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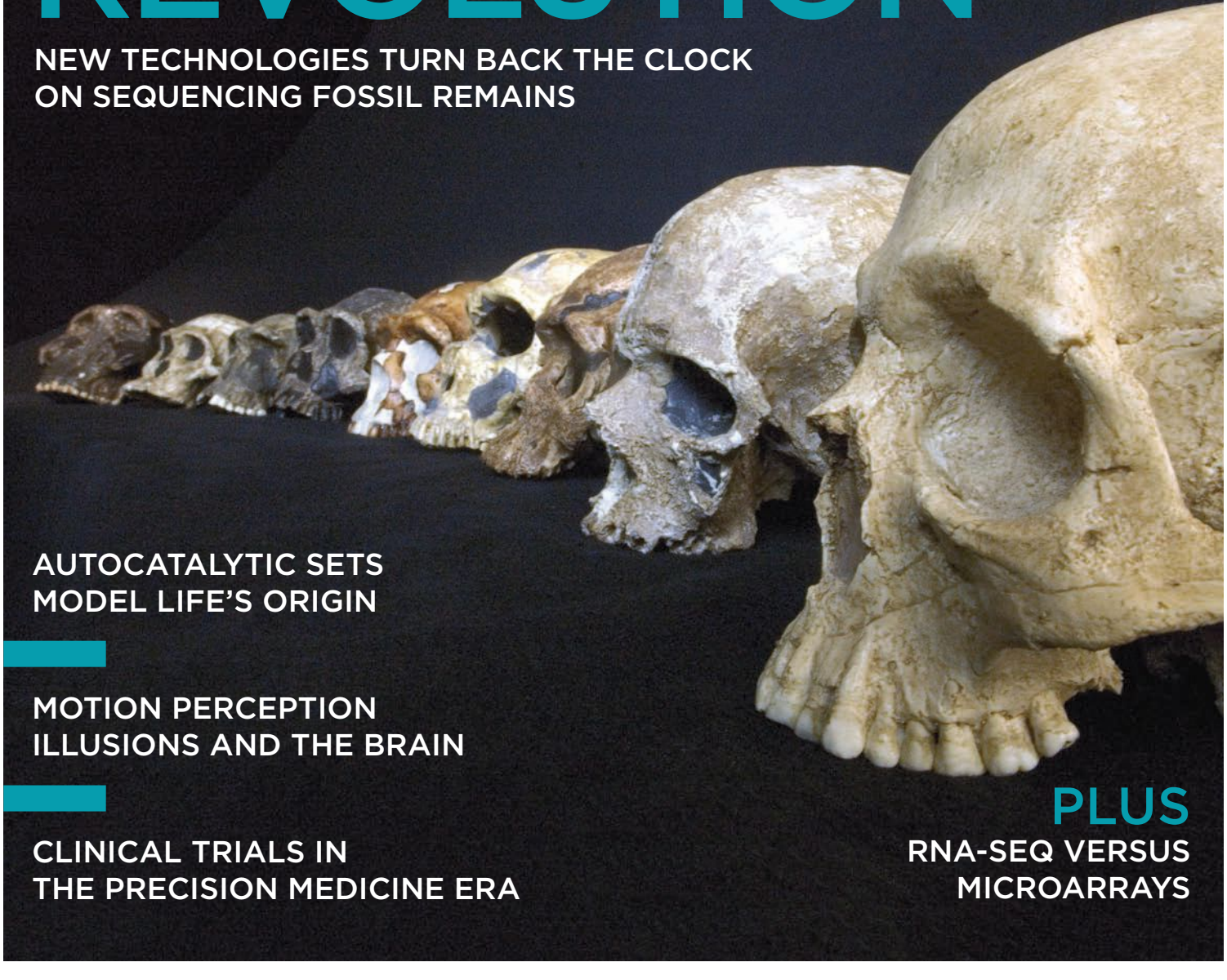

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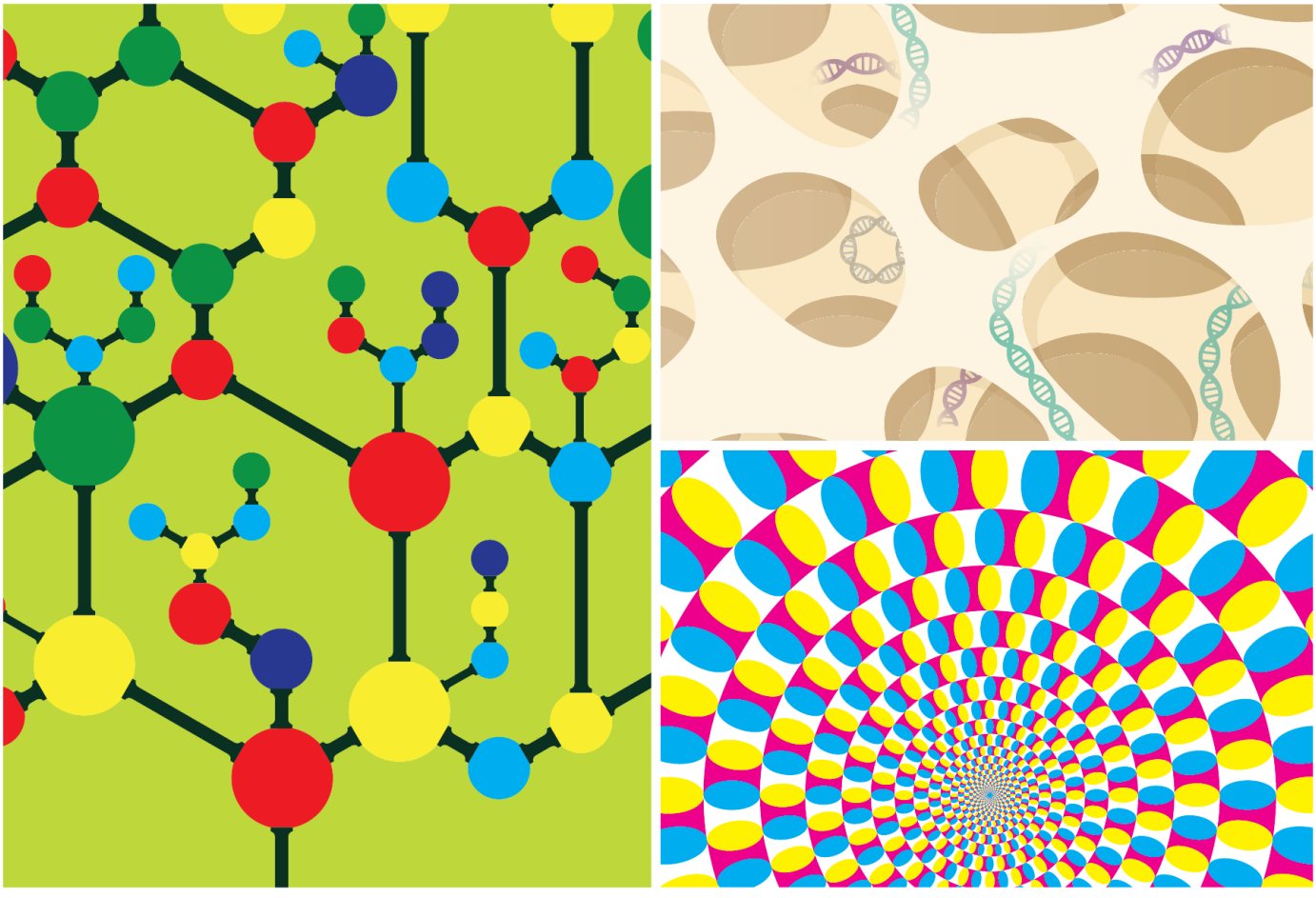

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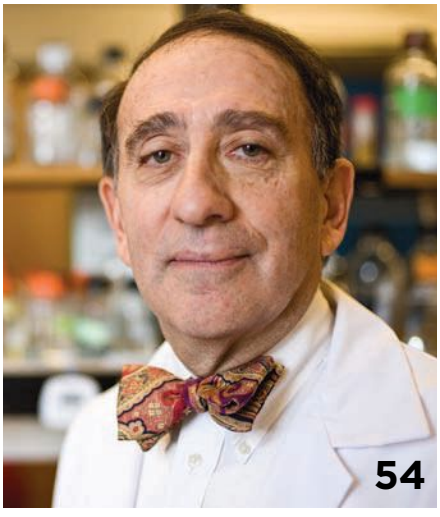

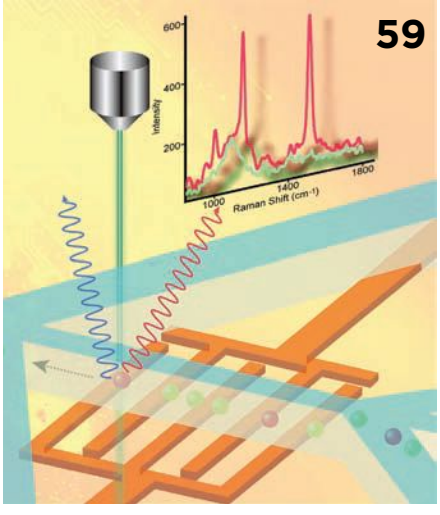

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CORRECTIONS:

In "Hearts on Trial" (May 2015), reporter Kerry Grens wrote that BAMI is a double-blind study when it fact it is open label; also, Cedars Sinai is in Los Angeles, not Beverly Hills.

In "Follow the Funding" (May 2015), the protein that Evris Gavathiotis studies was identified as PAX. The correct name is BAX.

