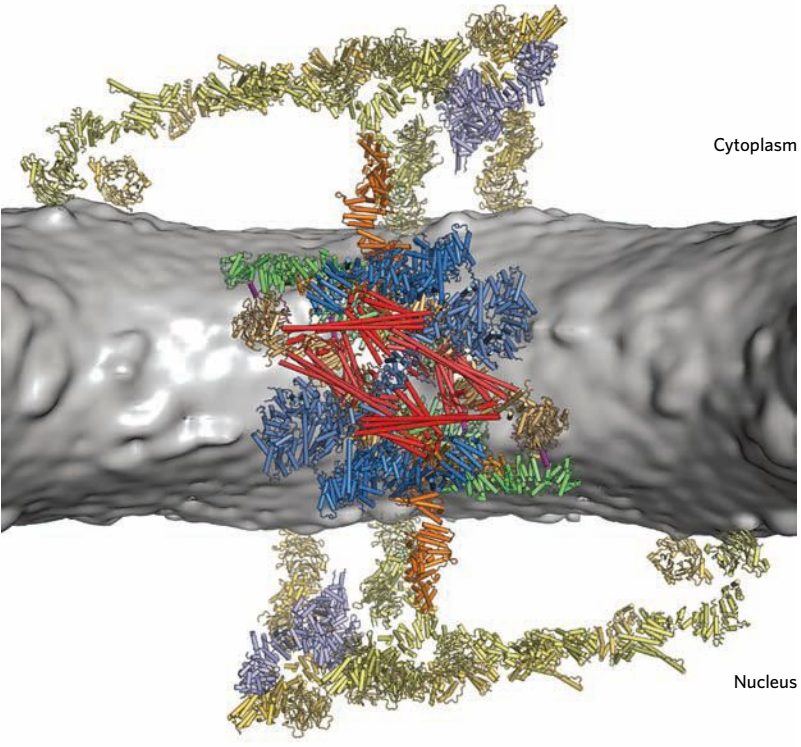

NPC ARCHITECTURE: These composite structures of the nuclear pore complex, which were generated by piecing together high-resolution crystal structures of the protein subunits, show its multiple levels of symmetry. Eight identical "spokes" evenly spaced around the pore give the pore its eightfold rotational symmetry (as seen in the top view, left), while the cytoplasmic and nuclear faces of the pore's symmetric core exhibit twofold rotational symmetry across the nuclear envelope (gray surface).

example, there is growing evidence that NPCs play an important role in regulating gene expression.[7] Inactive heterochromatin is localized to the nuclear envelope, while more actively transcribed genes are found near the periphery of the NPC, possibly to enhance the efficiency of mRNA export. Additionally, many nucleoporins and even karyopherins have been found associated with chromatin, and they appear to be directly involved in transcriptional regulation. The exact mechanism and consequences of these emerging functions are exciting avenues for future research.

Many nucleoporins and factors involved in nucleocytoplasmic transport also appear to have functions outside the nucleus. For example, the nucleoporin Sec13 is an essential component of vesicle coats and also a component of the amino acid–sensing TORC1 pathway. And, components of the Ran cycle appear to play a role in cell division. RCC1, the Ran guanine-exchange factor, remains localized to chromatin during mitosis, maintaining a higher concentration of RanGTP near chromosomes as microtubules segregate chromosomes into two daughter cells. Several proteins that regulate microtubules are recognized by karyopherins and kept in an inactive state, but are released by the karyopherins near chromosomes because of the elevated levels of RanGTP. Thus, Ran provides a signal for spatially controlled activation of microtubule formation near chromosomes. Many nucleoporins are also localized at important mitotic structures, suggesting that the transport machinery may have an even larger role in the progression of the cell cycle.

Given the NPC's role in ensuring the flow genetic information from the nucleus to the translational machinery in the cytoplasm and in protecting the genome, and given the many additional functions of nucleoporins in the cell, it is not surprising that a wide variety of human diseases have been associated with the dysfunction of the NPC or its component parts. Several cancers involve genomic rearrangements that have resulted in nucleoporin genes fused to other genes, for example.[8] And several mutations in nucleoporins have been linked to heritable diseases that result in lethal developmental defects.[9]

Researchers have also recently linked NPC function to neurodegenerative diseases including amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). Recent animal studies found that nucleoporins are extremely long-lived proteins in nondividing cells such as neurons, with half-lives on the order of months.[10] Thus, unlike most cellular proteins, damaged NPC proteins are not replenished by protein turnover in these cells, but instead would accumulate over time, meaning aging NPCs may have an important role in human disease. Taken together, it is clear that building on our current understanding of NPC structure and function could lead to novel ways to combat a wide array of human diseases. ■

Daniel H. Lin is a biochemistry and molecular biophysics graduate student at the California Institute of Technology in Pasadena. His advisor, André Hoelz, is a professor of chemistry at the California Institute of Technology, a Heritage Principal Investigator, and a Faculty Scholar of the Howard Hughes Medical Institute.

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SCIENCE, 352:AAF1015, 2016

The Rise of the Pangenome

As improved sequencing methods reveal never-before-seen genomic variation within species, some researchers are retiring the concept of a single reference genome.

BY CATHERINE OFFORD

In 1995, researchers published the world's first complete genome sequence of a free-living organism, that of the bacterium *Haemophilus influenzae*. The sequences of many model organisms, including *Escherichia coli* and *Arabidopsis thaliana,* followed shortly thereafter, and by 2001, the Human Genome Project had completed the first draft of a person's entire DNA sequence. With this new capacity to decipher the genetic blueprint for any organism, some researchers believed they held the key to explore the inner workings of every species on Earth—guided by just a single reference genome per species.

"It was not really in the accepted thought that you'd have to [sequence] more than one of anything," says Hervé Tettelin, an associate professor of microbiology and immunology at the University of Maryland. "The human genome had been done, a bunch of reference genomes had been done, and that was it. [Researchers] thought that we'd have all the models we needed to understand biology."

When researchers compared eight isolates of *Streptococcus agalactiae*, they found not only the small, within-gene variations predicted by conventional genetics, but an average of 33 completely new genes with every new genome sequenced.

But with genome sequencing becoming faster, cheaper, and more accessible all the time, it quickly became apparent that this attitude overlooked one very important aspect of biology—genomic variation among individuals. And for some species, such variation can be significant. In the early 2000s, for example, when Tettelin, then at the Institute for Genomic Research (TIGR), and his colleagues compared eight isolates of *Streptococcus agalactiae* (or group B *Streptococcus*, GBS), they found not only the small, within-gene variations predicted by conventional genetics, but an average of 33 completely new genes with every new genome sequenced. "It was a shock," says Tettelin. "We saw there were many regions—relatively large regions—of diversity."

Analyses of *S. pyogenes* (a group A *Streptococcus*) revealed a similar story: each new strain added, on average, 27 new genes. Tettelin and his colleagues realized that "we were far from having enough

genomes to characterize all the genes [for a given species].” This concerning realization prompted the researchers to propose the concept of the “pangenome,” defined as the entire set of genes possessed by all members of a particular species. “We were trying to find a way to represent this diversity,” Tettelin says.

Publishing on the idea for the first time in 2005, the TIGR team focused on the pangenome’s utility in describing the genomic content of microbial species.[1] But geneticists studying the sequences of plants, fungi, and animals soon began to face the same problem with their reference genomes: members of a species do not always share the same genes.

Over the past 10 years, large-scale sequencing projects have revealed startling levels of individual genomic variation across the tree of life, challenging the value of the modern reference genome—as well as the very notion of a species. To better capture the genetic makeup of any given taxon, many researchers now argue that the field of genomics should adopt a pangenomic framework in which diversity is central, rather than incidental, to our view of species.

“It’s exciting,” Tettelin says. “People are realizing this [diversity] is there. I think the approach is going to be used more and more.”

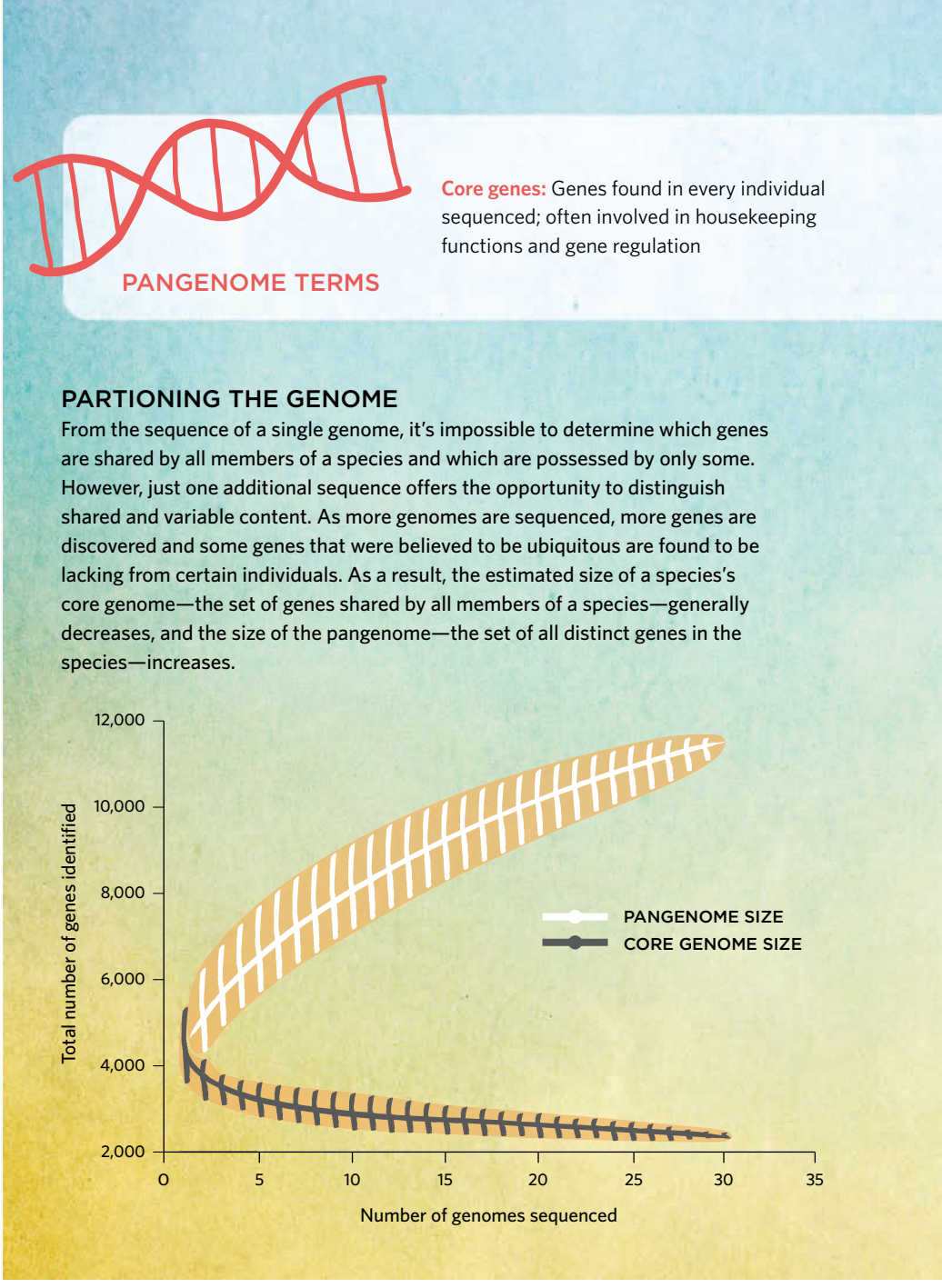
Getting a grip on bacterial diversity

By 2005, public databases stored the complete genome sequences for around 250 bacterial species, with “species” broadly defined as a group of organisms that share more than 97 percent sequence identity in their slowly evolving genes for 16S rRNA. But more than 80 percent of these sequences were assembled from a single bacterial isolate—an approach that the TIGR team’s data suggested fell far short of capturing all the genes found in a species. So in their paper, now cited more than 1,000 times, Tettelin and his colleagues laid out the founding principles for a new way of thinking.

Instead of a single reference genome, the authors argued for a description of bacterial species using the set of all genes identified as belonging to members of that species (or “operational taxonomic unit,” as bacteria grouped by close similarity in their 16S rRNA sequences are commonly known). Those genes could be subdivided into core genes, present in every strain sequenced; variable, or “dispensable,” genes that can be found only in some strains; and unique genes, restricted to just one strain.

The team also presented a method for estimating the total size of a taxon’s pangenome (sometimes referred to as the supragenome) based on the number of new genes discovered in each complete sequence. “We came up with the principle of doing all combinations of adding every genome to another,” says Tettelin. If the average number of new genes with each new genome shows no sign of plateauing, the pangenome is theoretically infinite, and said to be open; if the average number of new genes is asymptotic, the pangenome has a more predictable size, and is considered closed. (See illustration above.)

“It was pioneering work,” says Chitra Dutta of the Indian Institute of Chemical Biology, whose group recently developed



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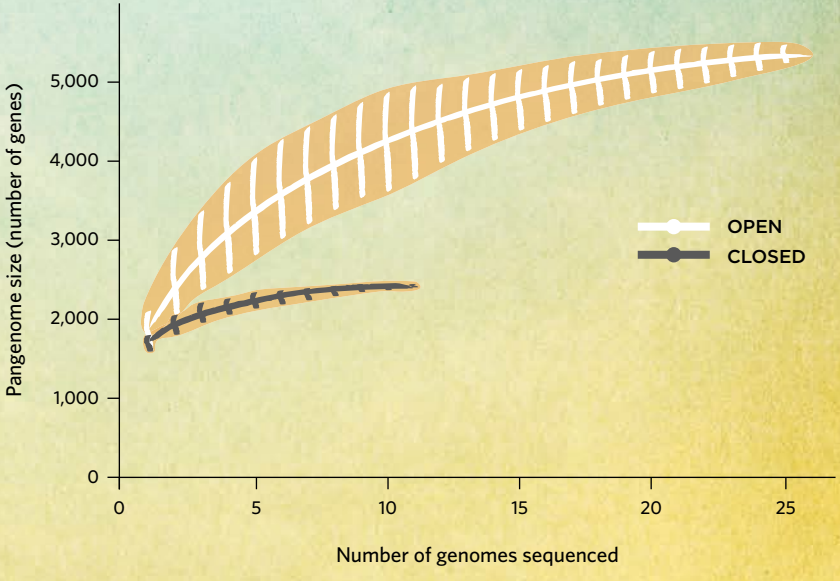
Variable genes: Genes found only in some individuals or strains of a species; often involved in adaptation to particular niches or new functions

Unique genes: Genes found only in one individual or strain

OPEN AND CLOSED

Provided genomes are sampled randomly from a population, the number of genes in the pangenome can be estimated by plotting the number of genes discovered with every new sequence. If this plot, known as a rarefaction curve, is asymptotic—i.e., after a few sequences, no more novel gene content is discovered—then the pangenome is said to be "closed" (gray). The slow-evolving bacterium *Bacillus anthracis,* for example, has a closed pangenome comprising approximately 2,900 core genes and just 85 variable genes. If the number of new genes shows no sign of plateauing, the pangenome is said to be "open," meaning that it is, at least theoretically, infinite (white). Many human pathogens, including *E. coli* and *Streptococcus agalactiae* have open pangenomes.



software to perform pangenomic analyses on microbial sequence data.[2] "They showed how to quantify the genomic diversity of the species, and provided a framework for predicting how many additional whole genomes might be needed to fully characterize the species. It was a giant leap forward."

The pangenome concept has since been widely adopted by research groups trying to keep track of newly discovered diversity within and among bacterial taxa. In addition to high recombination rates and mobile genetic elements, which have long been known to be drivers of prokaryotic diversity, horizontal gene transfer—direct or indirect exchange of genetic material among even unrelated organisms—is proving to contribute to individual diversity across the bacterial domain and beyond. (See "Bacteria and Humans Have Been Swapping DNA for Millennia," *The Scientist,* October 2016.)

Bacteria have "access to a toolkit within species and across species," Tettelin explains. For taxa with large, open pangenomes—often a reflection of high rates of gene transfer—that toolkit corresponds to a theoretically unlimited pool of easy-to-access new biological functions. "They're essentially champions at versatility and adaptability," he says.

MIT microbiologist Sallie Chisholm has spent decades studying this genomic fluidity in *Prochlorococcus*, a marine cyanobacterium with a global population of around 3 x 10^{27}. Each strain contains a modest 2,000 genes, but 10 years ago, Chisholm and her colleagues began discovering up to a couple hundred previously unknown genes every time they sequenced a new strain. The 2005 paper introducing the pangenome concept "captured my imagination as a new way to order all of that complex information, and to get an idea of where we were headed if we kept sequencing genomes," she says.

> The pangenome view changes the way you think about what an organism is.
>
> —Sallie Chisholm, MIT

In 2007, Chisholm and her colleagues used the TIGR team's method to determine an open pangenome for *Prochlorococcus*, with a size of nearly 6,000 total genes from an initial 12 genome sequences.[3] Eight years later, with 45 strains sequenced, they revised that estimate up to at least 80,000 genes—around four times the number of genes in the human genome—with an individual's core genome comprising only about 1,000 genes, or less than 2 percent of the total gene pool. "That's a lot of information shaping that collective," says Chisholm. "[The pangenome view] changes the way you think about what an organism is."

Understanding genomic versatility is particularly relevant to the study of disease-causing bacteria, which frequently have large numbers of variable genes. For any particular pathogen, "if the focus is to get a vaccine, we need to know all of the genes that this thing has access to, and all the genes that it expresses into proteins and, more importantly, surface-expressed proteins," explains Tettelin, whose early

GENOME BIOL, 8:R71, 2007.

BLURRED LINES

The classification of species has never been simple. Since the earliest use of the term in a biological context by English naturalist John Ray in the 17th century, the definition of species has been rehashed many times, based variously on criteria ranging from shared physical traits or a capacity to produce viable offspring to a shared niche or evolutionary history. But whichever definition is employed, the boundary between one taxonomic group and the next is not always clear-cut. While a reproductive definition effectively divides most multicellular animals into distinct taxonomic groups, many bacteria, plants, and fungi are much less genetically isolated from one another. Far from offering a neat solution, genome sequencing has revealed the extent of the problem by uncovering dramatic variation within species and surprising overlap between them.

In the face of such complexity, some researchers are developing a more nuanced view. In prokaryotes, where the lines between taxonomic units are fuzziest, pangenome analyses—which partition the genome into core and variable genes depending on their presence or absence among strains or purported species—could offer a more effective way to distinguish closely related organisms than more-traditional approaches. While most current methods compare the sequences of only one or a handful of genes—such as the 16S rRNA gene, or housekeeping genes in the case of multilocus sequence typing (MLST)—to determine relationships between organisms, pangenome analyses compare and contrast whole genomes across multiple individuals, providing an expanded insight into the similarities and differences between organisms.

These methods are already refining biologists' understanding of bacterial taxonomy. For example, analysis of the core genome in multi-drug resistant *Klebsiella pneumoniae* revealed that the group comprises two distinct genetic clades (*PNAS,* 111:4988-93, 2013). And recent analyses of *Shigella*—one of the leading causes of dysentery—suggest that the bacterium falls into a subgroup of *E. coli,* rather than forming an independent genus (*Front Microbiol,* doi:10.3389/fmicb.2015.01573, 2016).

Even in the eukaryotic world, where genomic fluidity is far less pronounced, pangenomic analyses have cast new light on conventional taxonomies. Sequencing of multiple individuals in several groups of eukaryotic organisms, from marine phytoplankton to crop plants, have challenged traditional notions of within-species diversity and raised the question of what "species" even means. If the goal of classification is to create biologically useful groups of like organisms, then such questions are important to resolve—yet for now, at least, they're still very much open.

COMPOSITE IMAGE © ISTOCK.COM

work in reverse vaccinology—the identification of candidate vaccines using a pathogen's genome rather than purely immunological or biochemical methods—depended on such information. "If a genome is representative of what's in the species, then when you have one, it's game over. But given the diversity we saw, we sort of knew that one genome, and maybe even a few genomes, was not going to be enough for us to find a new cocktail of vaccines that we could take to market."

Pangenomic analyses in the last decade have allowed researchers to begin to develop universal vaccines that could provide protection against all strains in a species, or even against several related species. In 2005, Tettelin and his colleagues' work on GBS led to the identification of a potentially universal vaccine based on a combination of four bacterial surface proteins.[4] And last June, researchers at the University of California, San Diego, published a pangenome study of hospital superbug methicillin-resistant *Staphylococcus aureus* (MRSA) from 64 strains as a starting point for developing a widely effective MRSA vaccine.[5]

To date, researchers have applied the pangenome framework to some 50 bacterial species, including model organisms, such as *E. coli*, and commercially significant microbes, such as the wine bacterium *Oenococcus oeni*. Scientists have used the same basic principles to consider shared and variable genes and gene families within larger groups such as genera, the human microbiome, and even the entire bacterial domain. Now, with the approach broadly accepted as a useful way to organize bacterial diversity, efforts are focusing on incorporating this more variation-centric view into metagenomics, phylogenetics, and even taxonomy itself. (See "Blurred Lines" at left.)

"We have learned a tremendous amount about the machinery of life from model organisms that have been studied in the lab, but we haven't really confronted head-on the role of the diversity," says Chisholm. "Now I think we're learning that this diversity is part of what life is all about."

Not just for bugs

As the pangenome concept continues to percolate through the microbiology research community, newly discovered intraspecific variation is beginning to influence genomic descriptions of other taxa. Although eukaryotic species—often loosely defined on the basis of evolutionary or reproductive isolation—are thought to have relatively low levels of horizontal gene transfer and genome rearrangements relative to their promiscuous prokaryotic cousins, sequencing of multiple individuals per species is revealing extensive genomic diversity that goes far beyond small, within-gene differences.

> In pangenomics—and genomics in general—we need to move into a phase of functionality.
>
> —Candice Hirsch, University of Minnesota

California State University San Marcos researcher Betsy Read realized the importance of such interindividual variation a few years ago, shortly after finishing the construction of a reference genome for the tiny but ubiquitous eukaryotic phytoplankton *Emiliania huxleyi*. From the Arctic to the tropics, "almost every bucket of water you pull from the ocean is going to contain *E. huxleyi*," says Read. Suspecting that the organism's ability to adapt to such varied conditions might depend on single-nucleotide polymorphisms within genes, the team got to work sequencing more isolates.

Thirteen sequences later, Read and her colleagues were surprised to find that genome size, originally estimated at some 30,000 genes, varied remarkably between strains, with some strains apparently missing more than 2,000 genes. When they conducted a pangenome analysis, the researchers found that just two-thirds of the genes they had identified were shared by all sequenced isolates.[6] In particular, Read notes, there was a high degree of variability in genes encoding metal-binding proteins—key components in *E. huxleyi's* adaptation to the environment.

Given the lack of evidence for horizontal gene transfer in *E. huxleyi*, the availability of the total gene pool to each individual is unlikely to mirror that of prokaryotes. But Read's team believes that the large pangenome relative to an individual's core genome underpins this single-celled eukaryote's adaptability. "*E. huxleyi*'s got this huge plasticity in its ecophysiology," she explains. "We think that this genomic variability helps to explain that."

E. huxleyi is hardly alone in harboring such diversity in its DNA. Larger-scale sequencing projects covering thousands of whole genomes of model eukaryotic organisms such as *Saccharomyces cerevisiae* and *Arabidopsis thaliana* have also revealed significant numbers of duplicated or novel genes. And in crop plants, whose genomes frequently contain large duplicated regions, a handful of studies already support links between the presence or absence of "variable" genes and disease resistance, metabolite production, and stress responses. "It's becoming increasingly acknowledged that gene difference does have an impact," says David Edwards, a geneticist at the University of Western Australia who studies variation in the mustard family of plants.

One of the first high-resolution plant pangenomes was published in 2014 by the University of Minnesota's Candice Hirsch and colleagues, who combined the sequences of 503 inbred lines of maize to categorize differences in gene content and other genomic variation.[7] "We found that the reference genome assembly contains less than a third of the total genes that exist in maize," Hirsch says. "That, for our community, was a pretty big breakthrough to grasp how extensive this variation really is." Separate pangenome studies have established core and variable genes for rice and the wild relative of soybean. And the planned large-scale resequencing of entire germplasm collections by organizations such as the Global Crop Diversity Trust promises huge volumes of data for future analyses.

In theory, the pangenomic approach to plant species could help identify genes involved in adaptation and inform strategies for the introduction or cultivation of environmentally resilient populations. But that sort of progress will require going beyond mere descriptions of variation, notes Hirsch. "In pangenomics—and genomics in general—we need to move into a phase of functionality," she says. "We need to move beyond the variation at the genome level, and ask how it impacts phenotypic variation." And, from a cultivation perspective, "can we introduce this variation artificially in an intelligent way?"

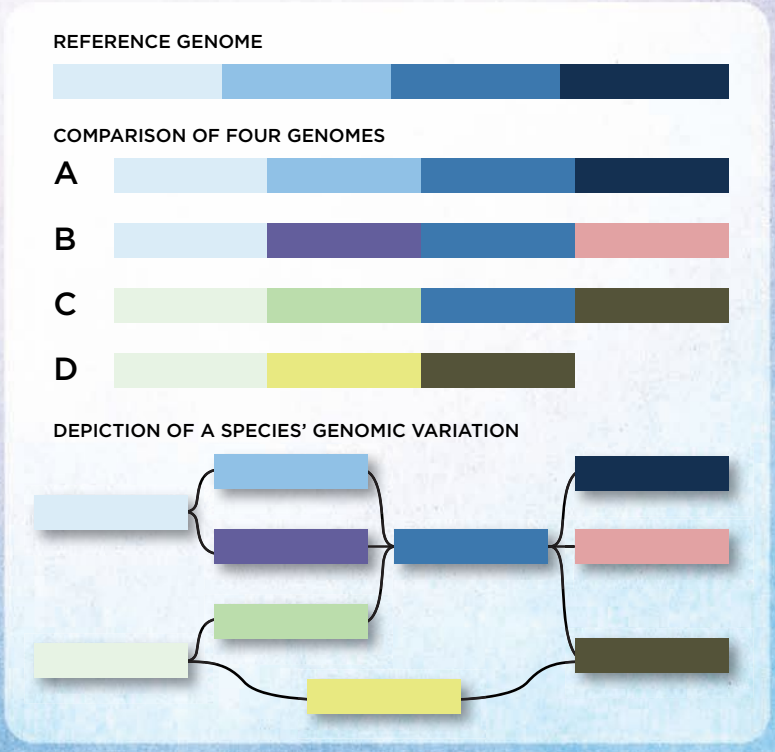
An answer to human variation?

Of course, one eukaryotic species that has no shortage of genome sequences is *Homo sapiens*. Between 2008 and 2015, the 1000 Genomes Project generated the world's largest public catalogue of human variation based on data from 2,504 individuals from 26 populations; in 2012, the U.K.'s 100,000 Genomes Project launched an even bigger effort, with an expected completion date of 2017.

The data from these and smaller projects are revealing that *Homo sapiens*, too, is fairly poorly described by a reference sequence built on just a handful of genomes. Although variation in genomic content between two humans is minuscule in comparison with that of microbes or plants, the earliest attempt at building a human pangenome in 2009—based on the human reference genome and just two others—estimated that up to 40 megabases of sequence, including protein-coding regions, were absent from the reference genome.[8] The same year, another team estimated that gene counts varied between any two randomly chosen people by 73 to 87 genes, largely because of variations in copy number.[9] And in 2015, a team in Denmark published the first attempt at a "national human pangenome" using sequence data from 10 sets

VISUALIZING THE PANGENOME

A reference genome built from the DNA of an individual organism can be visualized as a linear sequence (top). But there is a growing appreciation that this sort of representation fails to reflect the diversity among individuals of a species, which includes not just sequence variation within shared genes, but often different genes altogether (middle). To visualize the genomic content of a species, researchers use interconnected nodes representing all possible combinations of genomic segments or genes found in a species (bottom). Such an approach makes all known sequence information available simultaneously, instead of hiding some away as annotations describing how newly sequenced genomes differ from a linear reference.



of Danish father-mother-child trios, highlighting hundreds of thousands of new structural variants.[10]

With differences in gene number increasingly being associated with disorders including autism, Parkinson's disease, and Alzheimer's, there are strong medical justifications for taking a more variation-centric view of our species, says Mark Chance, director of the proteomics and bioinformatics center at Case Western Reserve University. He's part of a team that recently identified more than 300 small sequences absent from the reference genome but present in at least 1 percent of the human population.[11] "There's human and there's human and there's human," he says. "[The genome] does encompass a lot of variation."

Some researchers now argue for the adoption of a more pangenome-friendly, graph-based representation of the human genome to more accurately represent this variation. (See illustration above.) But the concept (and the terminology) of a pangenome has certainly not yet "caught on as much in the mammalian world" as in microbiology and plant sciences, Chance says, citing the sheer complexity of the task as one potential obstacle. Whether a version of the framework will become popular in the genomics of humans and other animals remains to be seen—and if it does, its form may differ markedly from the original.

But for Tettelin, who has watched the transition from single-genome to multiple-genome descriptions of species across the tree of life, the applicability of the pangenomic idea, first laid out by the TIGR team more than a decade ago, continues to broaden. "It certainly put us on the right track," he reflects. "Sometimes you embark onto things that lead you into dead ends. But in this case, we essentially landed on a highway instead of a dead end. Now we just have to speed up." ■

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2016

TheScientist

TOP 10 INNOVATIONS

This year's list of winners celebrates both large leaps and small (but important) steps in life science technology.

BY *THE SCIENTIST* STAFF

Oftentimes innovation is incremental. After all, even big, brash new ideas have nuts and bolts that can be endlessly tweaked to improve performance, efficiency, and utility. This year's Top 10 Innovations winners do include bold, new platforms that look primed to rev up discovery in basic biology, drug development, and clinical labs. But the list also features products that speak to the important, but often underappreciated, tinkering that drives life science innovation.

Just as geneticists might revel in the release of a new platform capable of generating long-read sequences with single-molecule resolution, synthetic biologists eagerly await the development of improved CRISPR-Cas9 guide RNAs and nucleases to facilitate ever more efficient and precise genome editing. Like biology itself, life science technologies are often more than the sum of their parts.

Also worth mentioning are the more clinically relevant innovations that made this year's Top 10 list. Synthetic human kidney tissue that brings properties of the organs to the petri dish and specially designed panels that quantify a host of biomarkers in various samples promise not only to enhance work in the lab, but to change lives in the clinic. Advances like these remind us that innovation and the pace at which it occurs serve more than manufacturers, developers, and academics—they can serve humanity.

From a machine that allows for single-cell Western blotting to a microfluidic device that streamlines mass spectrometry, this year's Top 10 Innovations are a celebration of transformative life-science advances, large and small.

ProteinSimple » Milo

Single-cell Western blotting is now available for purchase. Developed by Amy Herr's lab at the University of California, Berkeley, Milo is a benchtop instrument that allows researchers to search for specific proteins in about 1,000 single cells at once. Users simply pipette a cell suspension on top of a 1-by-3-inch glass microscope slide covered in a 30-micron thick gel layer dotted with 6,400 microwells. As the cells settle into the gel, some wells will remain empty, but about 1,000 will collect individual cells for analysis. Researchers then add reagents to chemically lyse the cells and denature the proteins. Next, they apply a charge to pull the proteins into the space between the wells and use UV light to activate chemicals in the gel that lock the protein bands into place.

"People who are doing conventional Westerns can't see heterogeneity, because they're looking at a bulk level," says Kelly Gardner, a former graduate student in the Herr lab and current director of marketing at ProteinSimple. "Milo gives you access [to] identify subpopulations." A description of the technology was first published in June 2014, and strong interest from the scientific community led Herr, Gardner, and their colleague Josh Molho to launch Zephyrus Biosciences, which was acquired by ProteinSimple's parent company Bio-Techne in March. The company declined to give the exact price of a Milo unit, but stated that Milo's cost is comparable to a benchtop flow cytometer, and that interested researchers can request a quote on the website. The company was also unable to provide user comment due to the newness of the product.

UNGER: *A novel use of a custom chip that eliminates the transfer step and allows efficient large-scale tests of thousands of single cells. As price keeps coming down, this should allow the detailed efficient testing of many problematic (e.g., poor flow) proteins, and give info about individual cellular responses, which is an important area of inquiry today.*

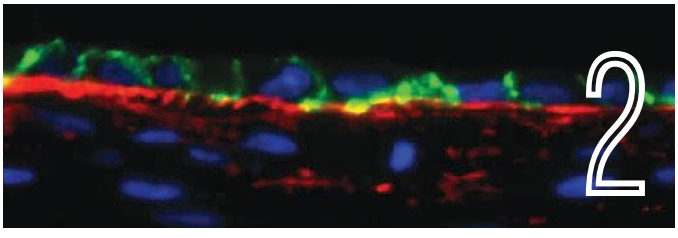
FISHMAN: *This is an example of the potential for scalability of a known technology at lower cost and using less space. This also saves researchers' time by testing protein-expression heterogeneity in their cells, simultaneously.*

Organovo » ExVive Human Kidney Tissue

A crucial stage of drug development is testing whether a candidate compound damages the kidneys, but existing cell cultures and animal models can only approximate the human kidney. ExVive Human Kidney Tissue from Organovo is a replica of the kidney proximal tube created using 3-D bioprinting. It offers drug developers a reliable means of testing for renal toxicity.

Currently, few preclinical tests can determine whether a potential drug is toxic in humans, making investing in clinical testing risky for developers. Identifying renal toxicity early on reduces that risk. More importantly, "you're really talking about doing no harm to the patients that are going to be in the clinical trial," says Organovo Chief Scientific Officer Sharon Presnell.

Bioprinting operates on a similar principle to 3-D plastic printing, explains Presnell, but "instead of putting beads of polymer into a printer, we're putting little aggregates of cells." Organovo, which won a spot in 2014's Top 10 Innovations for its ExVive Liver Tissue, produces tissue samples on a contract basis, and pricing can vary widely depending on the number and type of samples a client requires.



The replica kidney tissue could be applied outside of toxicology too, as a platform for experiments on kidney tissue that would not be otherwise feasible, Presnell says.

"It seems to have integrity like a native kidney tissue," says Caroline Lee, a metabolism and pharmacokinetics researcher at Ardea Biosciences who profiled transport protein expression in the artificial tissue. Lee found that directional transport proteins were oriented correctly along the membrane. "You can see drugs going in the right direction," she says. "It's pretty remarkable."

UNGER: *Based on quite a novel and bold approach to copying the detailed morphology and function of kidney tissues, this innovation offers major advantages over conventional cell culture methods, which have limited predictive capacity at the tissue level.*

FISHMAN: *This technology can be used instead of preclinical animal trials, reducing our reliance on laboratory animals to test new compounds. It also has the potential to transform drug development by better mimicking human kidney biology to test for the renal toxicity of new drugs.*

Pacific Biosciences » The Sequel System

At less than a third the size and weight—and half the cost—of Pacific Biosciences's original long-read sequencer, the Sequel System is the company's latest offering in single molecule, real-time (SMRT) sequencing.

Sequel, which debuted last fall, generates the same long reads and single-molecule resolution accomplished by the company's older SMRT sequencer, called the PacBio RS II. Compared with the RS II, Sequel "is a higher throughput version of SMRT sequencing, which allows the faster generation of more data to tackle larger genomes and biology requiring higher molecular depth as well as metagenomic samples in the same relative time frame," says Robert Sebra of the Icahn School of Medicine at Mount Sinai in New York City who has used the system since December 2015.

Sebra, who worked at PacBio from 2007 to 2012, has used SMRT technology for various applications over the past six years, including de novo human genome sequencing. "It's very flexible for both R&D and production sequencing," he says. "There's essentially no systematic error, enabling higher quality value sequence data in tandem with longer reads to help discover previously unknown genomic features."

Sequel is also particularly useful for metagenomics and infectious disease research. It was recently used to produce a reference genome sequence of a Korean individual, says Jonas Korlach, chief scientific officer at PacBio and coinventor of SMRT sequencing (*Nature*, 538:243-47, 2016). In October, leaders of the Genome 10K (G10K) and Bird 10,000 Genomes (B10K) initiatives announced their choice of SMRT sequencing as a principal technology.

With a list price of US$350,000, a PacBio sequencer is within reach for more labs. "Now, SMRT sequencing is for everyone," says Korlach.

FISHMAN: *The Sequel System is an improvement on Pacific Biosciences's earlier systems in that it provides higher throughput and more scalability at a lower cost.*

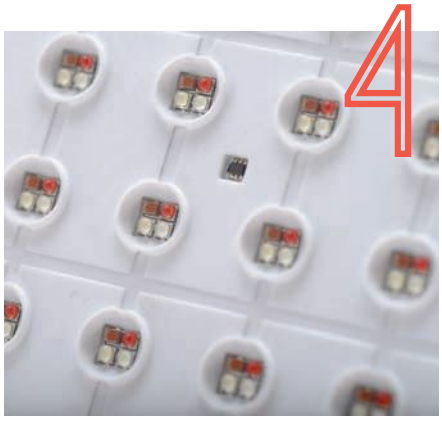
STRÖMVIK: *Though not affordable for small labs, high-throughput, long-read sequencing is essential for any group working on large and complex genomes, metagenomics, and metatranscriptomes.*

Axion BioSystems » Lumos

Axion BioSystems makes in vitro optogenetics more precise and more replicable than ever, thanks to its new Lumos light-delivery system, first shipped in December 2015. The apparatus contains 48 wells, each with four individually controllable LEDs that can flash different wavelengths of light—blue, green, orange, and red—with microsecond precision. When positioned above a microarray culture plate with a recorder fixed beneath, the setup allows researchers the ability to precisely stimulate, manipulate, and measure a variety of cultured cells.