In "All Is Not Quiet on the Western Front" (May 2015), Bio-Techne was incorrectly identified as the sister company of ProteinSimple. In fact, ProteinSimple was acquired by Bio-Techne.

The Scientist regrets the errors.

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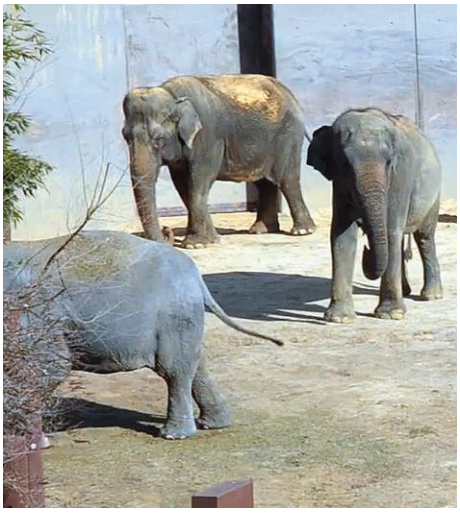
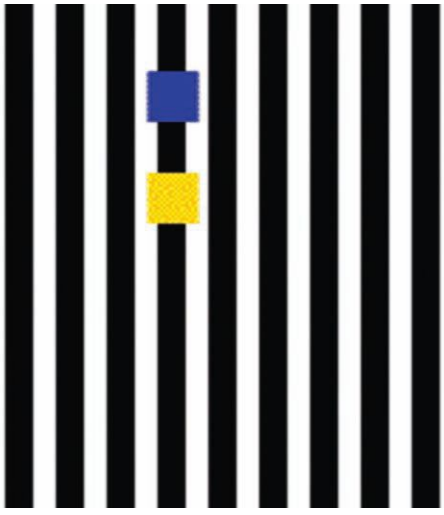




THIS MONTH AT THE-SCIENTIST.COM:

VIDEO
What's Stored in That Trunk?
TS Live takes a trip to the zoo for a story on the herpesviruses that stalk young elephants, often with fatal consequences.

VIDEO
Seeing Things
Challenge your brain with visual illusions that elucidate the evolutionary roots of motion perception.

VIDEO
What's Mine Is Yours
See the social interaction between wild baboons that may be the key to shared microbiomes among groups studied by the Amboseli Baboon Research Project.

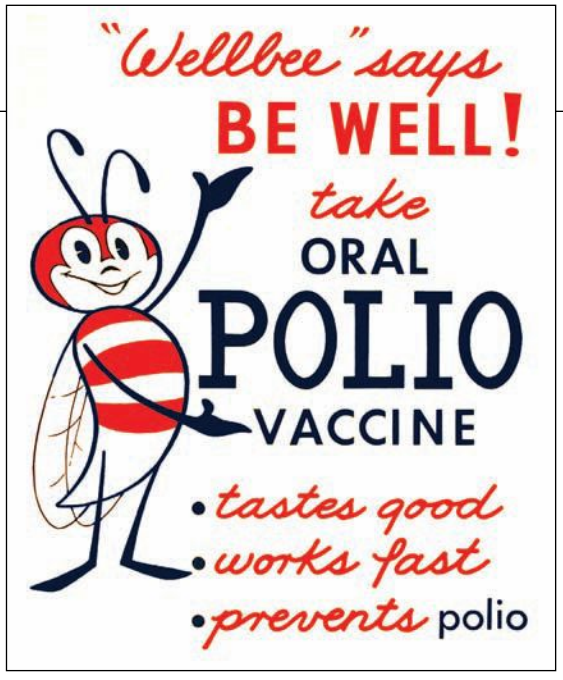
AS ALWAYS, FIND BREAKING NEWS EVERY DAY, AND LEAVE YOUR COMMENTS ON INDIVIDUAL STORIES ON OUR WEBSITE.

Coming in July

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

- Tracking disease with satellites
- Eradicating polio and guinea worm disease
- Balancing the human microbiome
- Doing research after retirement
- New saliva- and oral-based diagnostics

AND MUCH MORE



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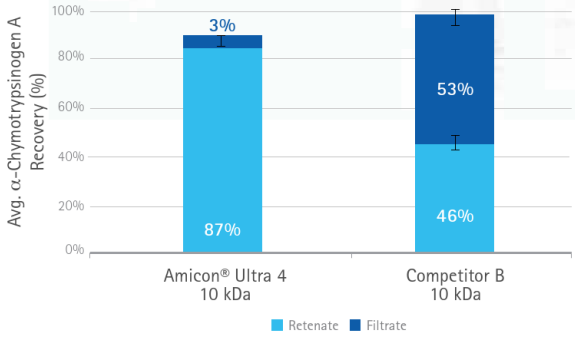

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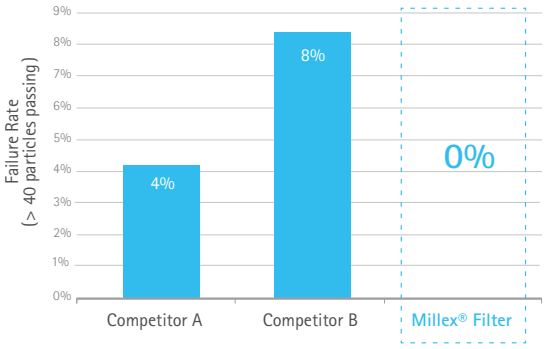

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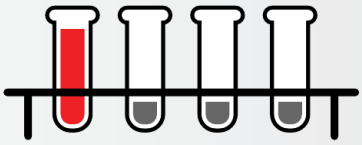

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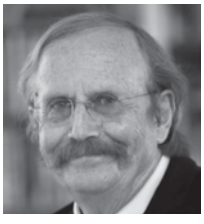
Contributors

Wim Hordijk, a self-proclaimed "wandering scientist," has worked on computational projects on six continents. A native of the Netherlands, Hordijk completed his graduate studies with Melanie Mitchell and Jim Crutchfield at the Santa Fe Institute in New Mexico, where he used computer simulations to study how individual units in natural systems can coordinate to make decisions. After earning his PhD from the University of New Mexico in computer science in 1999, "I knew I wanted to see more of the world," says Hordijk, who decided to use his research as an excuse to live in various locations around the globe. Among his favorite places are New Mexico, where he returns as often as he can, and New Zealand, where he did postdoctoral work with Mike Steel at the University of Canterbury in Christchurch on autocatalytic sets—groups of molecules that can catalyze all the reactions necessary to make each other and may have been the starting point for life. "The notion of biology and evolution is very much based on competition," Hordijk says. "The concept of autocatalytic sets is a whole different view of life. It's really more focused on cooperation than competition and selfishness. That's something that I like very much." Hordijk explores autocatalytic sets in "The Living Set" on page 30.



"Pasteur said, 'Chance favors the prepared mind.' Well, it also favors the unprepared mind, if you just notice things as they happen," says **Stuart Anstis**. Anstis began his studies in philosophy at the University of Cambridge in his native England, but when his advisor commented, "you're not exactly Bertrand Russell," he switched to psychology. While most of the professors Anstis met while searching for a graduate advisor were dull, he recalls, his conversations with neuropsychologist Richard Gregory were "sparkling, full of jokes, and just an inspiration." After graduate and postgraduate work with Gregory, during which Anstis discovered and explored the ramp aftereffect, which occurs when our eyes and brain adjust to dimming light by making it seem brighter, Anstis continued to study visual perception as a professor at the University of Bristol. Eventually, he worked his way westward, spending 10 years as a professor at York University in Toronto before joining the faculty at the University of California, San Diego, where he is today. Anstis's current research includes investigating how the mind fills in blind spots in our vision, even among people who have a large blind spot in the middle of their visual field. He has just completed a research tour in Japan. In "Seeing Isn't Believing" (page 45), Anstis explains visual tricks played on us by our brains as we perceive motion.

"What has been really fascinating about my career is the variety," says **Ian Tattersall**. "I don't have a typical day ever, and it's never been boring." Although he grew up in East Africa, in the geographic midst of key discoveries about the origins of modern humans, Tattersall did not become aware of those findings until his undergraduate days at Cambridge, where he says he "fell into" paleoanthropology by "happy accident." During his doctoral research with Elwyn Simons at Yale University, he studied the diverse extinct lemurs of Madagascar, which led him to a career exploring evolutionary experimentation in hominid evolution, both in the field and in the lab. Tattersall, now a curator emeritus at the American Museum of Natural History in New York City (where he has been since 1971), has curated permanent and special exhibitions on the topic and authored more than 20 books. In his 2012 book, *Masters of the Planet: The Search for Our Human Origins*, he sought to explain just how human beings became the peculiar creatures they are, but found he couldn't include the history of the field because "it got in the way." His latest work, *The Strange Case of Rickety Cossack*, serves as a companion volume that traces the tangled web of the discipline's history. "Within the next couple decades, we will develop a totally different understanding of human evolution," Tattersall predicts. "It's a tremendously exciting period, and new discoveries are being made all the time." Tattersall provides a taste of the complex history of paleoanthropology in his essay "Reimagining Humanity" on page 70.