Geneticist David Goldstein is poised to use the Lumos in his Columbia University lab to study the behavior of cultured human neuronal networks with mutations that cause different forms of epilepsy. "What we've been looking for for a long time now, in a precision medicine context for epilepsy, is a medium-complexity in vitro model . . . but [one that is] still high-throughput enough so we can screen compounds," he says.

Cultured neuronal networks tend to synchronize their synaptic firing, decreasing the amount of information that experimentalists can extract from their behavior. "To elicit more complex behavior that might reveal the effects of the mutations, what we want to be able to do is kind of tune activity in the networks while we're monitoring the response," Goldstein says, adding that he expects data from the Lumos to come in over the next year. "That's exactly what this system allows us to do."



The Lumos costs US$26,000. Pairing two light-delivery systems allows users to create a 96-well apparatus.

UNGER: *This system for high-throughput optical stimulation in multiwell plates is the first large-scale practical implementation in the emerging field of biophotonics.*

PLATT: *The platform . . . now puts unique control in the hands of the researcher. Using high-power LEDs that span several wavelengths increases the customization ability to go with specific light-manipulated protein manipulation.*

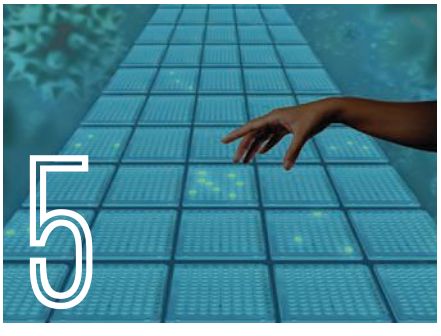
Thermo Fisher Scientific » LentiArray CRISPR Libraries

With its user-friendly nature, the CRISPR-Cas9 technique is often lauded as the technology that will democratize gene editing. Thermo Fisher Scientific's new LentiArray CRISPR Libraries, introduced in September, make applying the tool in screening assays even more accessible to researchers. The company has developed reagents that, when introduced to any variety of human cells—from HeLa to induced pluripotent cells—knock out genes, one-by-one, using CRISPR.

Northwestern University's Simone Sredni, who studies an aggressive childhood cancer called rhabdoid tumors, participated in beta testing the libraries by screening the effects of mutating 160 kinases in patients' tumor cells to find those that affected cell proliferation and growth. Her preliminary data came back in three months, and she identified some kinases whose impairment did indeed slow growth. "It really happened fast," she says, taking only a little more than a year to get to the point now where she is testing inhibitors against those kinases in vivo in animal models. "This is something I wouldn't be able to find if it wasn't for the screen."

The libraries come in a variety of flavors. Customers can choose from 19 different gene sets, customize their array, or conduct an unbiased screen of about 18,000 genes. "It's not only the most efficient screening technology on the market, but it gives us a lot of latitude in creating different types of experiments for different applications," says Jon Chesnut, the senior director of synthetic biology R&D at Thermo Fisher Scientific.

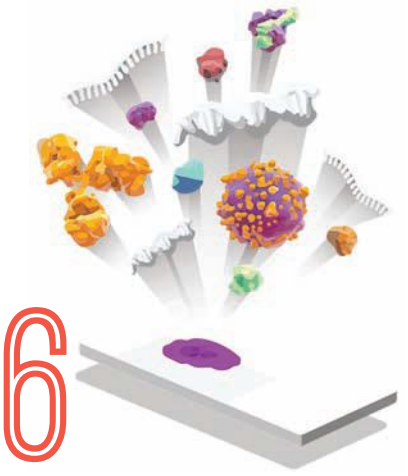
Starting at US$10,000 per library, it can be a bit costly, says Sredni, but for labs doing high-throughput screens, the price may be worth it.



STRÖMVIK: *Anything taking CRISPR technology to a high-throughput level is worth at least a look!*

WILEY: *This is a great enabling system. You can assemble this type of library yourself, but this product offers a streamlined way to start mapping genes to function.*

NanoString Technologies
nCounter Vantage 3D Panels



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In 2008, when NanoString debuted its nCounter analysis system—an automated microscope that tallies color-coded barcodes hooked to target molecules—the plan was to mature the technology from quantifying mRNAs to counting DNA sequences and proteins as well. This year, the company met its goal and unveiled its nCounter Vantage 3D Panels.

"The Vantage assays were expanded to allow the digital counting of mRNAs, DNAs, proteins, and even phosphorylation status of proteins all at the same time," says NanoString's senior vice president of R&D, Joe Beechem. In April, NanoString released the first Vantage assays, designed to digitally count RNAs in lung cancer and leukemia samples, proteins related to solid tumor biology and immune-cell signaling, and single nucleotide variations in DNA.

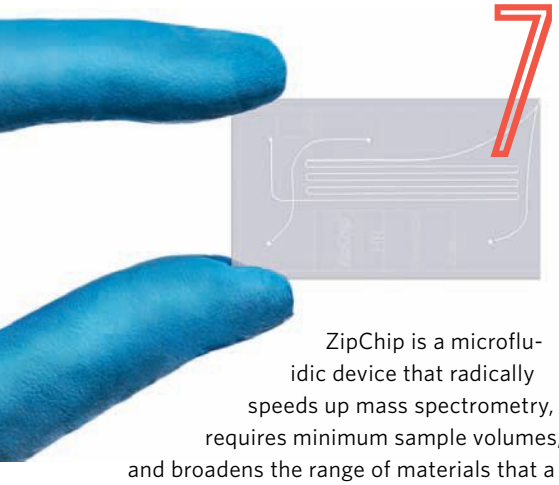
Gordon Mills, chair of the Department of Systems Biology at the University of Texas MD Anderson Cancer Center in Houston, helped develop the Vantage panels and uses them in MD Anderson's Zayed Institute for Personalized Cancer Therapy, which he codirects. "There are many platforms that one can take to the [human sample testing] laboratory," he says. "But none of them [except the nCounter Vantage system] had the robustness, the ease of use, and the potential to do DNA, RNA, and protein at the same time on a patient sample."

The nCounter analysis system ranges from US$149,000 to US$280,000, and the nCounter Vantage 3D Panels run from US$275 per sample and upward. In the near future, NanoString and Mills's laboratory plan to roll out new Vantage panels that add the dimension of measuring the spatial orientation of molecular components at the single-cell scale.

WILEY: *Potentially, this product is the biggest technological breakthrough by enabling the simultaneous detection of DNA, RNA, and protein abundance in the same small sample.*

STRÖMVIK: *Currently very geared toward cancer research, this system will hopefully also have other applications. One instrument can measure up to 800 different selected DNA, RNA, or protein molecules.*

908 Devices » ZipChip



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ZipChip is a microfluidic device that radically speeds up mass spectrometry, requires minimum sample volumes, and broadens the range of materials that a mass spectrometer can handle. The small box, less than a foot long, mounts directly onto a mass spec and works by processing samples through a microfluidic chip the size of a microscope slide.

Normally, preparing mass spec samples is time-consuming and error-prone. ZipChip reduces potential complications. "With our front end, we're able to analyze samples with almost no prep at all, even if they have salt, detergents, or different matrices present," says Chris Petty, cofounder of 908 Devices, the maker of ZipChip.

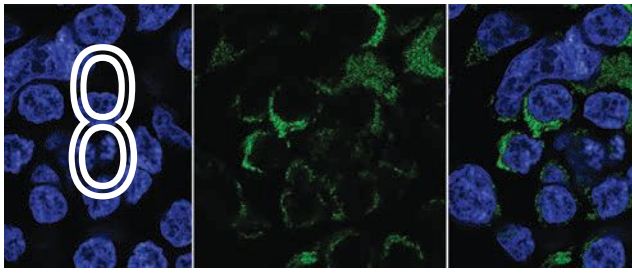
ZipChip uses capillary electrophoresis to separate sample components in just two to three minutes when liquid chromatography columns would require up to an hour, Petty says. The device provides better separation for samples, such as proteins, antibodies, and antibody-drug conjugates, that are difficult to separate with other techniques, says Petty. Plus, it only needs a few nanoliters of material. The device costs US$30,000. An autosampler adds another US$20,000 to the price tag.

Michael Pacold, who studies metabolomics at New York University, says that integrating a prototype of ZipChip into his lab has enabled him to take on a wider range of projects because he can gather data faster and from more sources. "A lot of clinical studies will only let you take a few microliters of plasma from a bank," he says. "Without something like the ZipChip, those studies were not accessible. Now they are."

PLATT: *The capillary electrophoresis, sample separation, and direct spraying to accompany mass spec units will allow for smaller volumes (nanoliters for ZipChip) and potentially lower the cost and improve sample identification while reducing preparation time.*

UNGER: *This uses integrated microfluidic technology to quite dramatically speed up separations as a front end to mass spec (MS) methods, without damaging the integrity of the sample. This should support the continuing widespread use of MS methods both for research and for production of bio therapeutics.*

Horizon Discovery
Turbo GFP Tagged HAP1 Cells



For the third year in a row, Horizon's HAP1 cells have earned a spot in *The Scientist*'s Top 10 Innovations. In 2014, CRISPR-generated knockout cell lines (then sold by Haplogen) won. Last year, Horizon made the list for cells with custom-made deletions. And in October 2015, Horizon launched its Turbo GFP Tagged HAP1 Cells, which made this year's list for their ability to fluorescently tag proteins of interest without requiring that the gene be overexpressed.

Daniella Steel, Horizon's senior product manager for cell lines, says one of the main advantages of using these cells over antibody labeling is simplicity. "Unlike antibodies, you don't need validation or optimization, and you can visualize these cells live."

Emma Lundberg of the KTH Royal Institute of Technology in Sweden recently received a batch of the cells for her work on the Human Protein Atlas project. She is in charge of mapping the subcellular locations of proteins using confocal microscopy and says that overexpression can sometimes lead to artifacts or misplacement of proteins. "The good thing is you know where you have your tag, and where you have it is expressed at the endogenous level," she says. "And HAP1 cells are easy to work with for imaging applications."

Custom-made cells cost US$3,400 and take about 16 weeks to develop. Horizon is also growing a collection of cell lines that tag proteins localized to particular organelles (those cost US$1,450). Lundberg says the price is reasonable considering how much time a lab would have to spend developing and validating its own cell line.

PLATT: *The promise of these genetically modified cells is that they will take advantage of CRISPR-Cas9 technology to tag genes with TurboGFP such that when they are expressed, they. . . can be followed without additional immunolabeling.*

FISHMAN: *TurboGFP uses CRISPR-Cas9 self-releasing tag to provide researchers with tagged proteins. The proteins are tagged endogenously, which is preferable to exogenous models, and provides them reliably and at lower cost.*

Photometrics » Prime sCMOS Camera

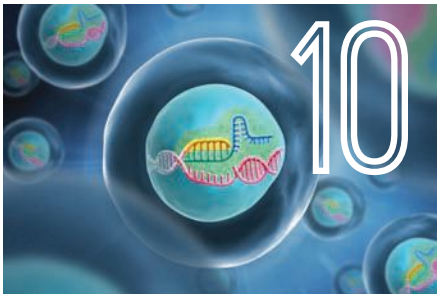
With modern microscopes, researchers turn to high-powered cameras to help them capture images of what's in their sample. "Every year, these cameras have gotten better and better and better," says Rachit Mohindra, product manager at Photometrics, a company that specializes in microscopy cameras and other imaging systems for life science research. "They're basically perfect." To improve on perfection is tough, he admits, but he thinks he and his colleagues have done just that with their 4.2 megapixel Prime sCMOS camera. Released at the beginning of 2016, the camera has a built-in algorithm to reduce shot noise—the variation inherent in measurements taken using light microscopes—without having to acquire many extra images and then average across them, or increase the light intensity, which can damage samples. "You're able to maintain your low levels of light, keep [target cells] alive for longer, and get nice data," Mohindra says. The Prime camera improves signal-to-noise ratio three to five times, he adds, which is "equivalent of being able to turn down the light by a factor of 10."

The Prime sCMOS camera's built-in algorithm also reduces the total amount of data collected by a researcher, hastening processing and analysis times. "It takes about 30 seconds per frame to process if you do it offline," Mohindra says. "When you have a camera that acquires at 100 frames per second, that's 5 minutes for 1 second's worth of data." But with the Prime camera, he says, researchers can process the data immediately.

"The real-time filtering and high frame rates of the Photometrics Prime sCMOS camera enable us to capture even more super-resolution microscopy data and to better characterize variability in the structure of chromatin," Kyle Douglass of École Polytechnique Fédérale de Lausanne in Switzerland noted on the company's website. The Prime sCMOS camera costs US$15,950.

STRÖMVIK: *Increasing demand for high-quality scientific imaging increases the information to be processed. Adding the field-programmable gate array-technology to the camera itself seems like the way to go.*

Thermo Fisher Scientific
GeneArt Platinum Cas9 Nuclease



Along with its LentiArray CRISPR Libraries, another arrow in Thermo Fisher Scientific's quiver of CRISPR reagents made this year's Top 10 Innovations—the GeneArt Platinum Cas9 Nuclease. Recombinant *Streptococcus pyogenes* Cas9 protein purified from *E. coli*, the GeneArt Platinum Cas9 contains a nuclear localization signal that aids in delivery to the nuclei of target cells.

"The things that we knew were important were consistent quality, activity, and purity," says Jason Potter, Thermo Fisher senior staff scientist and R&D manager of genome-editing tools and synthetic biology. "So we made sure, through extensive testing, that we had a very robust purification process."

Potter's team at Thermo Fisher published a paper last year showing that GeneArt Cas9 achieved cleavage efficiencies as high as 85 percent in a variety of cell lines (*J Biotech*, 208:44-53, 2015).

Matthew Porteus, a Stanford University stem cell biologist, uses GeneArt Platinum Cas9 in his studies of ex vivo gene editing to treat blood diseases—research that he's currently doing with mouse cells, but has partnered with Thermo Fisher to move into clinical testing. While using the CRISPR/Cas9 system is an efficient and specific way to accomplish genome editing, "our problem was that in the early preps of Cas9 protein that were commercially available, they had toxicity to them," he says. "[GeneArt Platinum Cas9] really became the gold standard protein that allowed us to do experiments that gave us results that we had not been achieving using any other reagent."

A 25 μg vial of GeneArt Platinum Cas9 Nuclease costs US$150, and customers can also tap into experts at Thermo Fisher who can help design experiments or talk through protocols. "The trick for genome editing is to take people by the hand and tell them exactly the pipetting schemes and what you need to do to be more successful," says Thermo Fisher vice president and general manager of synthetic biology Helge Bastian.

WILEY: *By avoiding the need for vector-based Cas9 expression, this reagent can greatly accelerate the typical CRISPR-Cas9 workflow, at least in certain cell lines.*

THE JUDGES

JENNIFER FISHMAN
Associate Professor in the Biomedical Ethics Unit and the Department of the Social Studies of Medicine and an Associate Member of the Sociology Department and the Institute for Health and Social Policy at McGill University. Fishman holds a Ph.D. in Sociology from the University of California, San Francisco.

MANU PLATT
Associate Professor at Georgia Tech University and Director of Graduate Admissions and Recruiting for the Coulter Department of Biomedical Engineering at Georgia Tech and Emory University. Platt studies tissue remodeling, systems biology, and a number of diseases using both computational and experimental approaches.

MARTINA STRÖMVIK
Associate Professor and Chair of the Department of Plant Science at McGill University's McGill Centre for Bioinformatics. Strömvik studies functional anatomy resulting from gene expression in crop and forest plants.

H. STEVEN WILEY
Senior Research Scientist and Laboratory Fellow at Pacific Northwest National Laboratory. Wiley published some of the earliest computer models of receptor regulation and is known for developing a variety of quantitative biochemical and optical assays as a basis for validating computational models of cell processes.

BARRY UNGER
Associate Professor of Administrative Services at Boston University. Unger has founded and participated in numerous companies, including Kurzweil Computer Products, Inc., which became Xerox Imaging Systems. He is also cofounder and chair emeritus of the MIT Enterprise Forum.

Editor's Note: *The judges considered dozens of entries submitted for a variety of life science products by companies and users. The judging panel is completely independent of* The Scientist, *and its members were invited to participate based on their familiarity with life science tools and technologies. They have no financial ties to the products or companies involved in the competition. In this issue of* The Scientist, *any advertisements placed by winners named in this article were purchased after our independent judges selected the winning products and had no bearing on the outcome of the competition.*

The Literature

EDITOR'S CHOICE IN CELL & MOLECULAR BIOLOGY

Tug-of-War

THE PAPER

R. Sunyer et al., "Collective cell durotaxis emerges from long-range intercellular force transmission," *Science,* 353:1157-61, 2016.

The physical features of a cell's surroundings—the texture of a substrate and the push-pull of intercellular forces—have often taken a back seat to scientific interest in the chemical environment of cells. But biologists are finding time and again that physical signals matter. In 2000, researchers observed that fibroblasts embedded in a gel migrate toward areas where the gel is stiffest—an apparent response to physical rather than chemical signals termed durotaxis. The discovery turned heads. The report attracted almost 2,000 citations over the next decade and a half, and it became a cornerstone of the emerging field of mechanobiology, says Xavier Trepat, who studies durotaxis at the Institute for Bioengineering of Catalonia in Spain.

Exactly how cells "durotax" has been a mystery. Available research implicates durotaxis in a number of important cellular processes, from neuron development to cancerous tumor formation, but "there are only a few papers out there proposing mechanisms," Trepat says, none of which give a definitive explanation.

Trepat and his colleagues studied the problem using traction force microscopy, which involves embedding fluorescent nanoparticles into a gel substrate and tracking the particles' displacement. As cells drag themselves around on the gel substrate, they leave a record of the forces they exert, allowing researchers to decode the physics at play.

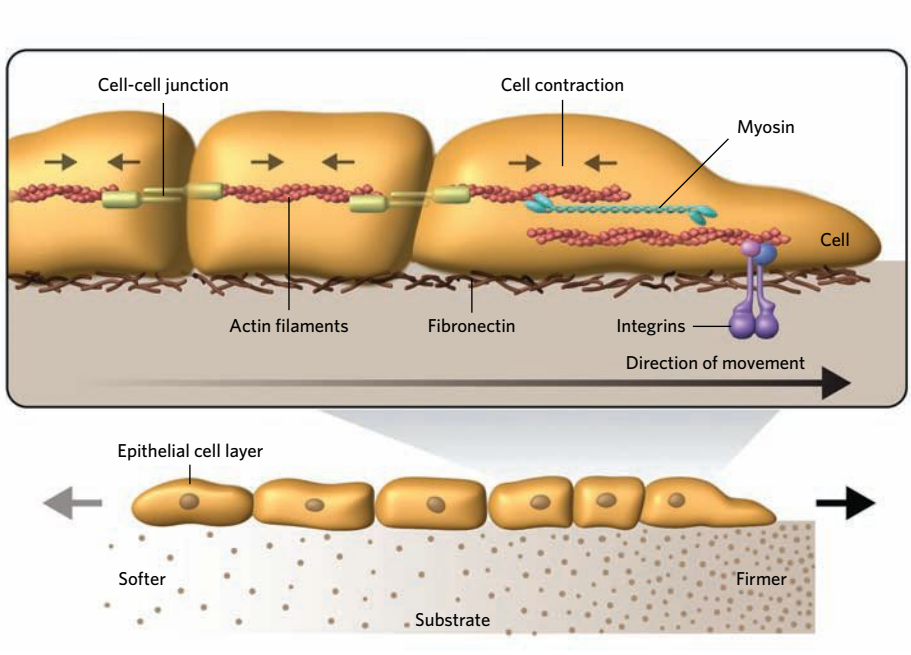
When Trepat and his team placed mammary epithelial cells on a gel substrate of graded firmness, they saw the cells durotax, but also noticed something strange. Only cells in a group moved toward the stiffer area, whereas solitary cells moved randomly. The team's effort to explain this quirk led them to a molecular and mathematical theory of durotaxis.