WIM HORDIJK; KEVIN WALSH; AMNH/DENIS FINNIN

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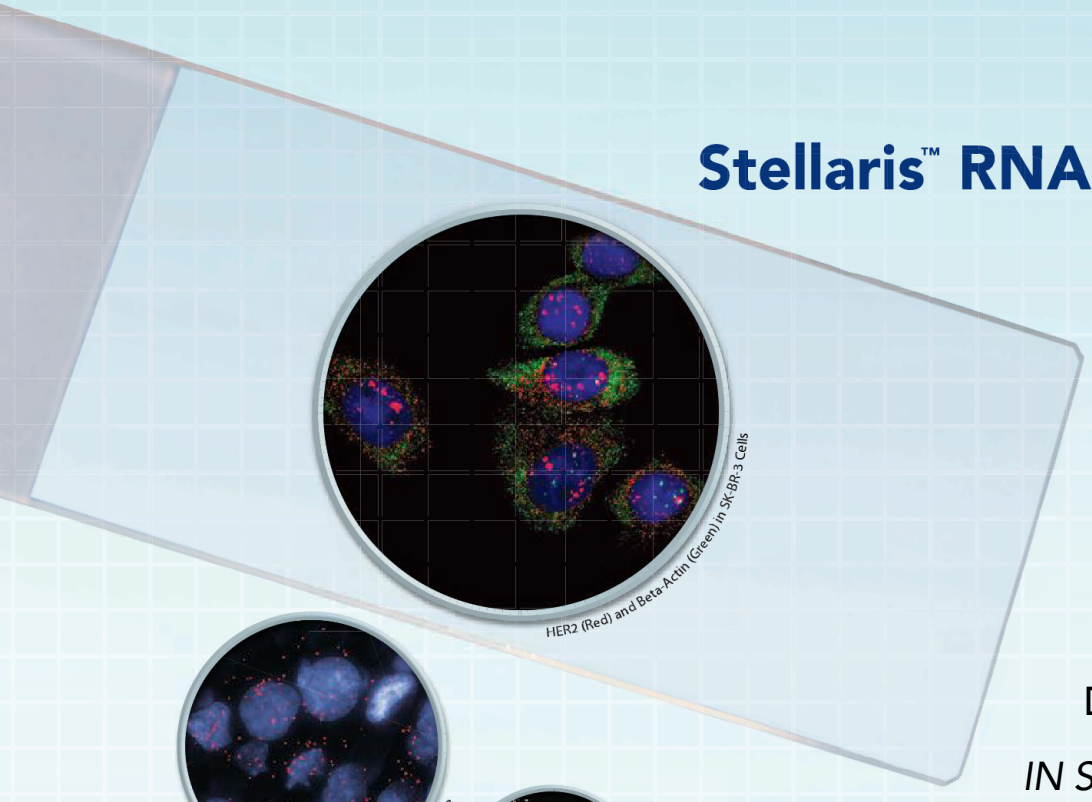

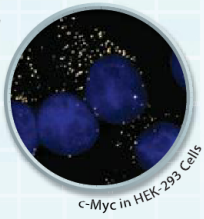

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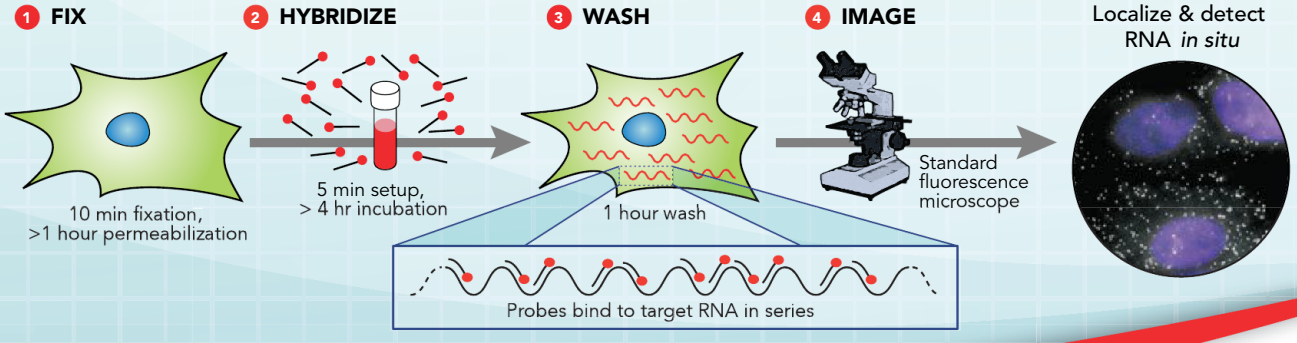

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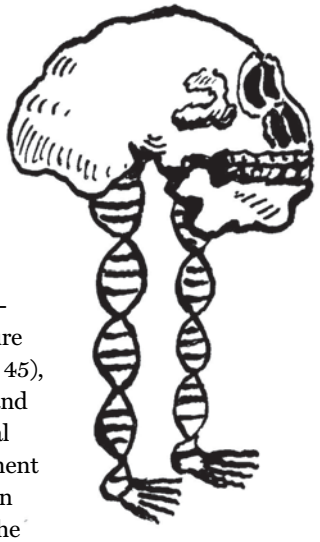
New Legs to Stand On

Reconstructing the past using ancient DNA

BY MARY BETH ABERLIN



ANDRZEJ KRAUZE

Origin myths are foundational to the world's cultures—depicted in cave paintings and later written or spoken. The fascination with origin stories lives on in modern humans, especially in a subgenre of such tales: how *Homo sapiens* got to be top dog. Dozens of print and film offerings inventively portray how the transition from tree-dweller to upright hominin might have played out—think *Clan of the Cave Bear, The Ugly Little Boy, Iceman, Quest for Fire,* and *The Croods,* to name but a few. Luckily, scientist debunkers are on the case.

Paleoanthropologist Ian Tattersall lays out the many factors, not least of which is our sometimes myopic fascination with our own species, that have complicated the drawing of any sort of reliable hominin evolutionary tree in an essay on page 70.

In the feature article "What's Old Is New Again" (page 36), Senior Editor Bob Grant reports at length on how recent advances in sequencing ancient DNA, some from hominin leg bones more than 400,000 years old, will help prune or reshape such trees. The bugaboo for paleogenomics has been that the older the fossil sample, the more fragmented and degraded the DNA. But new techniques for extracting, purifying, and sequencing the stuff have allowed the piecing together of genomes from fragments just 30 to 35 base pairs in length, a development that may one day allow researchers to derive sequence data from million-year-old fossils and possibly nail down for good the hominin origin story.

The ancient-DNA revolution, in essence, offers powerful new tools for recreating the biological past, for humans and for other organisms whose genetic material is decently preserved due to favorable environmental conditions. And the new methods even have something to offer to forensic scientists, whose crime-scene collections often include similarly degraded DNA. Adios, creative alibis.

Origin stories clearly fascinate Wim Hordijk, a peripatetic, independent computational biologist who seeks to model the emergence of life from the basic chemistry present on early Earth. In "The Living Set" (page 30), Hordijk considers the possibility that life began through the formation of autocatalytic sets—self-sustaining networks of chemical reactions that create and are catalyzed by components of the system itself.

Before you read neuropsychologist Stuart Anstis's feature "Seeing Isn't Believing" (page 45), move the opening image up and down. What's the neurological basis for the phantom movement you see? Anstis studies motion illusions and how they trick the visual system "because seeing the errors that a system makes can help us to understand how that system works normally. Visual perception goes far beyond our retinal images, which provide only partial sensory information. We use our knowledge and expectations of the world to fill in the gaps," he writes. You can read about the various illusions he and others have parsed, and then go to the-scientist.com and try them out yourself. Anstis's research is helping to write the story about how our eyes and brains evolved.

This issue also contains a number of articles that touch on RNA, an origin-story molecule if there ever was one. The study of RNA's functional roles is turning up some interesting surprises. Articles in The Literature department touch on transcriptional control of noncoding RNAs in embryonic stem cells and a muscle-regulating micropeptide coded by a supposedly noncoding RNA. A Lab Tools article, "An Array of Options " (page 63), weighs the pros and cons of RNA-seq versus microarrays for studying the transcriptome, while "RNA Structuromics" (page 29) describes a new method for determining RNA secondary structure in vivo.

We set two of this issue's stories, "What's Old Is New Again" and "A Plague on Pachyderms," in motion with a pair of TS Live videos that will premiere on the-scientist.com this month. Humanity's mythologies have monopolized the imagination for millennia, but as technological advances allow science to probe ever deeper into biology's past, present, and future, those origin stories, and even early logic-based attempts at explaining our world, may need rewriting. ■

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

Speaking of Science

I believe this is the first report of CRISPR/Cas9 applied to human pre-implantation embryos and as such the study is a landmark, as well as a cautionary tale. Their study should be a stern warning to any practitioner who thinks the technology is ready for testing to eradicate disease genes.

—Harvard stem cell researcher **George Daley**, in a *Nature* news story about a paper in *Protein & Cell* by a research team reporting the use of CRISPR/Cas9-mediated gene editing in human embryos (April 22)

This story underlines the urgent necessity for international dialogue over the ethics of germline gene editing in human embryos, well in advance of any progression towards theoretical clinical application. Recent calls for a moratorium on any such research to allow time for expert and public consideration of what is and is not ethically, socially and indeed legally acceptable with respect to human germline genetic modification should definitely be heeded.

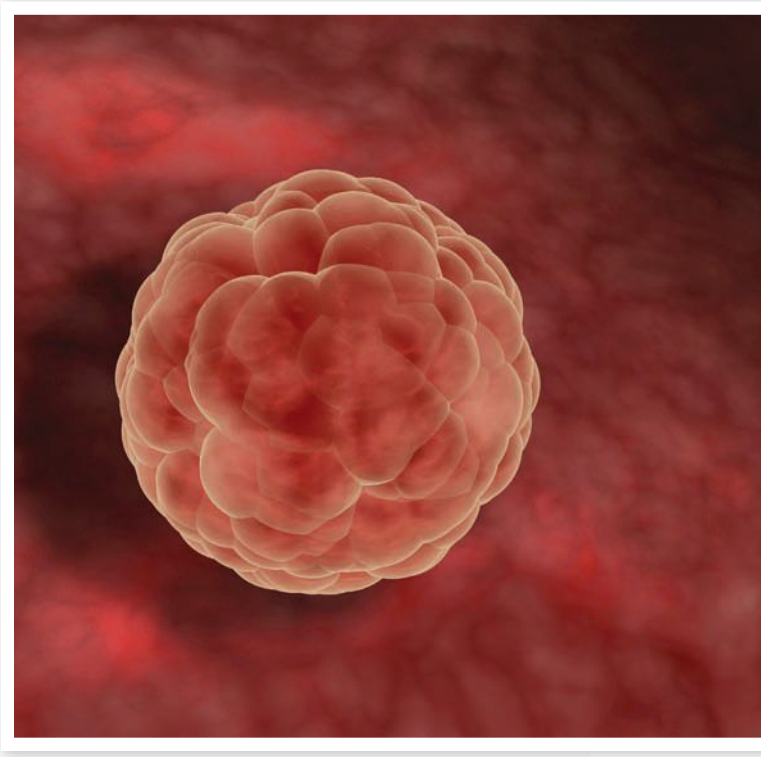
—**Philippa Brice** of health policy think-tank the PHG Foundation, reacting to the news of the paper reporting to have genetically engineered human embryos (*The Telegraph*, April 23)

Any time any "Top Journal" says "we're concerned about the ethics" you should actually read "we don't want to be involved in any mainstream media controversy."

—Science writer **Kelly Hills**, in a blog post about reports that *Science, Nature,* and other top-tier journals rejected, on ethical grounds, a paper by a research team reporting that they used CRISPR/Cas9-mediated gene editing in human embryos (April 23)

Understanding Earth's microbiome is a challenge that rivals going to the moon or developing cures for cancer.

—University of Arizona environmental microbiologist **Raina Maier**, in a *Scientific American* guest blog post about the dangers of altering the planet's microbiome (April 22)



A NEW BALL OF WAX: Scientists recently published a report that they'd used CRISPR/Cas9-mediated gene editing in human embryos, prompting a call for a moratorium on such research.

For most people, however, whole-genome sequencing is an absurd medical test. If you get entertainment value from a fortune-teller reading—or from a whole genome—that's one thing. But if you value your health, divert your resources to something more meaningful—maybe whole foods.

—Dartmouth Medical School professor **H. Gilbert Welch** and University of Washington medical geneticist **Wylie Burke**, in a recent *LA Times* op-ed about the realities of using whole-genome testing as a part of precision medicine (April 27)

Scientists are not dependent on the ideas of a single man, but on the combined wisdom of thousands of men, all thinking of the same problem, and each doing his little bit to add to the great structure of knowledge which is gradually being erected.

—Nobel Prize-winning nuclear physicist **Ernest Rutherford**, as quoted in 1959's *The Birth of a New Physics* by I. Bernard Cohen

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Notebook

JUNE 2015

THE MARYLAND ZOO IN BALTIMORE

A Plague on Pachyderms

In late February 2013, Mike McClure and the rest of the elephant team at the Maryland Zoo in Baltimore grew concerned about their calf, Samson. It was hard to pinpoint, but having raised the almost 5-year-old African elephant calf since birth, McClure knew something was wrong. "I looked at him and just noticed his behavior was off," he recalls. Maryland Zoo head veterinarian Ellen Bronson agrees: "He was just very slightly not himself."

The team sent blood samples out for analysis, and after a few inconclusive tests, a positive result confirmed their worst fears: Samson was infected with elephant endotheliotropic herpesvirus (EEHV), a virus commonly carried by adult elephants that can be deadly when acquired by a calf. "[The diagnosis] was devastating," McClure recalls.

Researchers first discovered EEHV in 1995, after a 16-month-old Asian elephant calf named Kumari at the Smithsonian National Zoological Park in Washington, DC, suddenly fell ill and died in a matter of days. Zoo pathologists Laura Richman and Richard Montali performed a necropsy of the young elephant and found significant amounts of hemorrhaging, especially in the capillaries of the heart, liver, and tongue. They also saw signs of inclusion bodies, protein aggregates characteristic of herpesvirus infections in other species, including humans. And then, late one night while peering through an electron microscope, Richman saw the telltale dark shape of a herpesvirus in Kumari's cells. PCR amplification and comparison to a DNA database eventually confirmed the diagnosis. Under the direction of Gary Hayward at Johns Hopkins University, Richman studied the virus, which she dubbed EEHV1, for her PhD project. EEHV1 primarily infects Asian elephant calves, but in the course of her research, Richman also identified a second type of the virus, EEHV2, which primarily affects African elephants.

SICK SAMSON: The juvenile elephant on the right, Samson, nearly died from an endotheliotropic herpesvirus infection at Baltimore's Maryland Zoo.

Mining the Microbiome

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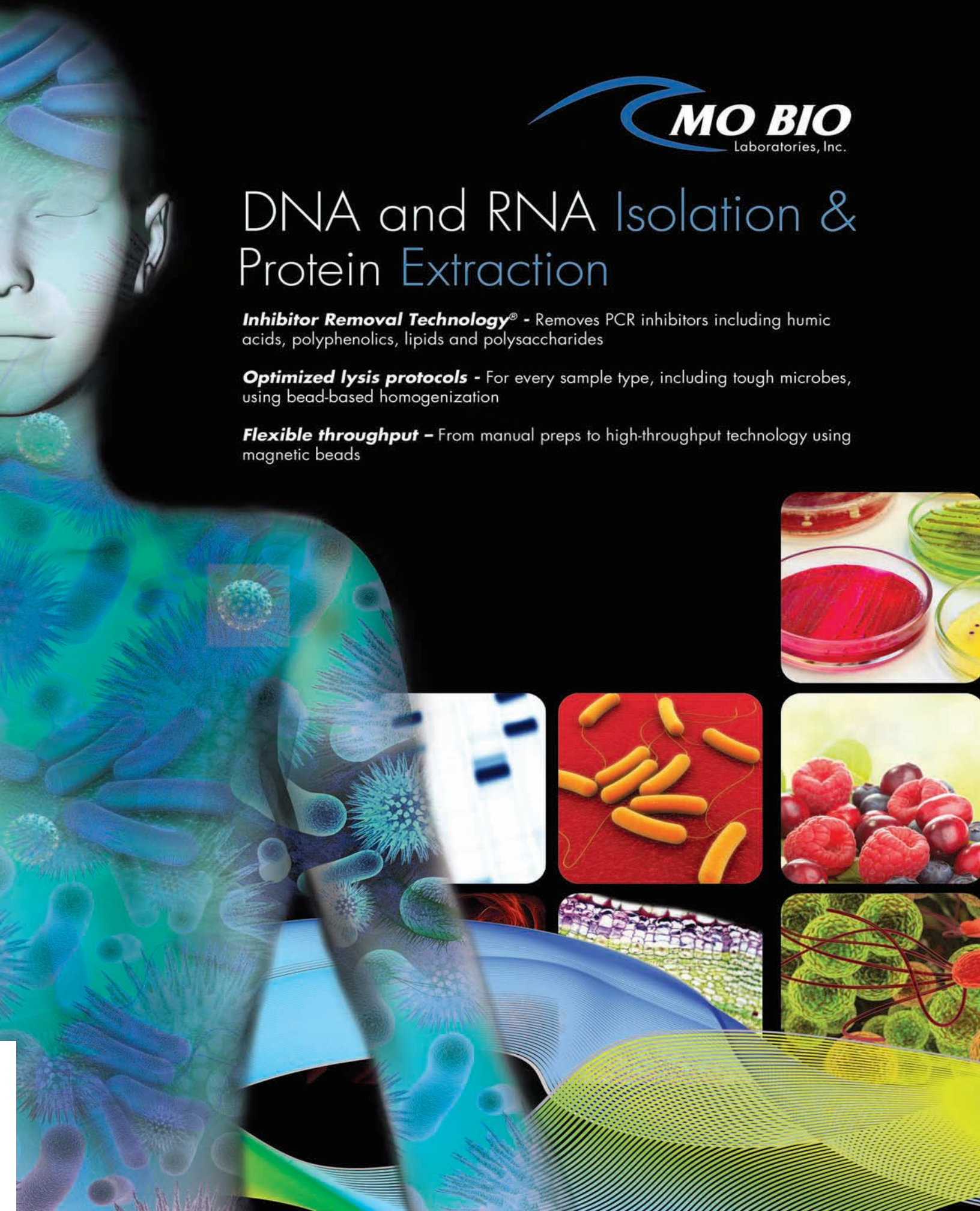
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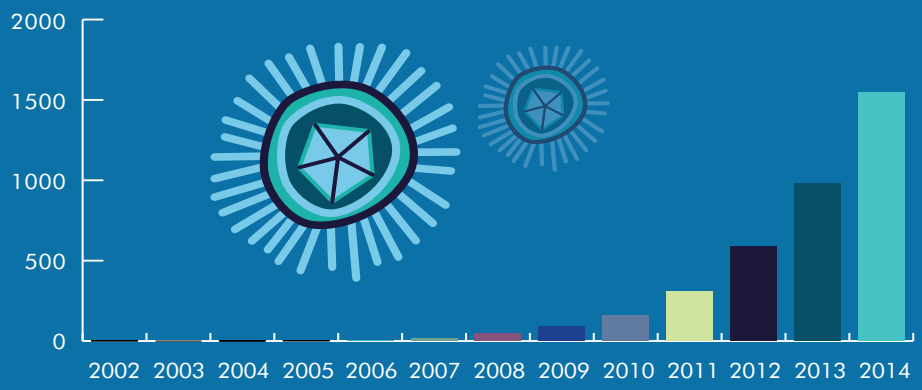
Mining the Microbiome

Trillions of microbes inhabit the human body—more than 10 times the number of our own mammalian cells. While the microbiome of the gut is the most well studied, researchers now recognize that single-celled organisms from all three domains of life form distinctive microbial communities in diverse tissues and organs, including those once thought to be sterile, such as the eye and the reproductive organs. To better understand how our microbial organisms affect our health and influence disease, scientists are now surveying these communities using increasingly affordable genomics techniques.