Researchers had already known that epithelial cells move around by linking onto each other with protein junctions and pulling on the substrate with myosin motor proteins, like someone climbing a rope. Using traction measurements, Trepat and colleagues revealed that epithelial cells in a group do not all move in coordination, but that each edge of the group pulls outward at the same time. Cells sitting on stiffer substrate get better traction, so they win this tug-of-war, and the whole group moves toward them. Durotaxis is not a complex signaling process, they realized, but the result of simple physics.

The discovery brings some clarity to a young field still rife with questions. "Cell migration and cell growth have been studied for decades now, but people have not thought about forces," says Kristian Franze, who studies mechanics in nervous system development and pathology at the University of Cambridge.

"This paper just makes so much sense," Franze says, but adds with a laugh that in the field of mechanobiology at large, "we barely know anything."

—Ben Andrew Henry



PULLING THROUGH: Groups of human epithelial cells migrate toward firmer ground due to linkages between cells and traction with the substrate. Cells adhere to their neighbors through intercellular junctions, which are connected to myosin motor proteins within each cell. The myosin motors cause the cells to contract and tug on one another, but cells residing atop firmer substrate get a better grip through integrin proteins and are therefore able to pull the group in their direction.

SNOWED IN: Spruce and pine parted evolutionary ways long ago, but their cold-adaptation genes are surprisingly similar.

EVOLUTION

Surviving the Cold

THE PAPER

S. Yeaman et al., "Convergent local adaptation to climate in distantly related conifers," *Science*, 353:1431-33, 2016.

FAMILY TREES

Related species sometimes adapt to similar environments with mutations in the same genes. But convergent evolution is usually observed in species that recently diverged or in traits that involve only a few genes and therefore may have fewer possible evolutionary paths. New research from the snowy North tells a different story.

WINTER SPECIALISTS

Interior spruces (*Picea glauca* and *P. engelmannii*) and lodgepole pine (*Pinus contorta*) each have hundreds of genes that contribute to one outcome: surviving the brutal winters of Canada and the northern U.S. "You would think that there would be many ways to get the same phenotype" with so many genes involved, says Sally Aitken, a population geneticist at the University of British Columbia. And yet, the two conifer groups share 10 percent to 18 percent of their cold-adaptation genes, Aitken and her colleagues report in a recent study.

COLD CONVERGENCE

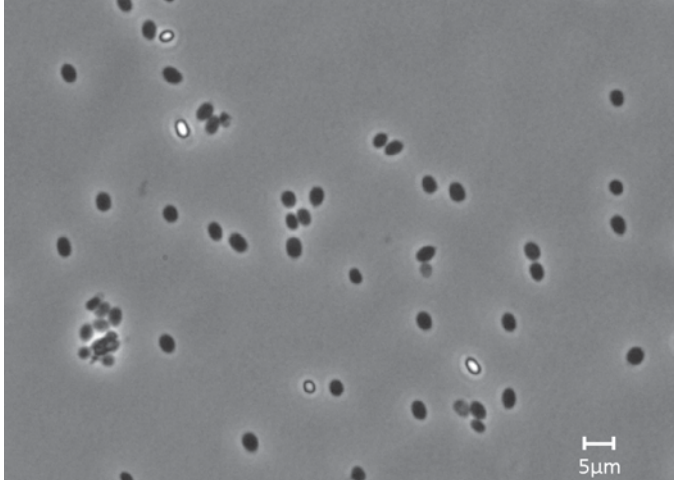
These species diverged when the Earth's climate was warmer, around 140 million years ago—leaving plenty of time to evolve unique genes. Although different variants of those genes arose in each species, evolution appears to have put them to use in the same way. The study suggests evolution "is repeatable even though these trees diverged so, so long ago," says Patrik Nosil, an evolutionary biologist at the University of Sheffield in the U.K.

FROZEN IN TIME

One possible explanation, Aitken says, is that the strong selective pressure of the climate constrained the emergence of new genes, forcing these species to rely on variants of old genes.

—Ben Andrew Henry

SALLY AITKEN; GEORGE KORZA



GERMINATION: Spores of *Bacillus subtilis* incubated in germination solution for 30 minutes turn dark. The three bright spores are still dormant.

MICROBIOLOGY

What a Spore Needs

THE PAPER

G. Korza et al., "Changes in *Bacillus* spore small molecules, rRNA, germination and outgrowth after extended sub-lethal exposure to various temperatures: Evidence that protein synthesis is not essential for spore germination," *J Bacteriol*, doi:10.1128/JB.00583-16, 2016.

THE HISTORY

For decades, scientists considered protein synthesis nonessential for the transition of dormant bacterial spores into active cells—a process known as germination. But a series of experiments from Sigal Ben-Yehuda's lab at the Hebrew University of Jerusalem in recent years suggested otherwise. Spores with disrupted translation didn't germinate, the group found, and with tagged amino acids "we could even identify proteins synthesized during this period [of germination]," Ben-Yehuda says.

SECOND TEST

After seeing Ben-Yehuda's results, Peter Setlow of the University of Connecticut Health Center wondered whether he would find the same failure to germinate if he incubated the spores at high temperatures, which would degrade their ribosomes. So his team warmed up spores of *Bacillus* bacteria to 75–80 °C for nearly a day, thereby wiping out ribosomal RNA (rRNA) beyond the limits of detection.

GERMINATION

Despite the spores' lack of translational machinery, "they could still germinate," Setlow says. "There were very slight effects." Setlow suspects all the proteins necessary for germination are made in advance of dormancy, obviating the need for protein synthesis. "All the proteins are there, and you just have to tickle it and say, 'Go,'" he says.

ANOTHER POSSIBILITY

Ben-Yehuda says a lack of rRNA is not definitive evidence against protein synthesis occurring, because rRNA can be made during germination. Her team showed previously that spores can make rRNA within a few minutes. Setlow says Ben-Yehuda's data are convincing, and each team's conclusions are consistent with the evidence. "That's sort of where we sit," he says. "We'll see what happens in the future." **—Kerry Grens**

PROFILE

Pushing Boundaries

Applying physics, chemistry, and cell biology, Satyajit Mayor seeks to understand how cell membranes work.

BY ANNA AZVOLINSKY

As a master's student in Bombay, India, in 1984, Satyajit Mayor worked in an organic chemistry lab devising probes to study synthetic biomembranes. He wanted to apply to molecular biology PhD programs in the U.S., but knew little about American universities except for the names of some of the top engineering and computer science schools where his friends had applied. Mayor, now professor and director of the National Centre for Biological Science (NCBS) in Bangalore, India, happened to be reading *Arrowsmith,* Sinclair Lewis's novel partly set at a medical research institute in New York City supposedly modeled in part on the Rockefeller Institute for Medical Research. "I looked up the school in the library and found that this was the place where Fritz Lipmann discovered coenzyme A and where many other famous scientists did important work," says Mayor.

"I had a chemist's intuition and perspective on biological systems. I wanted to know what the membrane looked like and how it interacted with the inside and outside of the cell."

He wrote to the university's graduate admissions office and, to his surprise, received a letter from the dean's office asking him to interview with two Rockefeller researchers who were visiting New Delhi. "It was beyond my means to buy an airline ticket, so I replied that I could meet the researchers if I was sent the airfare, thinking that would be the end of our communication." To his amazement, Mayor received an airline ticket to travel from Bombay to Dehli to meet Zanvil Cohn at the All India Institute of Medical Sciences. "Cohn was extremely warm and kind and described New York City as a wonderful place, completely selling Rockefeller as a fantasy land for doing science," says Mayor. "He said that I should plan to come if I was interested in doing biology. I told him that I had no experience with biology. His reply was, 'most of us didn't know any biology before starting to do it.'" Mayor accepted the offer and moved to New York in 1985 to enter the university's PhD program.

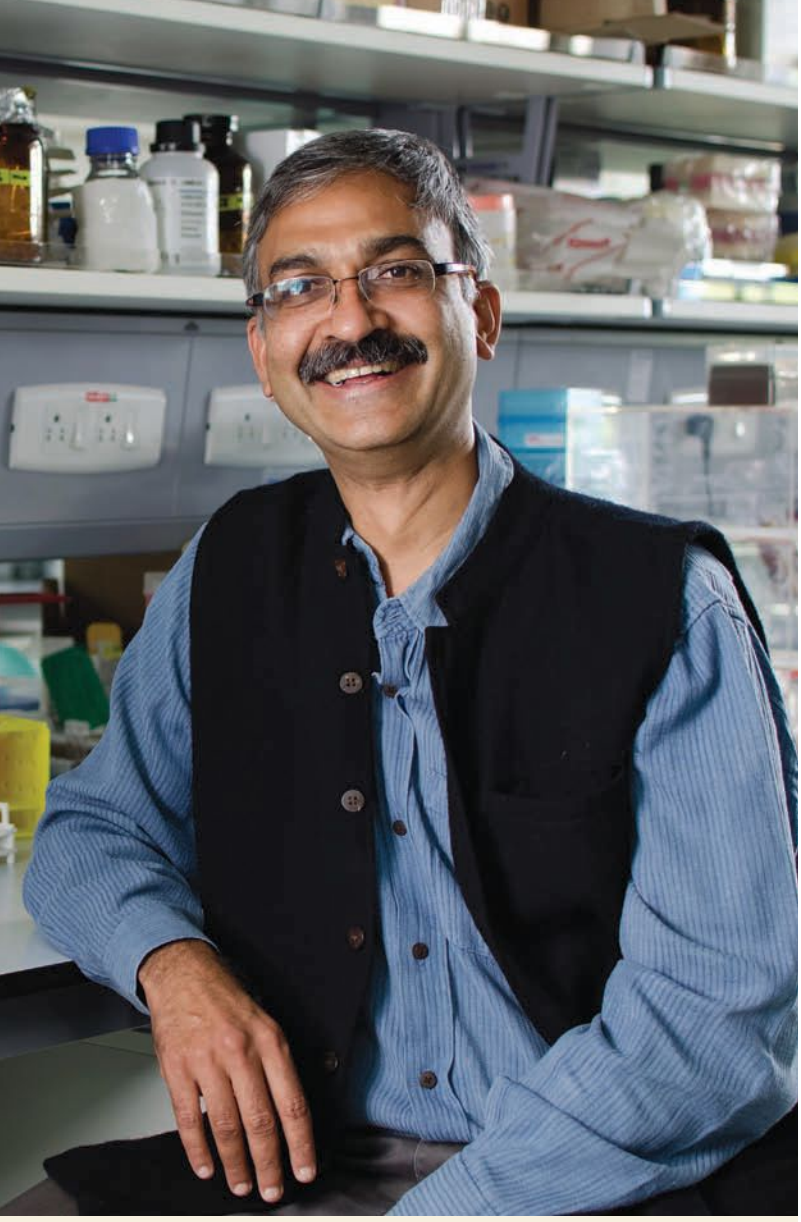
Mayor's doctoral work examined how trypanosomes construct the lipid anchor that attaches a variant surface protein to its cell membranes, helping the parasite evade the host immune system. Since then, he has studied the dynamics of the lipids and proteins that make up cells' plasma membranes. Here, Mayor divulges his first love—cricket, not science; the pressure to major in a "practical" science; and what happens when your results contradict published work.

MAYOR ON THE MOVE

Wild for wickets. Mayor was born in the city of Baroda (now Vadodara) near Bombay. He took up cricket when he was 12 years old, played for his high school, and was considered for a community team as a fast bowler. "I wanted to be a cricket player and I took the game seriously to the exclusion of other things," says Mayor. In 11th grade, he took a university placement exam, and, to his surprise, was accepted into an engineering program at the prestigious Indian Institute of Technology Kharagpur, far away from Baroda. "I sheepishly told my father that I wanted to play cricket and didn't want to do engineering," says Mayor. "He was extremely accommodating. He said that it was too early for me to go away from home and to play as much as I wanted." The following year, his final year of high school, Mayor took the exam again and was admitted to the Indian Institute of Technology (IIT) Bombay, much closer to his home.

Biology discovered. Mayor chose to study physics at IIT Bombay, but was steered away by school advisors who said that only those who didn't want to pursue a profession chose to major in a "pure science." He was persuaded to study mechanical engineering, the most popular discipline at the time. Mayor did not enjoy engineering and found an outlet reading about molecular biology discoveries in textbooks and *Scientific American.* "I was fascinated by this whole world inside the cell," he says.

Biomembranes discovered. "I read about Jacob and Monod's experiments and had been fascinated by Lipmann's *Wanderings of a Biochemist,* as well as other [books that] connected biology with chemistry, which slowly drew me to the chemistry department," says Mayor. In his third year of university, Mayor met Anil Lala, a young chemistry faculty member working on biomembranes. Lala told Mayor to switch to the chemistry department as an avenue to biology. Again, faculty advisors warned Mayor that he was making a big mistake to leave a predictable professional path for the uncertainty of a research career, but Mayor didn't waver. He transferred departments and began to do research in Lala's lab. "He was an inspiration to me. He had set up his lab in this biologically barren landscape and had a lively group of people who thought about biomembranes and proteins inserting across membranes and the structures of pro-



SATYAJIT MAYOR

Director and Professor, National Centre for Biological Science
Director, Institute for Stem Cell Biology and Regenerative Medicine (inStem), Bangalore, India

Greatest Hits

- With colleagues, identified how the parasitic protozoan *Trypanosoma brucei* assembles its GPI-anchored proteins at the cell surface
- Overturned prior work to show that, rather than forming discrete clusters within the plasma membrane, GPI-anchored proteins are diffusely distributed
- Showed that GPI-anchored proteins are endocytosed through a clathrin-independent pathway
- Helped to develop novel imaging techniques that revealed the nanoscale landscape of the mammalian plasma membrane
- Discovered that the organization of the mammalian plasma membrane is due in part to active reshaping of the membrane lipids by the cytoskeletal network of filaments

teins in membrane bilayers." Mayor worked on how bee venom inserts into host membrane bilayers, learning how to make synthetic lipid membranes and to use photoactive probes to study how molecules inserted into membranes. In 1984, he spent the summer at Bombay's Tata Institute of Fundamental Research, where he worked in Ramakrishna Hosur's molecular biophysics lab and published his first paper on the chemical interactions within peptides.

A pragmatic choice. Mayor says it took him a while to find his feet in molecular and cellular biology after he arrived at Rockefeller in 1985. "When it came to deciding what to work on, I felt that, if I wanted to go back and work in India eventually, perhaps studying parasites was one way to do research there," he says. So, he joined George Cross's lab, which studied *Trypanosoma brucei,* the parasitic protozoan that causes African sleeping sickness. In Cross's lab, Mayor researched the biochemical mechanism by which the organism synthesizes a surface coat that blankets the outer surface of its plasma membrane. This coat consists of various glycoproteins attached to the plasma membrane by glycosylphosphatidylinositol (GPI) anchors. Mayor characterized the chemical structures of two of the GPI-anchor precursors to understand glycolipid assembly of the surface coat protein of the trypanosome.

A chemist's eye. Mayor approached his graduate school research with the eye of a chemist. "I had a chemist's intuition and perspective on biological systems. I wanted to know what happens to the GPI anchored proteins once they get to the surface of the cell." By the time Mayor completed his PhD, he and Anant Menon, a Cross lab postdoc who is now a professor at Weill Cornell Medical College, had worked out how *T. brucei* builds the GPI anchor for surface proteins.

MAYOR MOTIVATED

Probing deeper. Toward the end of graduate school, Mayor realized he "wanted to know what the membrane looked like and how it interacted with the inside and outside of the cell." Mayor found that one of the few researchers tackling this question was Frederick Maxfield, then at Columbia University. "I felt that working in Fred's lab would give me a completely new dimension of looking at how a cell works. Fred was a pioneer of using video-enhanced fluorescence light microscopy to study intracellular processes as well as cell migration."

Guiding light. For his postdoc in Maxfield's lab, Mayor set up methods to visualize the movement of lipids within the membranes of living cells. "I wanted to understand the dynamics and movement and organization of the membrane and Fred was interested in endocytosis, which is how molecules are taken up from the outside by cells," says Mayor. "So we developed methods to visualize membranes using fluorescently labeled lipids. We found that we could get cells to take up these lipids and we could watch them go wherever they chose to go and make measurements of how they were distributed and measurements of their dynamics. This is the approach and perspective that I have kept ever since—making quantitative measurements and doing spatial and temporal analysis of what happens inside a living cell."

Against the grain. While at Columbia, Mayor used light microscopy to demonstrate that GPI-anchored proteins in mammalian cells are randomly distributed and diffuse throughout the plasma membrane. The work, published in *Science* in 1994, went against publications from cell biologist Richard Anderson's lab, which showed that the folate receptor, a GPI-linked protein, was highly clustered within the plasma membrane and associated with large patches of cave-like invaginations called caveolae. "The *Science* paper was almost like a negative result because Anderson's lab had proposed a new mechanism by which folate was taken up inside the cell." Anderson's team had used a primary antibody in conjunction with a secondary, fluorescence-linked antibody and visualized the receptors as clusters using both light and electron microscopy. Mayor, instead, directly labeled antibodies with a fluorophore and showed that the Anderson lab results were an artifact of the two-antibody technique. Mayor's contradiction of the Anderson lab's results was "really a turning point [in] coming to grips with the real, messy world of science," he says.

"I had become intrigued by Anderson's publications on the folate receptor because it wasn't clear to me how a protein anchored to the membrane by a lipid anchor on the outer leaflet of the bilayer could appear so highly clustered and localized in the membrane," says Mayor. "Our results led to a lot of arguments between the two labs. The confrontation of taking a popular concept and putting a new perspective on it was not easy. There were raging fights at conferences."

Homecoming. During that time, Mayor considered a return to his home country to start his own lab. "When you live away for more than 10 years, you begin to romanticize a country and forget that that country changes. I thought this would be a short, interesting interlude before I went back to the U.S.," he says. Mayor accepted a position at the National Centre for Biological Sciences (NCBS) that was being built in Bangalore and moved back in 1995. "When I got to India, I realized that it might be possible to combine my research and making a difference in this new institute. This was going to be an adventure."