Typical metagenomics studies focus on the 16S rRNA gene that encodes the small subunit of the ribosome, because this gene is shared by all microbes, but also contains hypervariable regions allowing differentiation of microbial communities. Other studies dive deeper, sequencing entire bacterial genomes or all the genes of a given environmental sample. Whole-community metaproteomic or metabolomic analyses can also yield clues regarding the community's composition and function. Finally, bioinformatic and statistical tools are applied to investigate dysbioses that might cause disease.

NUMBER OF PUBLICATIONS ON MICROBIOME IN PubMed*



*PubMed search of "microbiome"[All Fields] in March 2015 returned 3750 results.

SOIL

Distinct, diverse microbial species populate the soil of temperate, desert, tropical, and Arctic regions. Desert (pH of >8) and tropical forest (pH of <4.5) soils are home to the lowest levels of microbial diversity, while regions with near-neutral soil pH have the highest microbial diversity.[1] Soil biodiversity is also influenced by the chemical composition of soil organic matter within the soil microenvironment.[2]

WATER

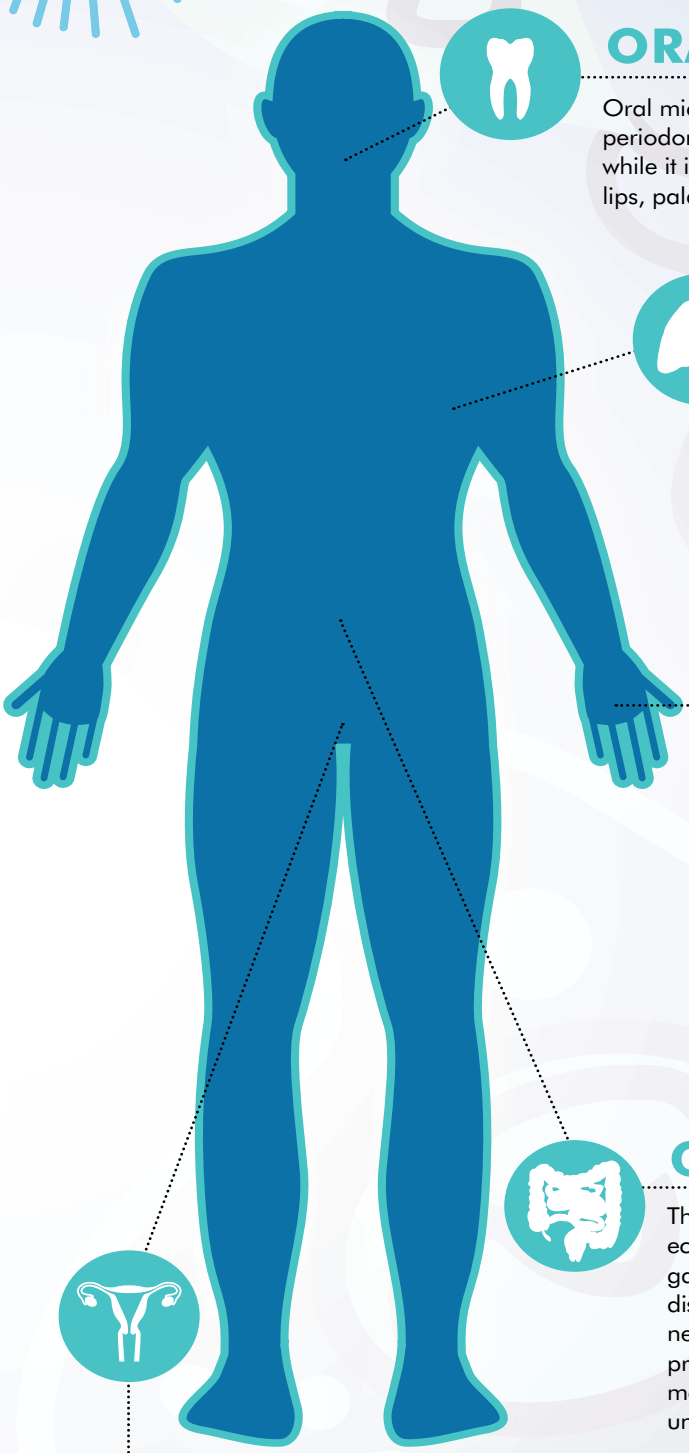
Microbial biodiversity in aquatic environments is subject to natural variation and is influenced by environmental factors—light, temperature, pH, waterflow, salt concentration, and aquatic species. Marine microbes impact the oceanic food web, chemical and nutrient cycling, and aquatic species health. Microbes also drive the degradation of dissolved organic matter in rivers and lakes, playing an important role in carbon cycling and the recycling of organic matter and nutrients.

PLANTS

While both root and leaf microbial communities play a role in plant fitness and adaptability, host-microbe interactions at the root may also be involved in the acquisition of nutrients from the soil. Studying how the microbiota can influence plant health in natural and agricultural ecosystems has implications for crop production, biodiversity management, and responses to climate change.

ARCHITECTURE

Scientists are also mapping microscopic life in urban environments to better understand public health implications of our surrounding microbial ecosystem. Analysis of 1,400 samples from surfaces of the New York City subway system identified more than 15,000 different species, including DNA fragments of bubonic plague and anthrax.[3] Analyzing the relationship between building design and microbial diversity is critical to understand the influence of the urban ecosystem on public health.



ORAL

Oral microflora play an important role in health and oral disease, including periodontitis and caries. The oral cavity is a distinct site for microbiota colonization; while it is warm, nutrient rich, and offers dynamic colonization surfaces such as teeth, lips, palate, and tongue, the antimicrobial properties of saliva inhibit bacterial growth.

LUNGS

While the lung was once thought to be a sterile environment in the absence of infection, recent studies identified diverse microbial communities in the healthy lung. The interactions of microbiota and host cells are being examined in the cystic fibrosis lung, to better understand the impact of pathogens in chronic infection and to guide therapeutic intervention. Alterations in the lung microbiome are observed in response to cigarette smoke and other environmental factors.

SKIN

The skin acts as a protective barrier for the body and is home to diverse commensal microorganisms that play a key role in host immunity. Within an individual, niche microbial colonies form that are dependent on variation in skin characteristics such as temperature, hair, sebaceous glands, and moisture content. The belly button is home to a diverse microbial community—more than 2,000 bacterial phylotypes were identified in a sample of 60 volunteers.[4] Disruption of the commensal skin microbiota has been implicated in cutaneous infections, atopic dermatitis, acne, psoriasis, arthritis, and chronic wounds.

GUT

The human gut is host to diverse microorganisms that form a complex ecosystem involved in host digestion, metabolism, and immunity. Alterations in gastrointestinal microorganisms have been identified in inflammatory bowel disease, irritable bowel syndrome, gastroenteritis, colorectal cancer, and neurological disorders. Scientists are actively studying gut microbial colonization, preservation of the healthy ecology of the gut, and the therapeutic potential of modulating the interplay between microbes and the immune system to better understand the role of this complex ecosystem in health and disease.

UROGENITAL

The urogenital region is host to a variety of bacteria that are influenced by factors such as age, genetics, sexual activity, circumcision, and pregnancy. The urogenital system is now known to host diverse microbiota, even in the absence of infection. Disruption of microbial communities correlates with pelvic infections, bacterial vaginosis, and preterm birth. The role of microbes in the immune response to HIV and sexually transmitted diseases is also being studied.

DNA Sample Preparation

While scientists have studied microbial communities for years, advances in DNA extraction tools combined with more efficient and affordable next-generation sequencing has revolutionized the characterization of different sample types such as tissue, blood, soil, air, etc. Successful downstream analysis is dependent on the preparation of pure, high-quality DNA.

SAMPLE COLLECTION

Good results start with proper sample collection and storage prior to DNA isolation.

*Tips:

Samples should be processed soon after collection or frozen at -20°C for long-term storage.

If using a preservative, be sure that it is compatible with the microbes in your sample and your extraction method. Many preservatives for tissue do not work with environmental samples, e.g. ethanol.

Numbers of microbes vary widely between sample types. Consider maxi kits or combining preps in order to isolate adequate yields of DNA, e.g. when working with low-biomass soil.

CELL LYSIS

Cells can be lysed by mechanical (e.g. bead beating), chemical (e.g. detergent), or enzymatic (e.g. lysozyme, proteinase K) methods. The accuracy of microbial community diversity is impacted by cell lysis methods. Enzymatic lysis may not provide a good representation of the microbial communities in the sample; mechanical lysis via bead beating provides a better representation of microbial diversity.[5]

*Tips:

Optimize bead type to maximize DNA yield and integrity. Use smaller beads (0.1–0.5 mm) for lysis of bacteria, yeast and fungi. Use larger beads (2.0–3.0 mm) for breaking down bulk tissue such as seeds, animal tissue, and plants.