MAYOR MAKES HIS MARK

New way to see. "In the mid-1990s, there was no way to visualize anything smaller than the optical limit; the only fluorescence tool we had was fluorescence resonance energy transfer (FRET)," says Mayor, who wanted to use reagents that were minimally perturbing to the cell. A visiting scientist at NCBS introduced Mayor to a FRET-based method of that used a single fluorophore instead of the usual two, but the technique had not yet been used for biological imaging. A fluorescent folate was an ideal probe to visualize its distribution in the plasma membrane, Mayor reasoned. His student, Rajat Varma, assembled the necessary tools and "the results were absolutely incredible and very different from our expectations," Mayor says. "Instead of a random distribution, the folate receptor distribution now looked like an incredibly rugged landscape, where previously there was absolute homogeneity." While FRET measures proximity between molecules at the 1–10 nanometer (nm) scale, light microscopy is limited by the diffraction limit and only determines the distribution of fluorophores at the optical limit of resolution of about 250 nm. "Therefore, these results indicated that although the folate receptor was present at a uniform distribution in the light microscope, it was anything but randomly distributed at the nanometer scale," says Mayor.

The work, published in *Nature* in 1998, provided a new imaging tool and demonstrated that lipid-linked proteins within membranes could be organized in submicron-size domains dependent on cholesterol. "In 2009, using a direct visualization by near-field scanning microscopy, a colleague showed that the picture we painted back in 1998 is exactly what you can see with the super-resolution methods, almost to the last molecule, which was very satisfying," says Mayor.

Moving parts. In 2002, Mayor's lab also observed the dynamics of folate receptor uptake by fibroblasts and discovered that GPI-anchored proteins are endocytosed through a pathway that does not involve clathrin-coated vesicles or caveolae, which cells use to internalize many contents from the environment.

Then, extending the observations of the dynamics of cell membrane components, Mayor's lab worked with soft matter physicist Madan Rao to show that GPI-anchored proteins are organized as nanoscale clusters that are dependent on the dynamics of the actin cytoskeleton at the inner leaflet of the cell membrane. In 2012, Mayor and Rao showed that the nonequilibrium organization of lipid-anchored proteins and of transmembrane proteins that bind to the actin cytoskeleton may be understood in terms of a theoretical framework based on the active mechanics of actin and myosin at the inner leaflet of the membrane. "This provides a patterning mechanism to create nonrandom distributions of membrane components," says Mayor. More recently, his lab has created an in vitro system that could reconstitute these membrane-cytoskeleton interactions to understand what they mean for the control of the organization of molecules in the membrane. ■

Vlad Denic: Failing Upward

Professor, Department of Molecular and Cellular Biology, Harvard University. Age: 40

BY BEN ANDREW HENRY

As a graduate student studying cell biology at the University of California, San Francisco, Vlad Denic was not interested in projects suggested by his mentor that were sure to result in a publication. Instead, he devised his own experiments, screening for genes involved in sphingolipid metabolism, among other research pursuits. After seven years, Denic pulled together a manuscript on a lipid-synthesizing enzyme only to have it rejected by *Cell* for lacking general interest. He submitted a different manuscript, this one on an artificial lipid-synthesis technique, to *Science* a year later and was rejected again, this time on the grounds that his findings were not novel enough.

"I'm okay with failure," Denic says now. "As long as it prompts me to come up with something new." His persistence paid off during an experiment with the enzymes that help produce very long fatty acid chains, important cellular building blocks. Denic observed that by adjusting the position of one amino acid, he could control the size of the chain, a mechanism he called a "molecular caliper."[1] The finding earned him a publication in *Cell* and drew his eight years of grad school to a close.

During those years, Denic built a reputation for inventive experimentation that helped him immediately land a faculty position at Harvard University in 2008. Initially, his lab focused on tail-anchored (TA) proteins, a broad and vital class of membrane-bound molecules involved in diverse cellular processes, from vesicle fusion to cell death. Denic, with his first grad students and postdocs, described the cellular machinery that ferries some of these proteins from ribosomes to their destinations in the endoplasmic reticulum, a process needed for efficient organelle assembly.[2]

But after a string of papers that answered key questions about this complex system, Denic began to look around for fresh projects. "Once [Denic] feels he understands the basics of some problem, he has absolutely no fear of moving into other areas," observes Manu Hegde, a long-time friend of Denic who studies TA proteins at the Medical Research Council Laboratory of Molecular Biology in the U.K. "He's a very original thinker."

As the TA protein studies wound down, Denic says, "we started shifting focus toward selective autophagy," a process by which cells tag, capture, and recycle damaged or toxic material. "We thought there would be many big mountains left to climb." His group discovered that yeast cells trigger autophagy on demand through the flip of a molecular switch, rather than at a constant rate, as had previously been thought.[3] The mechanism's existence suggested that autophagy could be ratcheted up or down to keep pace with a cell's needs, Denic and his coauthors reasoned. Lately, the team has been using CRISPR-induced random mutations to screen for uncharacterized genes in mammalian cells that play critical roles in autophagy.

Denic has spent the last few months on sabbatical exploring yet another new topic in a familiar city, San Francisco. Before he returned to the Bay Area, his Harvard lab identified a transcription factor that prevents misfolded and toxic proteins from building up in the cell. Denic is now exchanging ideas and techniques related to protein homeostasis with scientists at Calico, a biotech venture started by Google to study aging. When he returns to Harvard in the spring, Denic says he plans to investigate how the protein folding process erodes over time, a line of inquiry that may shed some light on the cellular consequences of aging. ■

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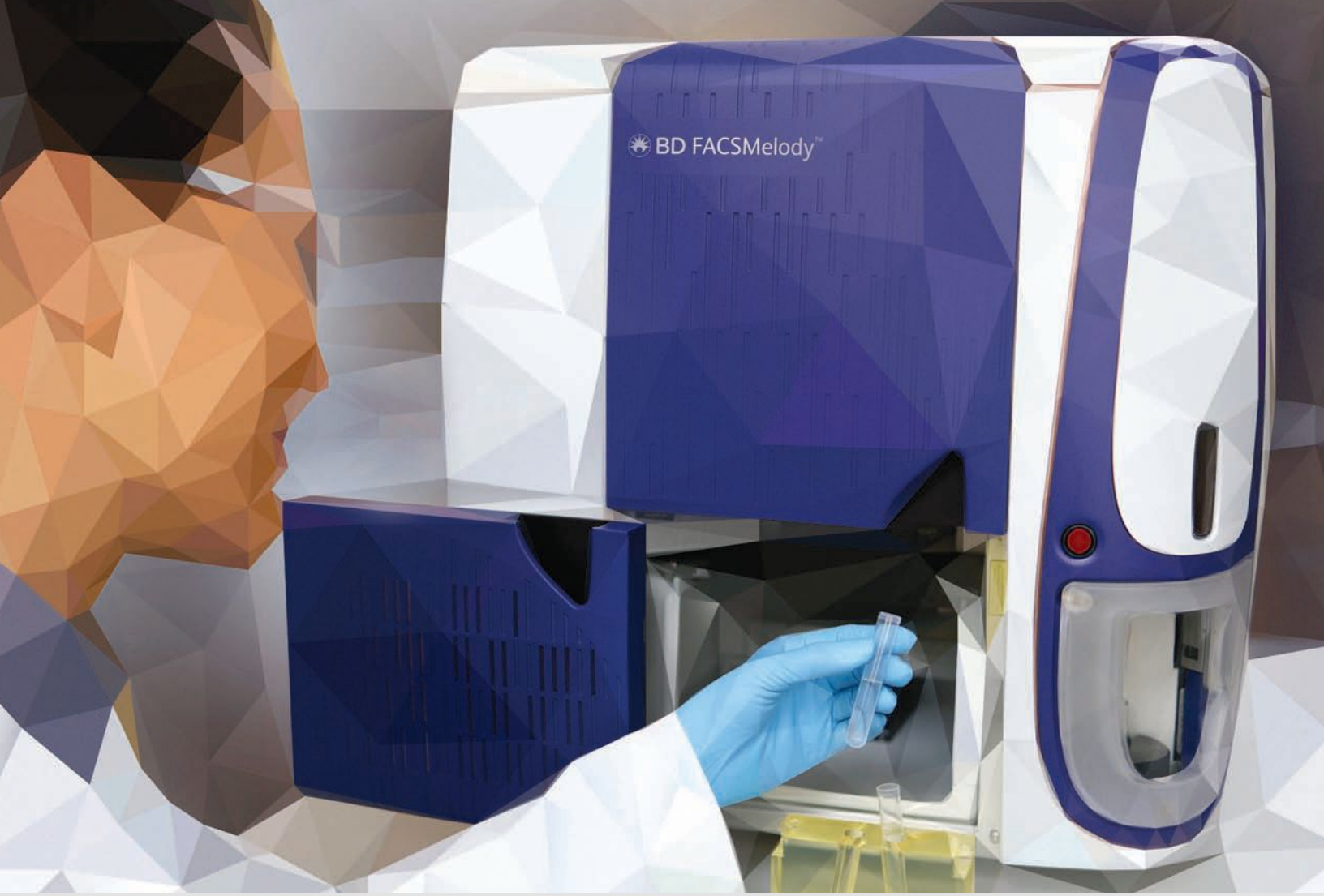

THE DIFFERENCE OF ONE SIMPLE SORT

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Where Fate Beckons

Using an ever-expanding toolbox, researchers track cell lineages as they develop.

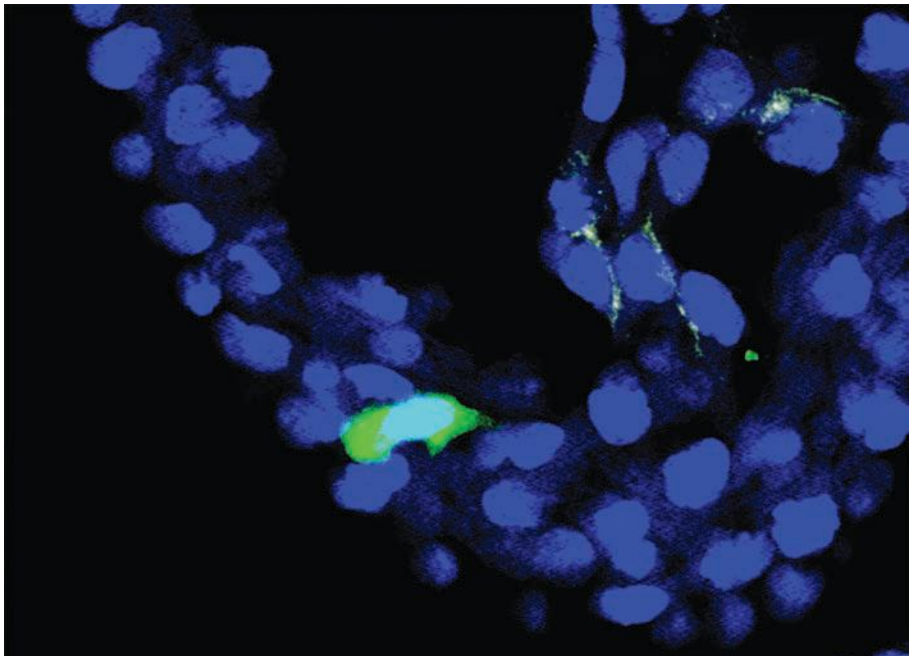
BY KELLY RAE CHI

The average human is made up of more than 30 trillion cells, not counting the hefty microbiome he or she carries. Although mostly red blood cells, our bodies comprise about 200 different types of cells. Understanding how organisms grow from one cell type to many different cell types is the overall goal of lineage tracing or fate mapping experiments, the first of which date to the late 19th century.

Historically, cell lineage tracing studies have taken large pools of cells from mature tissues and inferred their origins retrospectively by staining the cells with vital dyes or transplanting isotopically labeled cells into donor animals. In the past few years, however, an ever-expanding set of tools for single-cell analysis has allowed researchers to track each cell's fate as it happens—by focusing on embryonic development in animal models or by tracking the differentiation of specific populations of stem cells. For example, researchers can now use combinations of transgenic animal models and modern imaging techniques to view single cells buried deep within embryos. And next-generation sequencing, in particular, RNA sequencing, allows researchers to delineate changing patterns of gene expression as new cell types form. Lastly, the CRISPR-Cas9 system yields entirely new ways to drop genetic breadcrumbs into developing cells.

Such techniques will enable scientists to answer long-standing questions in development. "What we are able to do now is look at the time in the embryo where people think [key developmental] decisions are occurring, and see what we see," says Bertie Göttgens, a principle investigator at the Cambridge Stem Cell Institute in the U.K.

The Scientist spoke with the developers of four recent applications of single-cell lineage tracing experiments—examples that reflect the power biologists have in 2016 to answer fundamental questions about how tissues and organs form. Here's what they said.



MINGFU WU

HOMING IN ON THE HEART: Using a line of Brainbow mice created for developmental biology studies, researchers label individual cells (green) in the 9.5-day-old mouse embryo. (Blue is a general cell stain, for reference.) A clearing protocol adapted for embryos allows deeper imaging, including zeroing in on the heart as shown here.

BRAINBOW FOR THE HEART

RESEARCHER: Mingfu Wu, Associate Professor, Department of Molecular and Cellular Physiology, Albany Medical College, New York

PROJECT: Trabeculae are sheets of heart muscle cells that stretch across the inner surface of the heart's ventricles and boost gas and nutrient exchange during development. It is impossible to survive without them. In a new study, Wu's group examined how these cells first form, between embryonic days 9 to 10.5 in mice (*Cell Rep*, 15:158-70, 2016).

APPROACH: Wu's group used Brainbow, a transgenic technique originally devised to map individual neurons in the brain, to label cells stochastically using gene cassettes that code for four uniquely expressed fluorescent proteins. (See "Synapses on Stage," *The Scientist*, November 2013.) Developmental biologists have increasingly turned to Brainbow animals because labeled progenitor cells assume a unique color after Cre recombination and maintain that color as they continue to divide.

To understand how heart muscle cells form trabeculae, Wu's team used a Brainbow mouse line called *Rosa26-Confetti*. (The *Rosa26* locus is active across almost

all mouse cell types, as opposed to just neurons.) To induce Cre recombination, researchers injected pregnant mice with tamoxifen one to three days before harvesting the embryos.

The scientists then stained the embryo with specific markers to identify cell populations. To address the challenge of imaging the heart—which is deep within the embryo—the team made it transparent by chemically stripping away the lipids, which cause light to scatter and limit imaging depth. More specifically, they adapted the so-called Sca*le* method, first described in 2011 for clearing adult mouse brain. Clearing takes up to a week. The group demonstrated this protocol recently in the *Journal of Visualized Experiments* (doi:10.3791/54303, 2016).

FUTURE: Wu's newly adapted methods can be combined with CRISPR-Cas9 tools, he says. His group is now deleting or overexpressing genes in labeled cells. "This is a great tool to study gene function in a single cell," he says.

One common complaint about using four-color *Confetti* to label single cells (in an animal) is that the cells express green fluorescent protein less often, or sometimes not at all, compared with cells that express each of the other three colors. And the reporter is also relatively insensitive, meaning a large dose of tamoxifen is needed to induce labeling in a single clone, he adds. Wu's group is working on a more sensitive four-color reporter line, but until then, he also uses a two-color reporter mouse line called mTmG.

BLOOD VESSEL VS. HEART LINEAGES

RESEARCHER: Bertie Göttgens, Principle Investigator, Cambridge Stem Cell Institute, U.K.

PROJECT: Göttgens and his collaborators are working to solve a long-standing question in developmental biology: To what extent do blood vessel and heart lineages overlap, and when do they diverge? For the first time, Göttgens and his team followed the formation of a mammalian mesoderm—one of three germ layers that forms bone, muscle, and many other tissues—in a 7.5-day-old mouse embryo. In particular, they analyzed the transcripts of 1,205 single cells to answer how mesoderm lineages diversify during the developmental process of gastrulation (*Nature,* 535:289-93, 2016).

APPROACH: Very little specialized equipment was needed for this study—apart from access to a flow cytometer to isolate single cells. On the other hand, pulling off this study would not have been possible without an interdisciplinary team of scientists well-versed in developmental biology, genomics, and bioinformatics, Göttgens says.

On the development front, for example, biologists must be able to harvest embryos after the embryos have implanted into the uterine wall. This requires a trained eye. "You really need to know what you're looking for, because it's a tiny speck of white tissue embedded in a background of white tissue," he says. Expertise is also required for staging the embryos.

The group deployed single-cell RNA sequencing, which has rapidly grown in popularity now that a handful of techniques are available. The research team elected to use a particularly sensitive variant called Smart-seq2, which generates full-length coverage of the cell's transcriptome. (See "Scaling to Singles," *The Scientist,* May 2016.) In parallel, they sequenced the transcriptomes of embryos missing a particular transcription factor crucial for blood cell development to validate their findings.

TIPS: Göttgens suggests working with a bioinformatician to design the experiment from the outset to power the study with the right number of cells and to limit the potential influence of batch effects (in which two results differ because they were collected on different days).

Sorting technical noise from new biology is still a challenge. One way his group helped address this is by adding a standard set of reference mRNAs into their single cells (otherwise known as ERCC spike-ins).

The scientists used diffusion maps—a basic way to order snapshots of single cells along their developmental journey—but they plan to study developmental trajectories using a new algorithm called Wishbone, which uses single-cell RNA sequencing and mass cytometry data to specifically model branching steps during cell differentiation (*Nat Biotechnol,* 34:637-45, 2016). Although Göttgens admits that single-cell transcriptomics is in need of more data-analysis tools, he urges newcomers to dig in to lineage tracing anyway. "If someone wants to do it, they can start now," he says. "There's no point in waiting."