A short heating step (e.g. 10 minutes at 65°C) can assist the cell lysis of more-difficult microbes such as gram+ bacteria and spores.

Optimize the time and speed of homogenization to maximize the lysis of microbes while minimizing DNA degradation.

REMOVAL OF INHIBITORS

Many samples contain PCR inhibitors, released during cell lysis, that can interfere with quantification, inhibit amplification, and result in false negatives in downstream analysis, e.g. humic acids in soil samples; polysaccharides and poylphenolics in plant and seed samples; heme, lipids, and polysaccharides in stool and gut samples.

*Tips:

Remove inhibitors prior to DNA purification to avoid interference with downstream applications.

PURIFICATION

Once inhibitors have been removed from the solution of lysed cells and nucleic acids, DNA is further purified on a silica spin filter or using magnetic beads.

*Tips:

Optimize solutions to bind, wash, and elute purified DNA from silica spin filters. Magnetic bead–based technologies are ideal for high-throughput DNA isolation.

QUALITY CONTROL

DNA should be quantified and checked prior to analysis to confirm that it is free of inhibitors.

*Tips:

Run DNA on an agarose gel to check size and quality.

Quantify DNA (e.g. with a NanoDrop) to determine purity—compare both the A260/280 and A260/230 ratios and measure double-stranded DNA via a PicoGreen® assay to determine an accurate concentration.

Samples free of inhibitors will have a matching NanoDrop and PicoGreen® concentration, while samples containing inhibitors will appear to have a higher concentration via NanoDrop.

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4. J. Hulcr et al., "A jungle in there: Bacteria in belly buttons are highly diverse, but predictable," *PLOS ONE*, 7(11):e47712, 2012.
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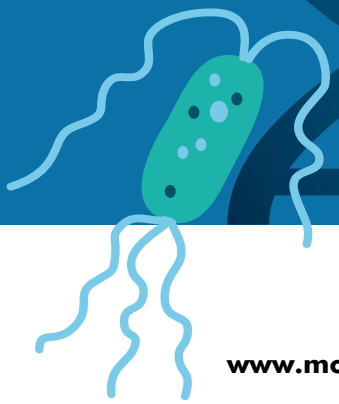
MO BIO Laboratories Inc., a privately held company located in the San Diego biotech corridor (Carlsbad, CA, USA), was founded in 1993 by Liz and Mark Brolaski. Today, MO BIO's growth and customer loyalty is challenging some of the world's leading molecular biology tool companies with innovative technologies and a commitment to value.

MO BIO is known as the World Leader in Soil DNA & RNA Isolation®, thanks to its patented Inhibitor Removal Technology®, which enables isolation of pure nucleic acids from challenging samples such as soil, water, plants and stool. Thus, MO BIO kits are the method of choice for microbiome projects, as these studies often require isolation of nucleic acids from challenging samples that contain a variety of PCR inhibitors. Microbiome projects relying on MO BIO products include the Earth Microbiome Project (EMP), the NIH-funded Human Microbiome Project (HMP), and many other related studies.

In 2014, MO BIO established the Microbiome Awards (www.microbiomeproject.com), which provide young, extraordinary scientists with funding and recognition to carry out scientific work in the field of microbiome research. The annual awards are meant to support and bring to light the many variations of studies in this field.

MO BIO's founders and employees are dedicated to the preservation of the environment and to bettering the quality of the Earth through science. MO BIO's team of scientists is committed to developing innovative tools for researchers that require pure, high-quality nucleic acids for use in next-generation sequencing and other downstream applications. Information about our kits for microbiome, soil, water, plants, stool, biofilm, microbial cultures, and more can be found at www.mobio.com.



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Richman and her colleagues developed PCR-based diagnostic tests for EEHV1 and EEHV2, and in the mid-2000s, the National Zoo began doing regular screenings of its own elephants. The lab also began testing samples sent in from zoos and circuses around the country. In 2007, for example, when a 6-year-old Asian elephant named Hansa at Woodland Park Zoo in Seattle started acting a bit lethargic, its keepers sent samples to National Zoo researcher Erin Latimer for analysis. Despite the keepers' suspicions that Hansa was suffering from an EEHV infection, the tests returned no sign of EEHV1 or EEHV2. Unfortunately, Hansa continued to decline, growing increasingly lethargic and refusing to eat. Within a week, the young elephant was dead.

The necropsy revealed characteristic inclusion bodies, suggesting that it was indeed a herpesvirus that had killed Hansa. But it clearly wasn't one of the two known types. Latimer, in collaboration with Hayward at Johns Hopkins and colleagues, then tested Hansa's samples with degenerate PCR primers that aren't specific to EEHV1 or EEHV2, instead binding to a broad array of herpesviruses. Sure enough, there was a different EEHV type, now called EEHV3, lurking in Hansa's tissues and blood. The researchers used the primers to test an older case in which a young Asian elephant had died after testing negative for EEHV1 and EEHV2. In those samples, they identified what is now EEHV4. "So by using degenerate primers we picked up two new EEHVs," Latimer says. And over the next few years, researchers would also identify at least three other EEHV types. "Every couple of years there's been a new one," Latimer says.

In an extensive survey of EEHV published in two papers late last year, Richman, Hayward, Latimer, and colleagues used DNA fingerprinting to compare known cases, confirming seven different types of the virus (*J Virol*, 88:13523-46 and 13547-69, 2014). "EEHV1–EEHV7 types are not just strains but distinct species that evolved separately tens of millions of years ago," Hayward tells *The Scientist* in an e-mail. "Each of them also has multiple subtypes and then also numerous easily distinguished strains that are amazingly diverged—the most we have ever seen in any herpesvirus."

The viruses are not limited to captive elephants; they also infect wild herds. Hayward's team examined benign lung nodules from wild elephants and confirmed that the viruses are natural pathogens of the giant mammals, which likely contract the virus as young elephants, then carry latent infections for the rest of their lives. Hayward speculates that perhaps elephants are more likely to survive a primary infection if they are still armed with maternal antibodies, acquired both across the placenta and from their mothers' milk. But in wild herds, which are increasingly fragmented due to habitat destruction, or captive elephants, in which group interactions may be disrupted, young calves may be exposed to the virus when they are least equipped to deal with it.

Fortunately, US zoos are getting a handle on EEHV. Between 1978 and 2008, the disease killed nearly 20 percent of U.S.-born Asian elephant calves, Hayward told *The Scientist*. But thanks to increased surveillance—every week Latimer runs tests on samples from several calves around the country—and immediate supportive care, only one captive elephant in the U.S. has died of an EEHV infection since. The Maryland Zoo's Samson is now one of at least a dozen EEHV success stories.

Even before the positive EEHV result returned, McClure and his colleagues started Samson on a heavy course of antiviral medications (in the course of his treatment, the young elephant downed more than 2,500 antiviral pills), injected him with anti-inflammatory drugs, and began hydration therapy. Each day, Samson would drink between 15 and 25 gallons of Gatorade-spiked water, and his keepers would deliver many more gallons of fluids rectally. It was touch and go for a while, but after about a week and a half the staff finally noticed significant improvements in Samson's behavior.

"When you have elephants, you're prepared for this disease," says Bronson, who

notes that captive elephants in the U.S. are now routinely trained from birth to accept the fluids, meds, and exams needed to monitor for and treat EEHV. "All these things we needed to save [Samson's] life started literally the day he was born."

—Jef Akst

Touchy Feely

On the Kenyan savannah, with Mt. Kilimanjaro's fragmented glaciers visible in the distance, baboons climb down from a scattered grove of trees to begin their day. Since the wee hours of the morning, a small team of field researchers has been waiting. Nearly every day for the past 40 years, scientists have shadowed these animals, scrutinizing not only their movements, but their eating habits, grooming behaviors, genital swellings, births, deaths, and sexual encounters.

Observers are given extensive training in keeping consistent and thorough records without disturbing the baboons (if you smile, don't show your teeth; don't wear red; don't eat in front of them). "One of the things we're very, very good at," says Duke University's Jenny Tung, who is an associate director of the Amboseli Baboon Research Project, "is collecting fecal samples." The researchers have to be fast to swoop in and grab the poop while it's still fresh and they know which baboon deposited it. Over the years, they've amassed 30,000 to 40,000 5-mL samples stored in multiple freezers. Such a wealth of waste yields myriad treasures—in particular, snapshots of the animals' gut microbiota.

In a recent study, Tung, Ran Blekhman of the University of Minnesota, Luis Barreiro at the University of Montreal, Amboseli associate director Beth Archie, and six colleagues analyzed fecal samples from the baboons to examine how social interactions affect the composition of the animals' gut bugs. "In the last few years, we've seen these hints that social relationships matter for shaping the gut microbiome," says Archie, whose lab is based at the University of Notre Dame. For instance, humans who live together harbor similar microbial communities—but is that a function of their diets, their relatedness, their physical contact, or some other factor?

Along with diet and kinship, the Amboseli project documents the social relationships among group members, giving the investigators the perfect opportunity to answer that question. Archie and a graduate student went into the field and, using Dixie cups, scooped up fresh fecal samples from 48 baboons. The baboons belonged to two different social groups, and, not surprisingly, each group had distinct microbiota. Because both groups ate pretty much the same things, diet probably didn't explain the differences. So the team looked at grooming behaviors within groups. Controlling for diet and relatedness, the researchers found that animals that groomed each other had more-similar gut microbes than those that didn't have such physical contact (*eLife*, 4:e05224, 2015).