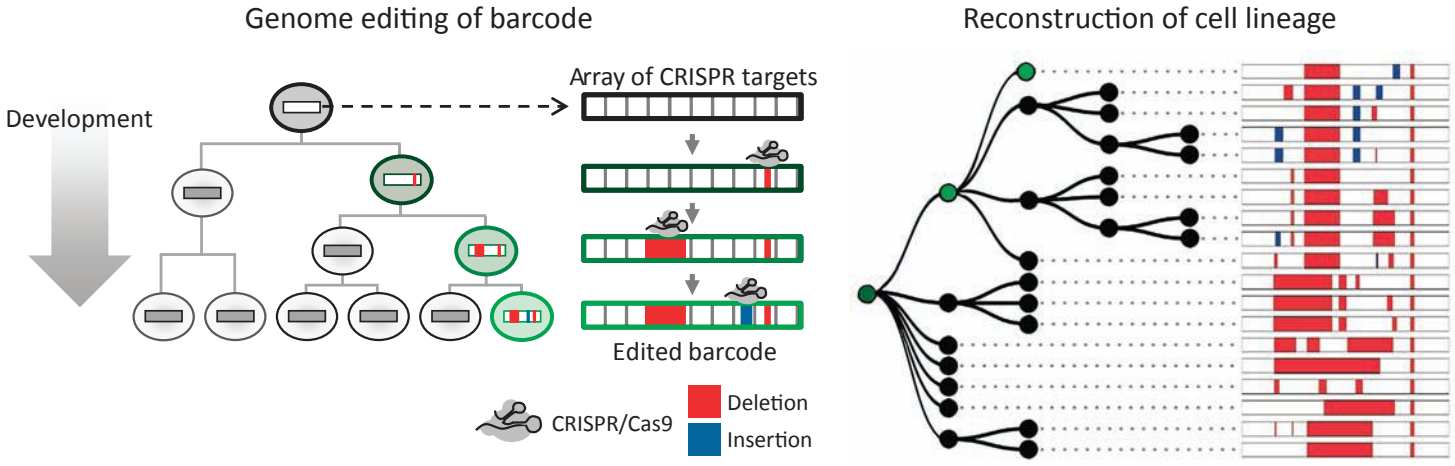
CRISPR FOR FATE-MAPPING

RESEARCHER: Jay Shendure, Professor, Department of Genome Sciences, University of Washington

PROJECT: In collaboration with Alexander Schier's lab at Harvard University, Shendure's group came up with a new way to trace cell lineages in cell culture and in whole organisms—in this case, developing zebrafish. The technique, called GESTALT (genome editing of synthetic target arrays and lineage tracing) is described in *Science* (353:aaf7907, 2016).

APPROACH: The basic idea of GESTALT is to introduce CRISPR-Cas9 into dividing cells and track the cells as they accumulate mutations in each subsequent generation of cells. The group's starting cells or embryos are equipped with a synthetic barcode approximately 300 base pairs in length. In their zebrafish model, the barcode contains 10 20-base-pair sequences targeted by Cas9 endonuclease, with each of the sequences matching a specific single guide RNA.

At the end of these experiments, the scientists amplify the barcodes and read their DNA or RNA, reconstructing the lineage trees computationally.



FATE MAPPING WITH CRISPR: Thanks to a new tool called GESTALT, researchers can now introduce a 300-base-pair lineage barcode into cells and, using the CRISPR-Cas9 system, allow edits to accumulate as those cells divide. New computational tools enable the reconstruction of those lineages (right).

A tag attached to the barcode acts as a unique molecular identifier to help account for potential biases introduced through polymerase chain reaction–based amplification.

The technique should be tractable in any organism where it is possible to do CRISPR-Cas9 editing. "We're already trying to adapt it to a number of other model organisms," says Shendure, who first worked on cell lineage tracing as a graduate student in George Church's Harvard lab in 2000.

Shendure's University of Washington team developed new computational tools for this study, including one for lineage reconstructions that are not feasible when using standard evolutionary phylogenetics tools. The code is publicly available via the software repository GitHub (under "Cas9FateMapping"). In addition, the group's barcode sequences and related constructs are available on Addgene.

FUTURE: The single biggest limitation is that the technique does not yield information about the spatiotemporal location of the cells, Shendure says. His group was able to dissect the organs and tissue for rough estimates, but a better way to address this question is with RNA sequencing. The team is also working on a method to improve the introduction of the editing reagents—at later time points in development, for example—to refine their control of mutation rates.

LOCATING PRE-HEMATOPOIETIC STEM CELLS

RESEARCHER: Fuchou Tang, Assistant Professor, Biodynamic Optical Imaging Center, Integrated Science Research Center, Peking University

PROJECT: In a study published in *Nature*, Tang and his collaborators isolated pre-hematopoietic stem cells (HSCs) for the first time and showed their unique transcriptome signatures before and during their maturation into HSCs (533:487-92, 2016). HSCs go on to form a diverse set of immune and other blood cells.

APPROACH: Isolation is a key hurdle in single-cell analysis (see "Singularly Alluring," *The Scientist*, June 2014) and even more so for Tang's team because pre-HSCs had never been isolated. To verify that the individual cells they picked were pure, the researchers cultured them for several days to determine, using cell markers, whether they formed mature HSCs. Then, they transplanted these cells into recipient mice and confirmed that the cells can form all of the blood cell lineages in vivo. Lastly, they transplanted the pre-HSCs a second time to confirm that they could form all of the correct lineages. This in vivo work was a real challenge, Tang says. A simpler strategy to confirm the isolation is to repeat the sorting of single cells and to fix and stain them, he adds.

Tang has been at the forefront of developing transcriptomics methods for single cells, and for relative beginners to deploy these methods, "now it is quite easy and straightforward," he says. That said, certain factors boost success. For instance, researchers should be able to isolate the cells rather easily and keep them alive while suspended in a liquid. It also helps if the cells are 6 µm in diameter or bigger, and if you have a good species-specific reference genome.

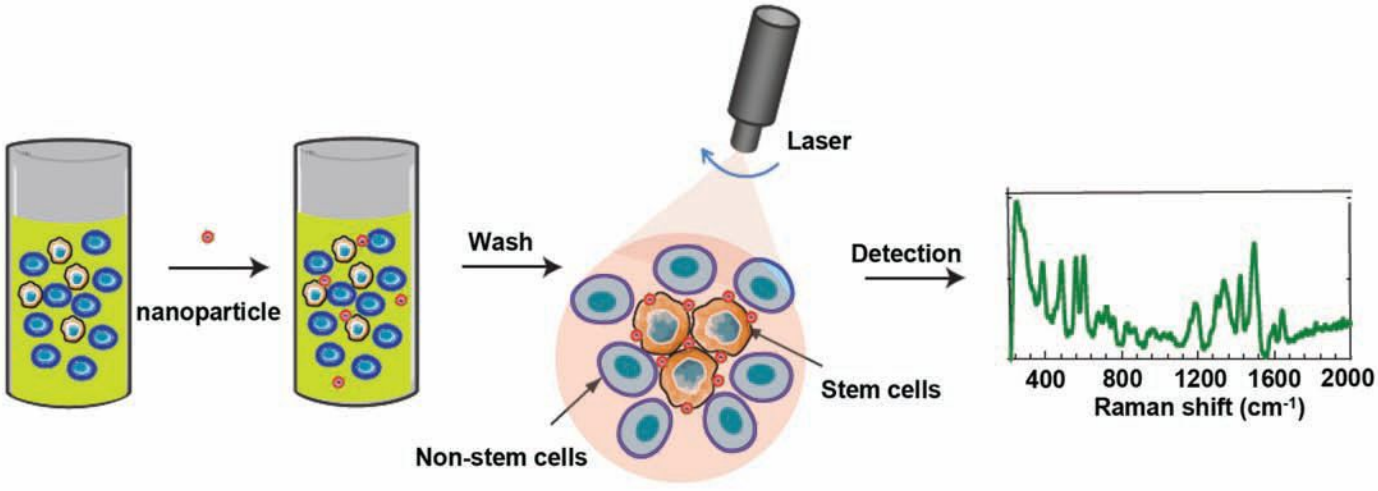
TIPS: The analysis of RNA-seq data is time-consuming and labor-intensive. "When people design such projects, they have to realize that they need much more manpower and time on this 'dry lab' side than on 'wet lab' side," Tang says. "It is not enough to be able to use the standard bioinformatics tools out there these days." For example, his group looked at specific signaling pathways and transcription factors expressed in their pre-HSCs. In addition, researchers should consider following up with interesting candidate genes by knocking them out individually in mice, Tang says. ■

COURTESY SHENDURE AND SCHIER LABS

Visualizing Cellular Vibes

Cell identification techniques that rely on observing molecular vibrations with Raman spectroscopy

BY JYOTI MADHUSOODANAN



SIFTING STEM CELLS: Gold nanoparticles carrying antibodies to stem cell surface markers bind to the cells and enhance spectral signals detected by surface-enhanced Raman spectroscopy (SERS).

When a laser beam shines on a single cell, chemicals within the cell can absorb, reflect, or scatter the light waves. The scattered light generates a unique signature at various wavelengths that can be used to identify specific molecules, depending on the molecule type—protein, sugar, or nucleic acid—and the chemical bonds present within its structure. For decades, researchers have used these signatures, known as Raman spectra, as chemical fingerprints to characterize cells.

Unlike flow cytometry, microfluidics, and other cell sorting methods, techniques that rely on Raman spectroscopy do not require labels, and they can be more sensitive and specific than flow cytometry for many applications. Variant versions such as surface-enhanced Raman spectroscopy (SERS)—where chemicals are adsorbed on the surface of a metal, boosting the emitted spectral signals—have upped the sensitivity and specificity of Raman-based assays by enhancing the electric field near molecules. Using antibodies bound to nanoparticles made of metals, such as gold or silver, can produce as many as six or seven SERS spectra in a single assay, providing a higher level of discrimination than fluorescent labels can.

With quickly improving tools and analytical methods, Raman spectroscopy is gaining popularity among life scientists. "Raman spectroscopy instrumentation has been developing very rapidly in the last five years, with very much improved detectors and light sources reducing the cost, but retaining performance of these systems," says Duncan Graham, a professor of chemistry at the University of Strathclyde in Glasgow. Faster cameras allow spectra to be captured in microseconds rather than seconds, and better software and statistical tools make it possible to analyze large data sets produced by sorting and analyzing cells, he adds.

Here, *The Scientist* takes a tour of four strategies that deploy Raman spectroscopy to detect and study cells.

TESTING STEM CELL THERAPIES

INVESTIGATORS: Chunhui Xu, associate professor of pediatrics, and Ximei Qian, assistant professor of biomedical engineering, Emory University

PROJECT: Removing residual cancer-causing stem cells from preparations derived from pluripotent human stem cells.

PROBLEM: Human pluripotent stem cells (hPSCs) are a promising route to stem cell–based therapies for a wide range of diseases. Cells derived from hPSCs often contain residual undifferentiated cells, however, and these contaminating remnants can form tumors when transplanted into animals. Flow cytometry and other assays can only detect these cells if they form 0.1 percent or more of a population, but a mouse study of tumor formation by undifferentiated cells estimated that concentrations of even 1 in 10^5 cells (0.001 percent) have the potential to form teratomas (*Cell Cycle*, doi:10.4161/cc.8.16.9353, 2009). Tumor formation after transplantation of hPSCs into animal models is the "gold standard" test for purity and safety, says Qian, but this pro-

J. HAN ET AL., *BIOMATERIALS*, DOI:10.1016/J.BIOMATERIALS.2016.07.033, 2016.

cess takes about three months. It is also expensive and nonquantitative, making it a poor choice for a clinical assay.

SOLUTION: Xu, Qian, and colleagues wanted to use SERS to detect residual stem cells, as they had done with circulating tumor cells in 2011. The team coated gold nanoparticles with antibodies to two stem cell surface markers known as SSEA-5 and TRA-1-60, and then covered the exposed nanoparticle surfaces with polyethylene glycol (PEG) to reduce nonspecific binding. They tested the nanoparticles on samples of hPSCs mixed with known numbers of fibroblast cells to confirm that the SERS signal was proportional to the number of stem cells present. The assay could detect one stem cell in a background of one million fibroblasts, 2,000- to 15,000-fold more sensitive than flow cytometry assays. The technique worked equally well in a culture of hPSC-derived heart muscle cells. "The high sensitivity and specificity is the key advantage of this assay over other methods," Xu says.

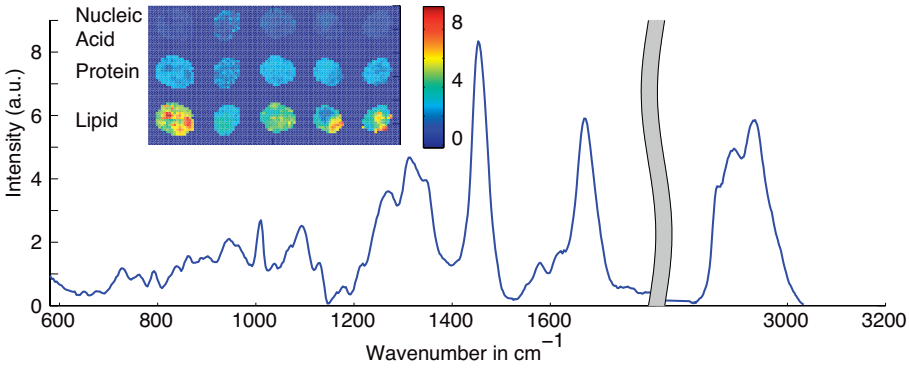
The assay proved to be as sensitive as the 2011 study of tumor cells, she adds. Optimizing the technique for different cell types requires finding the right ligand density per nanoparticle—in this case, 25 antibodies per particle for the SSEA-5 marker (*Biomaterials*, doi:10.1016/j.biomaterials.2016.07.033, 2016).

SCANNING FOR SPECIFICITY

INVESTIGATORS: Iwan Schie, postdoctoral researcher, and Christoph Krafft, research group leader, Leibniz Institute of Photonic Technology, Germany

PROJECT: Improving the specificity of single-cell classification by gathering mean Raman spectra

PROBLEM: Most studies rely on a single Raman spectrum reading of a cell. But that's not always accurate for eukaryotic cells, which are generally larger than the diameter of the laser focus. Single readings from a cell are affected by factors such as whether the cell is actively replicating DNA, if it's undergoing apoptosis or necrosis, and the quantity of lipid droplets or proteins. In addition, "you can get variations depending on if you focus the laser on the nucleus or the cytoplasm," Krafft says. Acquiring multiple spectra from each cell to identify a mean macromolecular signature is ideal, but this is time consuming and more expensive, so researchers may sample fewer cells to compensate.



PATCHY CELLS: Cell images (inset) reconstructed using mean Raman spectra acquired from the entire cell (graph) show that macromolecules are unevenly distributed throughout cells.

SOLUTION: Rather than taking multiple individual readings, Schie and Krafft found that scanning a laser beam along an arbitrary line or other defined shape inside a cell can generate a continuous Raman signal from the sample. Most commercial spectrometers are typically developed to analyze materials rather than cells, so the team designed a Raman microscope with several different optical lenses, a multimode optical fiber, motorized table, and other flexible components that make the instrument usable as a microscope as well as for acquiring single point spectra and spectra from multiple points along a line (*Biomed Spectrosc Imaging*, doi:10.3233/BSI-160141, 2016). The device costs approximately 50,000 Euros ($55,000), unlike commercial instruments that typically run €150,000–€200,000 ($163,000–$217,000), according to Schie.

The team assayed two different pancreatic cancer cell lines and a T-cell line using this setup and compared the results to Raman imaging in which single spectra from many points are combined to produce an image of a cell, and Raman spectra acquired from single points in cells. Integrated spectra yielded less variation between cells of the same type, so fewer cells had to be sampled to train a robust classification model. Using only 20 cells in a training model was sufficient to achieve an accuracy of more than 90 percent. All three methods could distinguish between the two cancer cell lines and the T-cell line. Because taking readings along a line samples cells continuously rather than taking discrete measurements, "the variation between two cells of the same type is not as high as if we acquire single spectra from random sites in cells," Krafft says. This method could be an effective, high-throughput way to use Raman spectroscopy to detect circulating tumor cells, he adds (*Analyst*, doi:10.1039/C6AN01018K, 2016).

TRACKING CANCER CELL DEATH

INVESTIGATOR: Mostafa El-Sayed, professor of chemistry, Georgia Institute of Technology

PROJECT: Using SERS to observe how heated nanoparticles can target and kill tumor cells

PROBLEM: Targeting nanoparticles to tumor markers and heating them with a laser can kill cancer cells, but researchers do not yet know the molecular mechanism. "Understanding precisely how a potential therapy kills cells is important to identify and develop new drugs," El-Sayed says. Previous explorations

COURTESY IWAN SCHIE

have monitored changes in mitochondrial membrane potential using flow cytometry or confocal microscopy, but those methods did not reveal whether the nanoparticles entered cells by passive or active transport, or whether cell death was apoptotic or necrotic. Varying results have been attributed to differences in particle size and shape, or the laser intensity and exposure time used to generate heat.

SOLUTION: Instead of employing an additional method to monitor cellular changes, El-Sayed and his colleagues used actively targeted gold nanoparticles to trigger heat-induced cell death and, with the help of SERS, to monitor how this death occurred in real time. Nanoparticles used for SERS are typically ~50 nm wide, but smaller nanoparticles absorb more radiation than they scatter, converting it to lethal heat, El-Sayed says. So the team used ~29-nm-wide gold particles, coated them with PEG, and linked them to peptides that increase the particles' uptake into the perinuclear region of tumor cells. They treated cells with a 0.2 nM concentration of nanoparticles, shined a 785 nm laser beam on the sample, and collected SERS spectra for two hours throughout the exposure.

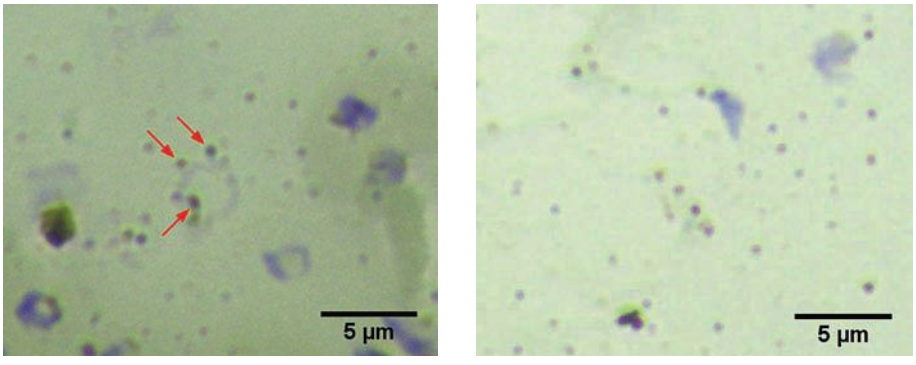
The initial changes in the spectra revealed that laser exposure broke down disulfide bonds in proteins. Later in the process, more protein and lipid structures were disrupted by the laser-induced heat, which eventually caused cells to die. The SERS signatures produced during this process were similar to those seen when cells were killed by heating them in an oven. This trajectory was unrelated to nanoparticle size or laser intensity, but a minimum threshold of particle concentration and laser intensity was needed to spur heat-induced cell death (*J Am Chem Soc*, doi:10.1021/jacs.5b10997, 2016).

MEASURING MALARIA

INVESTIGATOR: Quan Liu, assistant professor of bioengineering, Nanyang Technological University, Singapore

PROJECT: Using SERS to diagnose and quantify the severity of malaria

PROBLEM: Field-based, rapid diagnostic tests for malaria are low cost and readily available, but they often lack the sensitivity and specificity needed to detect the small numbers of parasites found in the early stages of infection. Lab-based techniques such as flow cytometry, used to sort infected red blood cells, are more sensitive, but they can only analyze individual or small batches of cells and are primarily used for research. "Each method has its own niche," says Liu. "We address the [divide] between cheap price and high sensitivity, which is a challenge for most existing methods."



MEASURING MALARIA: Silver nanoparticles (red arrows, left photo) formed in situ near the malaria parasite's vacuoles produce a stronger SERS signal for hemozoin, a degraded form of hemoglobin, than nanoparticles that are added to lysed blood containing hemozoin released from vacuoles (right).

SOLUTION: During infections, the malaria parasite *Plasmodium falciparum* feeds on hemoglobin in red blood cells and degrades it in to hemozoin, a molecule that serves as a disease marker and can be detected using Raman spectroscopy. Liu and his team turned to SERS for its high sensitivity, but also because the necessary detection equipment can be built at a low cost, he says.

The researchers used two different methods. In the first, they synthesized silver nanoparticles in the lab and mixed them with hemozoin crystals collected from lysed red blood cells. Hemozoin adhered to the nanoparticles and produced characteristic spectra that could be detected with a sensitivity of 0.01 percent, i.e., approximately 500 parasites/µL of blood. (Flow cytometry, in contrast, has a sensitivity of two percent, or approximately 100,000 parasites/µL.) In the second method, they synthesized nanoparticles inside the parasites themselves. They lysed red blood cells by sonication to release the enclosed parasites, but avoided using the lipid solvent Triton-X, so that the parasites—and their vacuoles containing hemozoin—remained intact. As a result, nanoparticles that formed near or inside these vacuoles adsorbed higher amounts of hemozoin than they did when cells were lysed and hemozoin was released into several milliliters of solution. With this version, the team could detect approximately 2.5 parasites/µL of blood.

The first method caused less variation in SERS measurements, and the correlation between SERS readings and disease severity was stronger. But the second assay was significantly more sensitive to low parasite loads and could be used as an early diagnostic tool, says Liu (*Sci Rep*, doi:10.1038/srep20177, 2016). Engineering the sample and nanoparticle preparation process to occur on a microfluidic device could turn these assays into a more effective portable, low-cost field test for malaria, he adds. ■

Q. LIU ET AL., *SCIENTIFIC REPORTS*, DOI:10.1038/SREP20177, 2016

Waiting for Oligonucleotide Therapeutics

Successful late-stage clinical trials could mark the maturation of a new drug development platform, but the path to commercialization is not without hurdles.

BY CATHERINE OFFORD

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Cold Spring Harbor Laboratory molecular geneticist Adrian Krainer was at a National Institutes of Health workshop in 1999 when he first learned about the crippling neurodegenerative disease spinal muscular atrophy (SMA)—the leading genetic cause of death in infants. The disease has no treatment, and more than 90 percent of infants born with SMA die before the age of two. At the workshop, Krainer recalls, researchers presented their findings on two genes associated with the disease, *SMN1* and a duplicate gene, *SMN2*, both coding for survival motor neuron (SMN) protein, an essential component in the production of spinal motor neurons.

Despite the apparent similarity of the genes, SMA researcher Christian Lorson, then of Tufts University School of Medicine in Boston, and colleagues had found that a single nucleotide difference was causing the RNA transcripts of each gene to be processed differently, Krainer says. While *SMN1*—which is usually absent or defective in SMA sufferers—produces functional protein, *SMN2* contains a mutation that causes exon 7 to be regularly left out of the transcript during splicing. The resulting messenger RNA (mRNA) is unstable and quickly degraded, resulting in low levels of SMN.

The research piqued Krainer's interest. He had been studying general mechanisms of splicing and exon skipping and saw the potential to restore proper splicing of *SMN2* transcripts as a way to compensate for *SMN1* loss in infants who suffer from SMA. After the workshop, he and his colleagues took up the challenge. In the early 2000s, they developed a splicing enhancer and a synthetic strand of nucleotides that bound close to exon 7 in SMN2 transcripts. On binding, this complementary, or antisense, compound would promote splicing of the nearby exon into the final mRNA, boosting levels of SMN protein.

A decade of improvements, modifications, and collaboration with antisense therapeutics company Isis (now Ionis) Pharmaceuticals resulted in the development of nusinersen—a drug delivered via spinal injection that is expected to become the first therapeutic for SMA. In August, Ionis halted its Phase 3 trial in SMA patients after interim analyses showed that infants receiving a spinal injection of nusinersen were better able to kick, stand, and walk than infants undergoing a sham procedure. "Obviously, we're extremely excited," says Krainer. "It's sort of beyond anyone's most optimistic expectations."

Nusinersen is more than just a breakthrough for SMA. It's among a handful of late-stage therapeutics in a class of molecules being hailed as the third major drug-development platform after small molecules and biologics: oligonucleotides. These short, chemically synthesized nucleic acids—between 10 and 30 nucleotides in length—have served as vital research tools for more than half a century, playing central roles in DNA sequencing, polymerase chain reaction (PCR), and molecular cloning. Oligonucleotides have also long been recognized as potential therapeutics thanks to their ability to modify gene expression, and in recent years, the number of clinical trials testing oligonucleotide therapies has spiked. Many believe that the platform will soon be ready to treat a wide range of genetic diseases, including those, such as SMA, that were previously undruggable.

"The promise is already here," John Rossi, an RNA biologist and cofounder

of Dicerna Pharmaceuticals, wrote in an email to *The Scientist*, adding that he sees "the next few years as becoming the era [of oligonucleotide therapeutics]."

A slow start

The first oligonucleotide technology explored for therapeutic purposes was antisense technology, which relies on single-stranded sequences of nucleotides that are complementary to RNA transcripts in human cells. Antisense therapies bind to an mRNA or a pre-mRNA—a transcript awaiting splicing and other modifications—and either block protein translation to eliminate a gene product or, as in the case of nusinersen, alter splicing to restore stability or function to a protein. The technology led to the first oligonucleotide therapeutic marketed in the U.S.: fomivirsen, a drug approved in 1998 that is injected into the eye to treat cytomegalovirus retinitis, an infection afflicting immunocompromised patients.

In the early 2000s, researchers also began to take therapeutic advantage of a recently discovered mechanism of gene silencing: RNA interference (RNAi). By employing double-stranded small interfering RNAs (siRNAs) to hijack this natural pathway in cells and degrade target mRNA, researchers hoped to block the translation of proteins associated with a range of genetic conditions, from cancers to macular degeneration. Unfortunately, lack of efficacy and immune reactions to the treatments led to a series of disappointing clinical trials in the late 2000s. (See "The Second Coming of RNAi," *The Scientist,* 2014.)

Besides fomivirsen, which was discontinued in the U.S. a decade ago following improvements in HIV medications, only two oligonucleotide drugs had reached the market prior to 2016. The first, pegaptanib, is a protein-blocking RNA known as an aptamer that was approved in 2004 to treat age-related macular degeneration, but soon met the same fate as fomivirsen, being overtaken by a more effective monoclonal antibody treatment. The second, mipomersen, is an antisense oligonucleotide that was approved in 2013 for the treatment of a genetic form of high cholesterol but floundered in the face of poor marketing and a failure to gain regulatory approval in Europe.

"[These drugs] were never commercially successful," says Dirk Haussecker, an independent biotech consultant and author of *The RNAi Therapeutics Blog*. "They're approved, but nobody's taking or prescribing them."

Oligonucleotide therapeutics are being hailed as the third major drug-development platform after small molecules and biologics.

Despite the slow start, oligonucleotides have both therapeutic and commercial promise. In addition to current antisense and RNAi-based therapies in clinical trials, researchers are now developing oligonucleotides that target microRNAs—small, noncoding RNAs that regulate gene expression posttranscriptionally. These include anti-miRs that block the activity of specific microRNAs and miRNA mimics that upregulate it (see table on following page). As of August, there were more than 70 oligonucleotide therapeutics in ongoing or recently completed clinical trials in the U.S. alone, and a recent analysis by a market research firm predicted that the global antisense and RNAi therapeutics market will reach $4.58 billion by 2022.

Researchers, pharmaceutical companies, and investors now anxiously await results from several late-stage trials, anticipated in 2017, as well as nusinersen's approval, for confirmation that oligonucleotide therapeutics are finally reaching maturity. "Every year it seems like we've gotten closer," says Phillip Zamore, director of the RNA Institute at the University of Massachusetts School of Medicine and cofounder of RNAi therapeutics company Alnylam Pharmaceuticals. Even RNAi drugs, which joined the game later than antisense, are catching up, he adds. "Now there are Phase 3 clinical trials."

Delivering success

One of the main difficulties in developing oligonucleotide therapies is that nucleic acids are broken down by endonucleases in the bloodstream and within cells, making systemic delivery of naked molecules ineffective. While chemical modifications can help antisense oligonucleotides resist this degradation, siRNAs used for RNAi-based therapies are much less stable, and challenges delivering these molecules were blamed for the disappointing drug trials a few years ago. After that, the industry realized that, "to be a successful RNAi company, you had to become a drug delivery company," says Zamore. "When that was solved, then things took off."

Early methods for packaging oligonucleotides used liposomes or lipid nanoparticles that, like unpackaged oligonucleotides, localize to cells in the liver. This natural hepatic affinity pointed the way to some of the most successful drug development in the field, says Barry Greene, president and COO of Alnylam. "It turns out that what some view as a shortcoming—that is, the ability to deliver to hepatocytes—is a treasure trove of opportunities," he explains. "We're only at the tip of the iceberg in terms of the kind of genetically valid targets that are in fact produced in hepatocytes."

Consequently, most oligonucleotide therapeutics companies have focused on the liver. Ionis has antisense oligonucleotide therapeutics in more than 10 clinical trials targeting diseases in the organ, from thrombosis to hepatocellular carcinoma. And Alnylam's most advanced therapeutic is an intravenous, nanoparticle-carried drug called patisiran, an RNAi treatment that knocks down the translation of a mutated protein made in the livers of patients with a rare disease called hereditary amyloidosis. Results from Phase 3 trials of the drug are expected in 2017 and, if successful, could pave the way for the first approval of an RNAi therapeutic.

More recently, Alnylam has conjugated its siRNAs to the sugar molecule N-acetylgalactosamine (GalNAc) to give the drugs even greater affinity for hepatocytes, improve potency, and reduce off-target effects. Early-stage testing of fitusiran, a GalNAc-conjugated siRNA targeting a liver-produced protein associated with hemophilia, is ongoing, the company says. Meanwhile, Ionis has already adopted a similar

method to conjugate GalNAc with antisense oligonucleotides destined for the liver—an advance that Ionis claims improves delivery and provides a more than 30-fold increase in potency over unconjugated approaches currently in trials. RNAi company Arrowhead Pharmaceuticals is also using GalNAc to help deliver oligonucleotides to the liver, but instead of conjugating it directly to the siRNA, the company's dynamic polyconjugate (DPC) technology links the sugar to the siRNA via a polymer that helps protect the cargo until it enters a cell. A recent clinical study on hepatitis B therapeutic ARC-520 found that the drug inhibits the production of viral protein in infected patients by up to 99 percent after a single dose.

"For anything where the target is made by hepatocytes, the problems are largely solved," says Zamore. "I think the next big challenges are delivering RNAs to a wider range of tissues and cell types with similar specificity."

Although targeting other tissues is challenging, some companies are now making strides to do just that. Ionis, for example, has developed modified antisense oligonucleotides that show effective targeting to cells in the kidney. Arrowhead, meanwhile, is working on DPCs that will target cells in non-liver tissues such as tumors by incorporating ligands that target cell type–specific surface proteins. "It's a matter of finding the right receptor-ligand combinations and to figure out how to conjugate the proper ligands," explains Krainer. "Down the line, I think one could have a set of conjugates that would allow you to target particular organs."

The waiting game

Despite considerable progress in oligonucleotide delivery, clinical successes have been balanced with setbacks that serve as reminders that the path to commercialization is never guaranteed. In August, for example, biopharmaceutical company and Ionis partner OncoGeneX announced that its antisense drug custirsen failed to improve survival of patients with prostate cancer in late-stage clinical trials. And earlier this year, Ionis reported dangerous reductions in platelets among patients in Phase 3 trials for two unconjugated antisense therapies, one for a rare cardiac condition, and one for elevated triglycerides. Although most drugs in Ionis's pipeline are now conjugated, which the company hopes will avoid such side effects, the announcement triggered a 40 percent plunge in share prices in May. (Shares are now recovering as the company makes progress with nusinersen.)

ORDERS OF OLIGONUCLEOTIDES

Multiple therapeutics using small, synthetic nucleic acids, or oligonucleotides, have been designed since the first antisense drugs of the 1990s and early 2000s. Although only four therapies have been approved by the US Food and Drug Administration to date, recent and expected approvals from regulators in the U.S. may give the field the boost it needs to launch this class of molecules as the third major drug development platform after biologics and small molecules.

Therapy	Description	Target	Mode of action
Antisense oligonucleotide	Single-stranded DNA or RNA	Messenger RNA (mRNA) or pre-mRNA	Blocks protein translation or affects splicing
Aptamer	Single-stranded DNA or RNA	Proteins, small molecules, toxins, or even whole cells	Inactivates or modifies target. (See "Antibody Alternatives," *The Scientist*, February 2016.)
Small interfering RNA (siRNA)	Double-stranded RNA	mRNA	Hijacks natural gene-silencing pathway in cells that uses interference (RNAi) to trigger target degradation
Anti-microRNA	Single-stranded RNA	microRNA	Inactivates microRNA, leading to changes in gene expression
MicroRNA mimic	Double-stranded RNA	mRNA	Augments activity of endogenous microRNA to block translation

More worryingly for the development of RNAi therapeutics, Alnylam halted development of its GalNAc-conjugated therapeutic revusiran for hereditary amyloidosis this October after reporting more deaths among patients receiving the drug in a Phase 3 trial than among those on a placebo. Despite attempts to reassure investors that other drugs in its pipeline use a newer GalNAc technology, the company saw its market value nearly halve overnight.

Even for drugs that make it through trials without major complications, there's still the slog of obtaining regulatory approval. Antisense drug eteplirsen, developed by Sarepta Therapeutics as a treatment for certain Duchenne muscular dystrophy sufferers, attained priority FDA review in late 2015. But the clinical data supporting the drug's approval—based largely on a trial including just 12 people—came under fire from regulators. The FDA finally granted approval this September, but the decision has proved controversial. "This has been delayed numerous times," Krainer notes. "Clearly, the drug is safe and there's no other treatment, but there are questions about efficacy."

With these sorts of ups and downs, the commercialization of oligonucleotide therapeutics is risky, Krainer says. "You could have a good drug that fails a clinical trial just because the right outcome measures weren't selected," he explains. "It's expensive enough that I don't think one gets to play the game too many times. Perhaps you have one shot or two—luck is involved and there's no guarantee."

But as more companies report the results of late-stage trials, a little good news could go a long way, says Ron Renaud, CEO of gene-upregulation biotech RaNa Therapeutics. "There's so much science and so much knowledge and so much benefit to be gained by everybody. The whole notion of a rising tide lifting all ships definitely resonates with a lot of us in these companies."

Just like previous drug platforms, which all took time to reach maturity, oligonucleotide therapeutics may now be on the brink of delivering on the promises of the last 20 years. "People are pretty impatient, but when you look at technologies like these, just the general platform can take decades to develop," says Krainer. "It was no different for making monoclonal antibodies; . . . it took a really long time. These therapeutics encompass a wide variety of technologies and each of them has to be given a chance to prove its worth." ■

Main companies currently involved in clinical or preclinical research	**Drugs that have been approved or are in late-stage clinical trials**
• Ionis Pharmaceuticals • Sarepta Therapeutics	• Fomivirsen (approved 1998) • Mipomersen (approved 2013) • Eteplirsen (approved 2016) • Nusinersen (Phase 3 trial halted in August due to positive results) • Alicaforsen (currently in Phase 3 trials) • Aganersin (currently in Phase 3 trials)
• SomaLogic • RIBOMIC • Aptagen	• Pegaptanib (approved 2004) • Pegpleranib (currently in Phase 3 trials)
• Alnylam Pharmaceuticals • Dicerna Pharmaceuticals • Arrowhead Pharmaceuticals • Arbutus Biopharma • Quark Pharmaceuticals	• Patisiran (results from Phase 3 trial expected mid-2017) • QPI-1002 (currently in Phase 3 trials)
• Santaris (owned by Roche) • Regulus Therapeutics	None
• Mirna Therapeutics • Regulus Therapeutics	None

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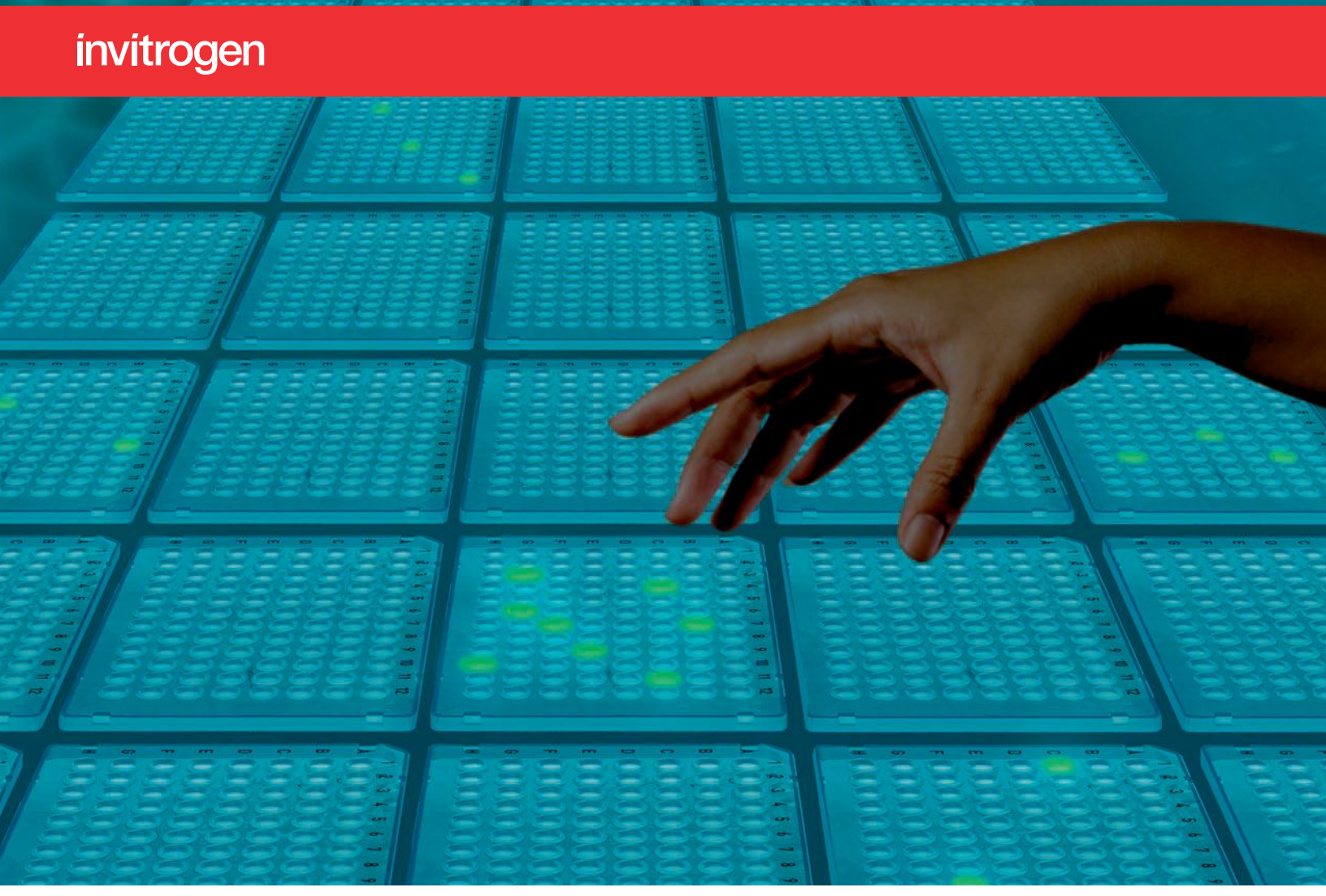

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Clinical CRISPR

The revolutionary genome editing tool will likely be used widely in patients. Is science and medicine ready?