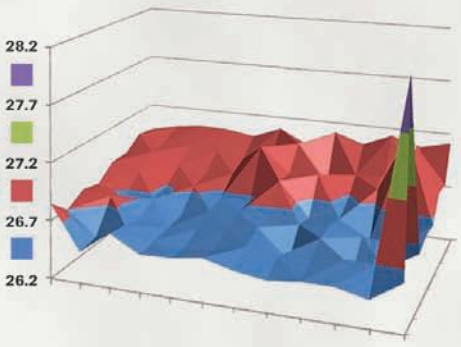
This study delves beyond the assumption that it's diet that's driving much of the structuring in microbial gut communities in mammals. Instead, the work shows that interpersonal contact is a key component in sharing microbes.

—Susan Lynch,
University of California, San Francisco

"It's confirmation of a very long-standing hypothesis, but no one has been able to show it before," says Andrew Moeller, a graduate student at Yale University who studies the evolution of primate microbiomes. The results back up the idea that social interactions help maintain diverse microbial communities over time. "If you think of one host, one lineage, parent to child, parent to child, with no social transmission, you'll have a loss of organisms," he says. "But once you have social transmission, those bottlenecks are completely erased."

Archie is now asking what this sharing means to the life of the animal, and how changes in social dynamics affect

ANDRZEJ KRAUZE



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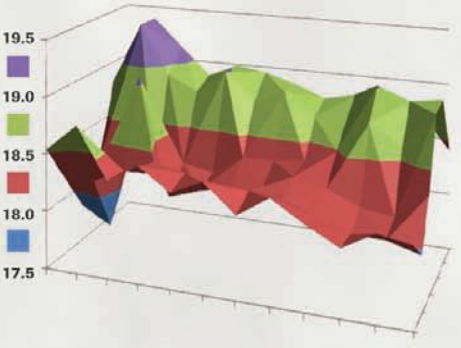

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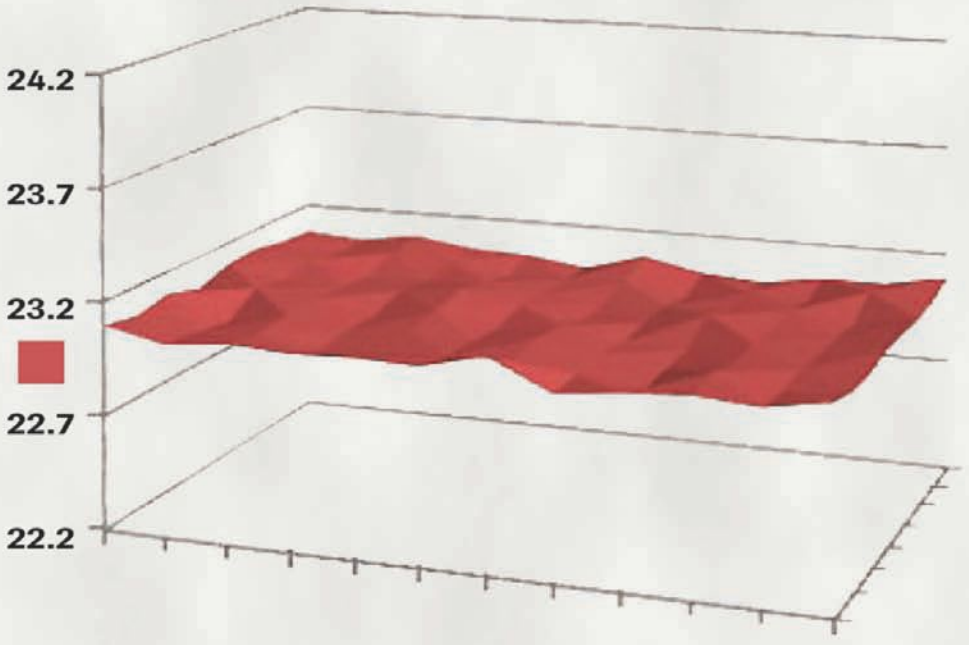

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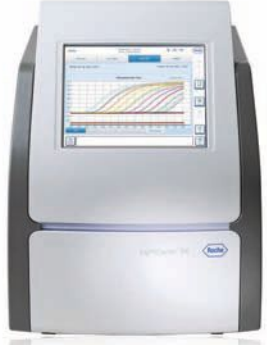

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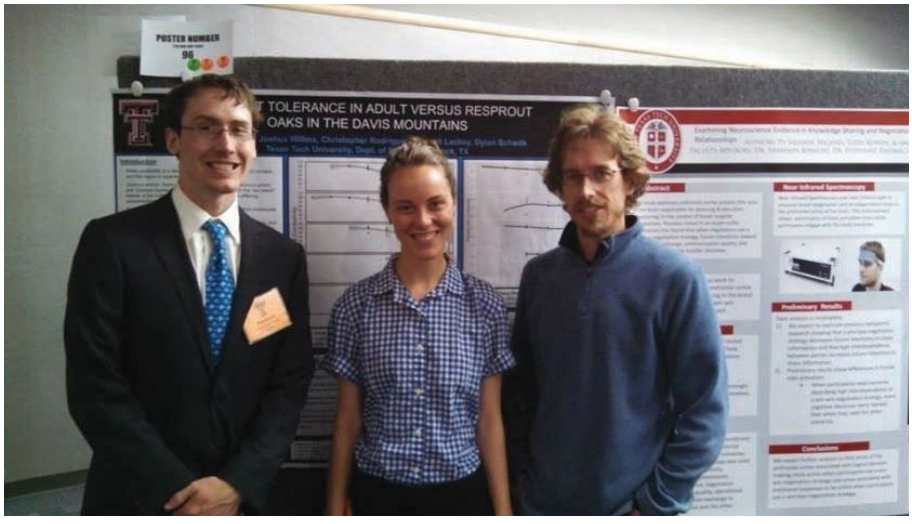
the composition of baboons' microbiomes. Her recent study on physical contact hinted that the differences between animals' microbes have some functional consequences. "The technique we used allowed us to see not only what species were there but what genes those bacteria carry," says Archie. "So we saw differences in the genic composition of [microbial assemblages within] social groups, suggesting they have different functions."

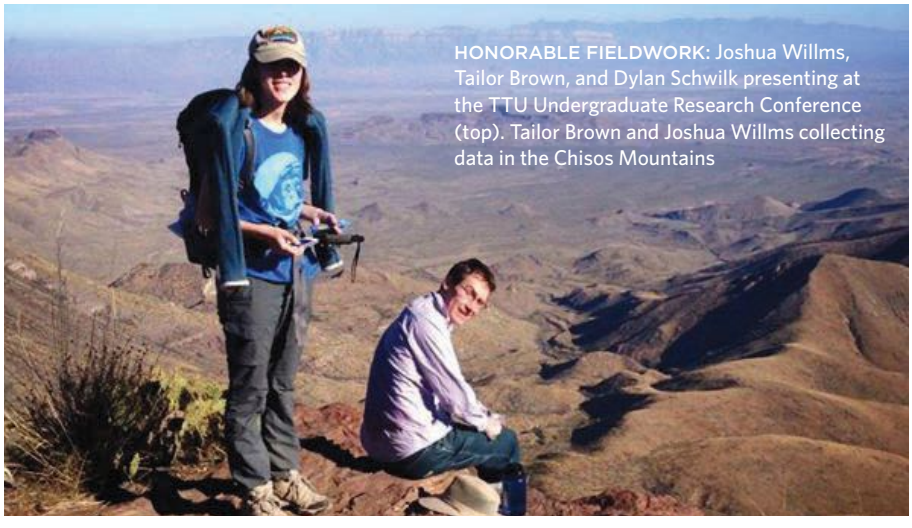
"What I really liked about this study is that it delves beyond the assumption that it's diet that's driving much of the structuring in microbial gut communities in mammals," says Susan Lynch, who studies the human microbiome at the University of California, San Francisco. Instead, the work "shows that interpersonal contact is a key component in sharing microbes."

Lynch adds that some research in humans has investigated the role of person-to-person touch in determining microbial composition. One study, for instance, found that babies born by C-section have microbes at multiple sites in their bodies that are more similar to those on their mother's skin, while babies born vaginally tend to have microbial communities most similar to their mother's vaginal microbiome (*PNAS*, 107:11971-75, 2010).

The significance of microbial sharing among humans is not entirely clear—nor is the significance of losing physical contact and, therefore, not swapping bacteria. "For me, what I thought was really interesting were implications for modern society," says Lynch. "We're becoming increasingly virtual, and how much of that is changing microbial patterns, and how is that associated with disease or health status?"

—Kerry Grens





HONORABLE FIELDWORK: Joshua Willms, Tailor Brown, and Dylan Schwilk presenting at the TTU Undergraduate Research Conference (top). Tailor Brown and Joshua Willms collecting data in the Chisos Mountains

COURTESY OF JOSHUA WILLMS

Memorial Research

In 2012, Christopher Rodriguez, a student at Texas Tech University (TTU) and a newly minted TTU/Howard Hughes Medical Institute Undergraduate Research Scholar, called his father Chris to tell him how excited he was about his fieldwork studying trees in the arid Franklin Mountains near El Paso. "Well, that sounds boring," the senior Rodriguez, a computer engineer, remembers thinking. Yet his son's enthusiasm and gift for explaining scientific concepts soon ignited a spark of interest in the father. "He had a way about himself to get people really excited about science, particularly about the stuff he was working on," Rodriguez's father says.