BY JOHN PARRINGTON

CRISPR-Cas9 genome editing has the potential to transform medicine in several important ways. First, the technique makes it possible to manipulate genes in a variety of mammals to create models of human health and disease. Previously, only mice could be engineered in this way, but genome editing has made it possible to precisely modify the genomes of almost any mammal.

Because pig hearts or monkey brains are far more similar to their respective human organs than those of mice, this should have a major impact on our ability to understand the genetic basis of heart disease and various mental disorders. But such developments are likely to be controversial because of opposition by some people to experimentation on primates.

Another way that genome editing affects medicine is by facilitating the study of physiological or pathological processes in human cells in culture. Using genome editing to precisely manipulate the genomes of human cells in vitro is making it possible to identify the genes involved in normal human physiology and in various types of human disease. I discuss these and other transformative applications of CRISPR-Cas9 genome editing in my new book *Redesigning Life: How Genome Editing Will Transform the World*.

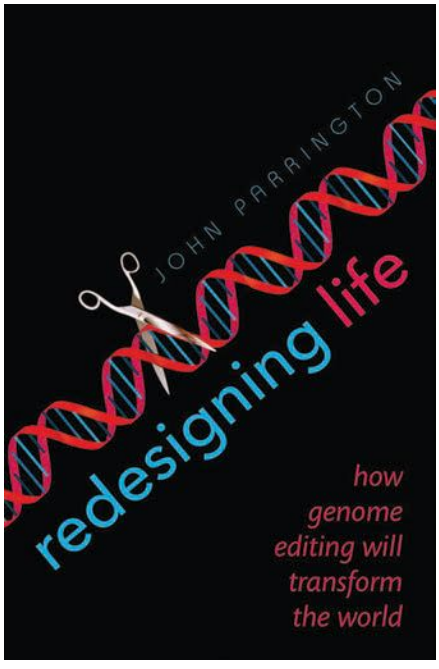
One particularly exciting development exists at the intersection of genome editing and stem cell technology. Pluripotent stem cells have the potential to develop into any cell type in the body. They can be isolated from human embryos as embryonic stem (ES) cells, or by activating specific genes in adult human cells to generate induced pluripotent stem (iPS) cells.

Recently, scientists have coaxed ES and iPS cells to develop into organoids—structures resembling tissues from the eye, gut, kidney, pancreas, prostate, lung, stomach, breast, and even the brain. And genome editing is making it possible to manipulate such organoids so that they can provide insights into human embryo development or serve as disease models and drug-screening platforms.

Su-Chun Zhang of the University of Wisconsin–Madison said in a statement released this summer that, "This marriage between human stem cells and genome editing technology will revolutionize the way we do science." A team led by Pablo Ross at the University of California, Davis, recently used CRISPR-Cas9 to engineer pig embryos so that they could no longer grow a pancreas. Injecting human iPS cells into the embryos encouraged the growth of a rudimentary human pancreas. "Our hope is that this pig embryo will develop normally, but the pancreas will be made almost exclusively out of human cells and could be compatible with a patient for transplantation," Ross recently told *BBC News*.

Engineering stem cells to create human organs for transplant surgery is one potential direction for genome editing; another is to use the technology to correct genetic defects that underlie some human diseases. Recent studies have shown the potential of genome editing to repair the genetic defects in the genes for dystrophin and huntingtin, which cause Duchenne muscular dystrophy and Huntington's disease, respectively. Citing successful animal studies, US regulators have green-lighted clinical trials that use genome editing to treat cancer and are considering trials of CRISPR-based treatments for a form of hereditary blindness.

Some aspects of CRISPR's incursion into the clinic are proving controversial.



Oxford University Press, August 2016

There is currently a debate taking place about the potential risks of this type of gene therapy. As Laurie Zoloth, a bioethicist at Northwestern University, recently told *Nature*: "Any first use in humans we have to be extraordinarily careful." One particular concern is whether genome editing is accurate enough not to result in potentially adverse off-target effects in other parts of the genome than the targeted gene defect. Could introduced human cells, such as those iPS cells introduced into pigs, affect brain development or have other troubling, off-target effects in a recipient animal? Another bioethicist, Mildred Cho of Stanford University, believes that studies in animals can only take clinical research so far. "Often we have to take the leap of faith," she told *Nature*. ■

John Parrington is an associate professor in cellular and molecular pharmacology at the University of Oxford. Read an excerpt of Redesigning Life *at the-scientist.com.*

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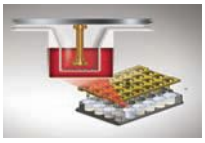

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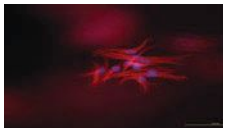

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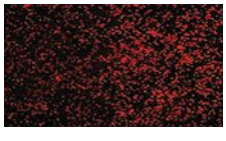

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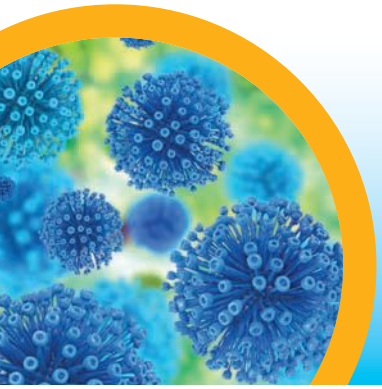

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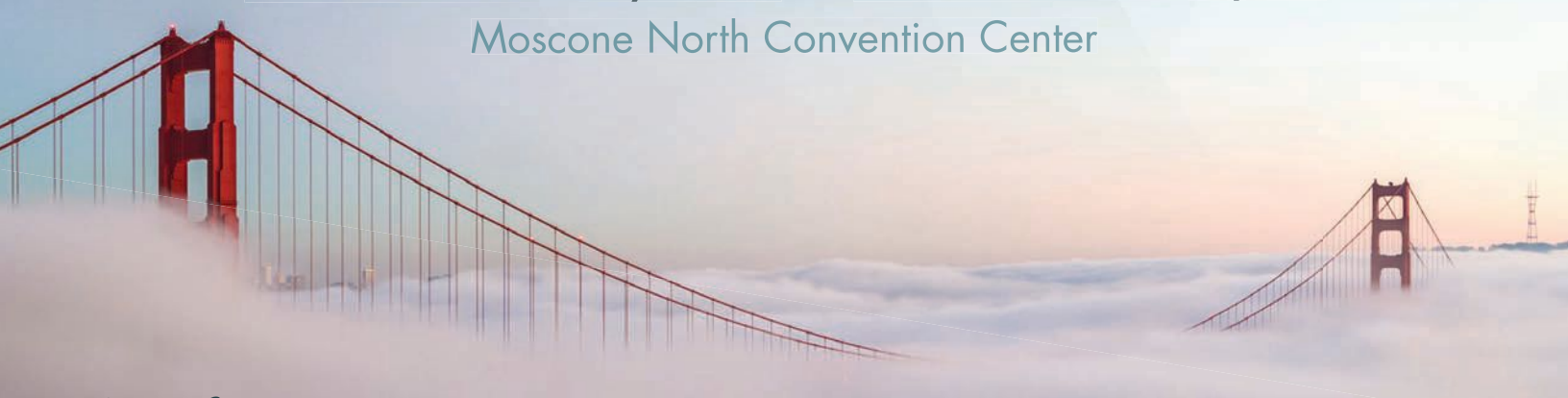

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The Pattern of a Pore, 1992

BY BEN ANDREW HENRY

When he was a postdoc in the 1970s, Ronald Milligan came across a grainy electron micrograph in a textbook that depicted the nuclear pore complex, a relatively unstudied but crucial structure responsible for shuttling molecules in and out of the nucleus. The blurry image, published in 1974 by Alexander Fabergé of the University of Texas at Austin, showed something remarkable: the pores seemed fashioned out of eight identical parts. "I remember taking it back to my advisor and saying, 'Look at this!'" he recalls, and they launched their pursuit of a better image.

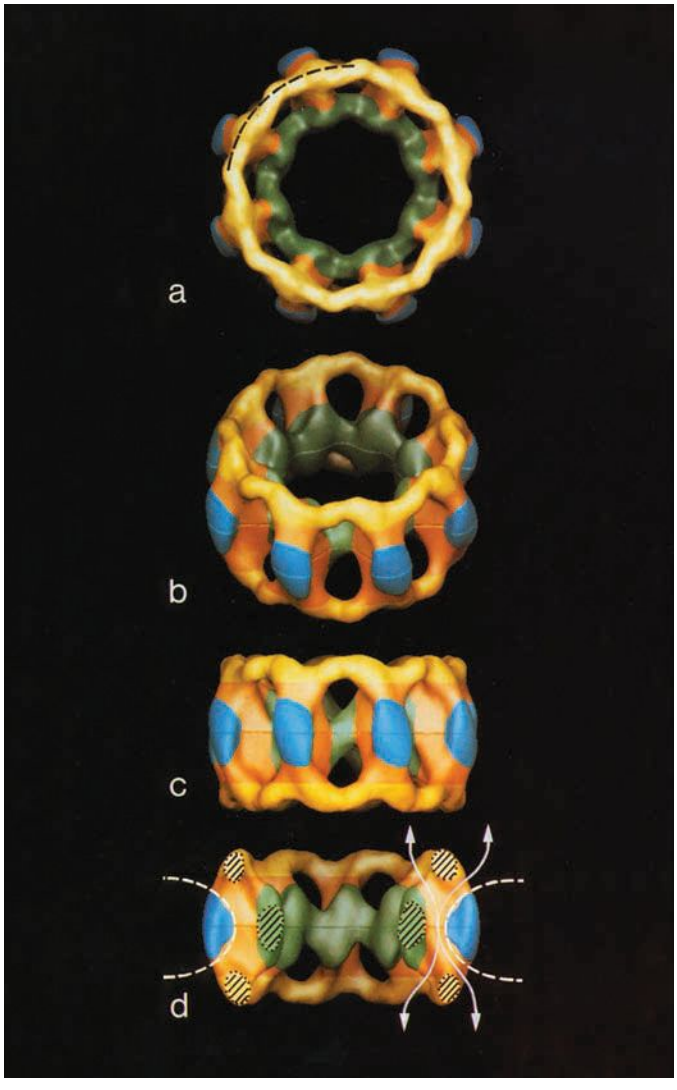
Milligan was taken in by indications that this tiny gateway had a symmetrical design. From sea anemones to flower petals, symmetry is everywhere in nature, and scientists were only just beginning to open a window onto the molecular world, revealing its varied architecture through advances in microscopy. Even those early images of the nuclear pore "are so dramatic," Milligan says. "Everyone is drawn to them. They're like little flowers."

Images produced by Milligan and others over the next decade revealed only a fuzzy ring, and "very few of them gave these hints of eightfold symmetry." It was not until 1992 that Milligan finally resolved the basic shape and structure of this mysterious portal in the nuclear membrane. Milligan had just established a molecular microscopy lab at the Scripps Research Institute, and along with one of his lab's first postdocs and a colleague at the University of California, San Diego, he came at the problem with state-of-the-art imaging analysis technology.

By extracting nuclear pores from the surrounding membranes of frog oocyte nuclei and applying early tomography techniques, the team ended up with a three-dimensional map of the nuclear pore and its constituent protein subunits. That first glimpse of symmetry from years earlier came into focus: the nuclear pore complex was not only made up of eight identical structures repeated around its circumference, it was also symmetric across the plane of the membrane (*Cell*, 69:1133-41, 1992).

A number of evolutionary concepts could explain the attractive design of this cellular machinery. Christopher Akey, a biophysicist at Boston University who coauthored another imaging study of the nuclear pore complex a year after Milligan and colleagues (*J Cell Biol*, 122:1-19, 1993), notes that iteration is the easiest way to build a structure as large as the nuclear pore, like building a wall brick by brick. "It's more economical to make smaller proteins—even if you have to make a lot of them—that self-assemble," Akey explains, because the odds of an error in any one protein are lower.

Despite the structure's apparent symmetry, Milligan knew there was more to the story. "We suspected that in the cell, because there was directed transport in either direction, [the nuclear pore] couldn't possibly be totally symmetric," he says. He and his colleagues hypothesized "elaborations added to the basic architecture that confer sidedness and enable directed transportation." The idea was borne out by later research from other labs showing smaller structures on either side of the pore, completing Milligan's picture of this unique structure. (See "Nuclear Comings and Goings" on page 24.) ■



STATE OF THE ART: Using a novel combination of imaging techniques, Ronald Milligan, a cell biologist at the Scripps Research Institute, and two of his colleagues published this model of a *Xenopus laevis* nuclear pore in 1992. Rather than examining one electron micrograph at a time, the researchers averaged many images together for more-reliable results. And by tilting their samples at various angles, they were able to translate flat images into a three-dimensional model, revealing an unexpected dimension of symmetry.

CELL, 69:1133-41, 1992, COURTESY RON MILLIGAN

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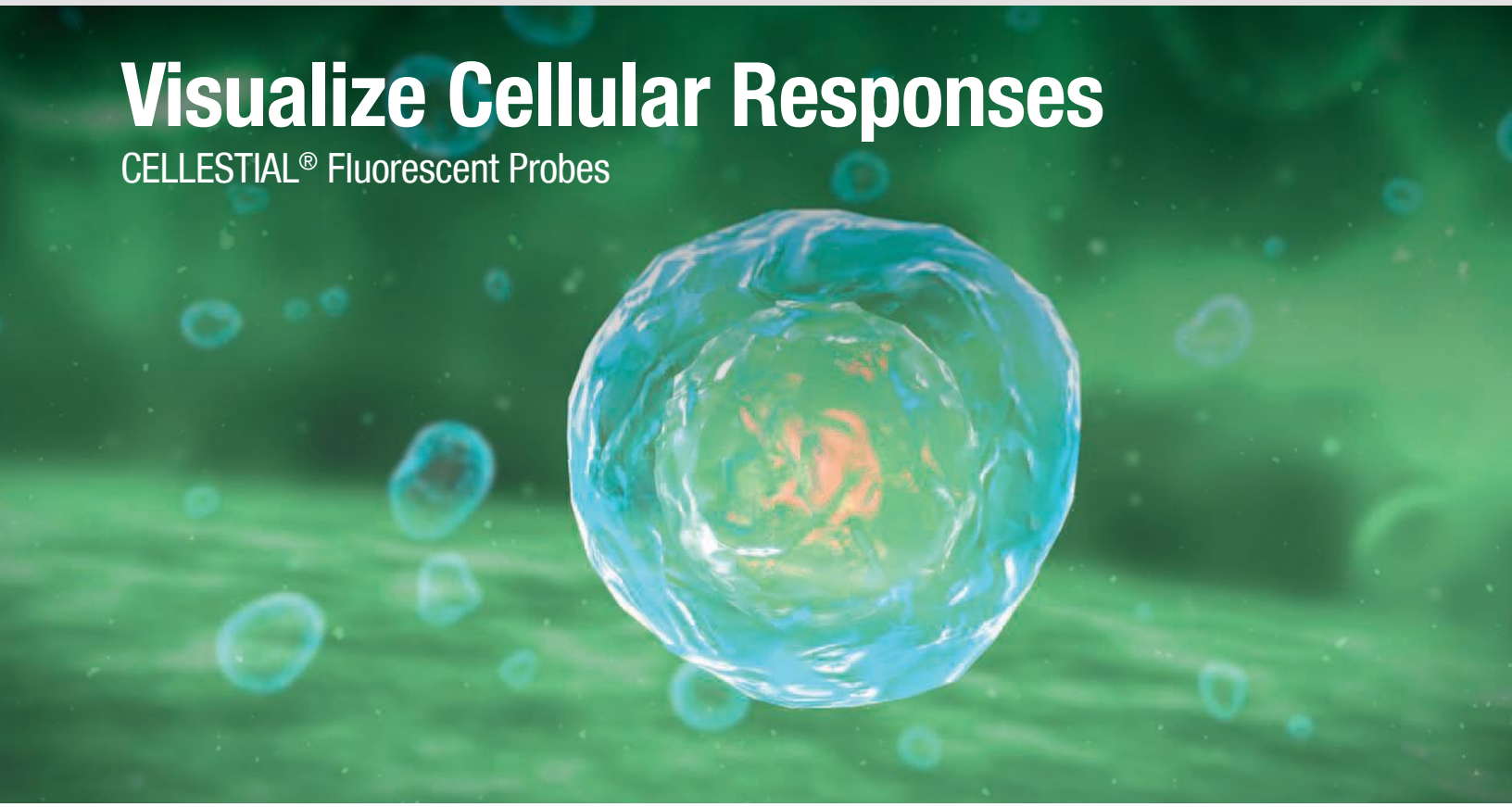

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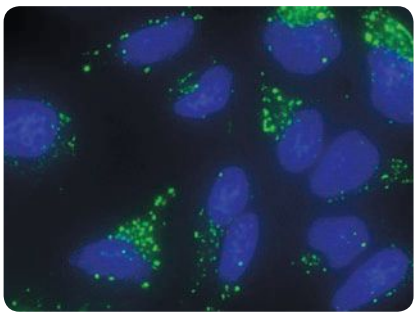

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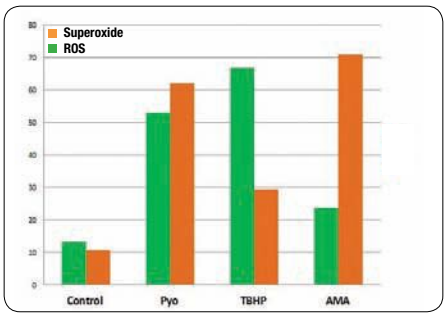

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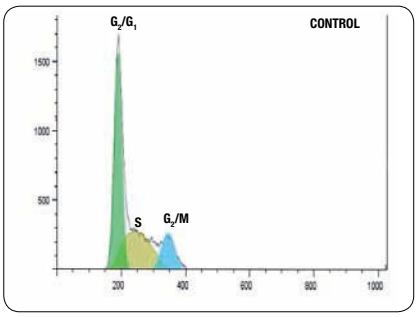

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