Like many of the students in what is now the Center for the Integration of STEM Education and Research (CISER) Undergraduate Research Scholar program, Rodriguez planned to attend medical school, even though he was also enrolled in the college of education. Fascinated by the physics of water transport in plants, Rodriguez joined forces with his mentor, plant ecologist Dylan Schwilk, to investigate how the drought tolerance of oak resprouts, the young plants that grow back after adult trees have been consumed by fire, might compare with that of mature trees. Rodriguez spent the summer before his junior year making long trips with Schwilk to mountainous field sites. During their long drives they discussed education and research, and Rodriguez peppered his mentor with questions while quickly learning how to work in the field.

In October 2012, a few months into the project, Rodriguez was gravely injured in a motorcycle accident. Rodriguez's father was stunned by the dozens of students

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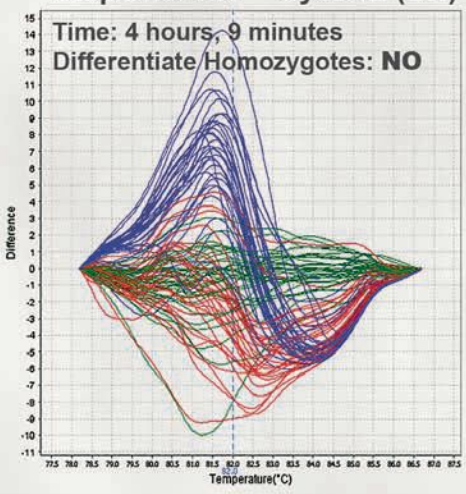
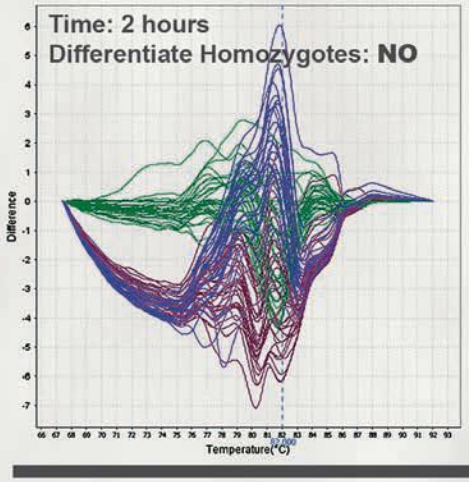


ViiA™ 7 System (LTI)

Time: 2 hours
Differentiate Homozygotes: **NO**



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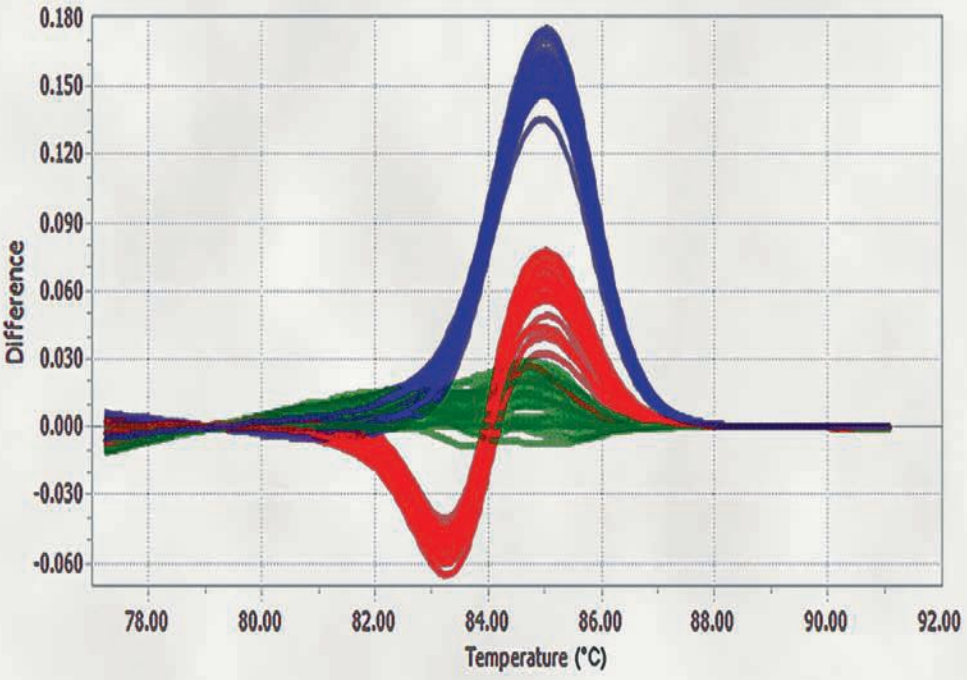

HRM analysis of an Add1 SNP (G→T)/68 bp human genomic DNA (10 ng). Master mixes recommended by the corresponding instrument manufacturers were used. *Roche R&D data on file.*

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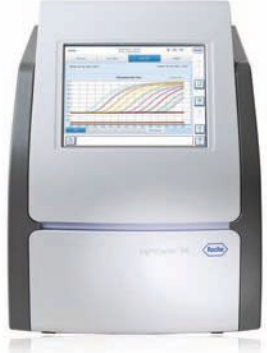

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who came to visit his son in the hospital. Several said that Christopher had helped them pass exams they would have otherwise failed by putting aside his own work to study all night with them. Despite the stream of well-wishers, Christopher died on October 6, three days after his accident. After his death, the community of undergrads with whom he had shared his passion for science and research decided to honor his memory by completing the project he had started.

"I wanted [the students] to be able to heal, and the science way of doing that would be to preserve a piece of Chris's thought and actions from the research that he was doing," says former TTU/HHMI student scholar Joshua Willms, who organized the effort. Schwilk was skeptical about how the enthusiasm of Rodriguez's student colleagues would play out in the lab, yet Willms found a way to involve the scholars in every part of the research. "Most of the time it was very simple things like resetting a temperature sensor or running a centrifuge, but everybody got to do some of the things that Chris had been doing and would have done," Willms says.

Many of the undergrads lugged water to the field site, cut stems underwater to maintain the proper water pressure in the twig, and brought them back to the lab. There, other students cut the stems into segments slightly wider than the diameter of a standard pencil and flushed them with water to get rid of any gases in the water-transporting xylem tissue. They then spun the stem segments in a centrifuge to simulate water deprivation, creating increasingly intense drought conditions with faster spins that allowed the students to quantify how the plants respond to ever-drier conditions. Schwilk recalls peeking into his lab one day to see half a dozen undergraduates, most of whom he didn't know, scurrying around in lab coats, ferrying oak stems between water containers. "I just retreated to my office," Schwilk recalls. "I didn't want to get in the way."

I wanted the students to be able to heal, and the science way of doing that would be to preserve a piece of Chris's thought and actions from the research that he was doing.

—Joshua Willms, former TTU/HHMI scholar

The results, collected by Schwilk's undergraduate student Tailor Brown with the help of Willms's team of scholars, revealed that oak resprouts shuttle water through their xylem tissue more quickly than their adult counterparts, which transport water more slowly through thinner vessels, making the adult oaks highly drought resistant. The findings are "on the cutting edge" of the new understanding that

drought tolerance can change throughout a plant's life, says Anna Jacobsen of California State University, Bakersfield, who first saw the work presented at the Ecological Society of America meeting in August 2014.

At the time, Jacobsen did not know about the small army of undergraduates who contributed to the project, but she says that their involvement provides a powerful lesson for mentors and researchers. "Typically, our response as academics . . . is that the students who come to us have to be passionate about the research that they're doing," she says. "To have students that are passionate about a project not because of the research, but because of a person . . . I think is really powerful."

"I loved being a part of a group that came together to honor a loved one in that way," Graysen Ortega, one of the students who contributed to the project, wrote in an e-mail to *The Scientist*. Saba Nafees, a student who spent an hour talking with Rodriguez about his research shortly before the accident that cost him his life, says that Rodriguez's passion for his research not only inspired her to organize his memorial service, help connect people with the research effort, and recruit her friend Brown to the project, but also motivated her to work harder in her own research endeavors. The students who worked on the project told her "they all feel very uplifted and feel like they're learning and contributing to something larger than life," Nafees adds.

"I see students inspiring one another," says University of Portland professor Amelia Ahern-Rindell, the current president of the Council on Undergraduate Research. "I see them helping each other over hurdles." She's not surprised by the healing power of contributing to research in memory of a friend, but she still thinks "it's just incredible that the students came together to support one another in this way."

For Rodriguez's family, it was "very humbling and very exciting" that his schoolmates took it upon themselves to finish the work he had started, his father says. The listing of Rodriguez as an author when the work is presented at conferences has also made him very proud.

The students' tribute remembers a young man with a noticeable passion for education, science, and research. "He was just exclaiming with delight at the end of a weekend trip," Schwilk says of Rodriguez. "He didn't realize that there were so many unknowns and that there was so much mystery in science." —Jenny Rood

Adapting to Arsenic

In parts of Argentina, people have been drinking poison—arsenic, to be specific—for thousands of years. The river running through the Andean village of San Antonio de los Cobres (SAC) has arsenic levels up to 80 times the safe limit established by the World Health Organization (WHO); it seeps into the groundwater from volcanic bedrock. Arsenic levels in the region's tap water were as high as 20 times the WHO's limit before 2012, when a filtration system was installed. The villagers are descended from indigenous Atacameño people who have lived and drunk the water in northern Argentina for as long as 11,000 years. Since 1994, Swedish biologist Karin Broberg, of Stockholm's Karolinska Institute, and colleagues at Uppsala and Lund Universities have been trying to figure out how generations of SAC's now nearly 6,000 residents have been able to survive this chronic arsenic exposure.

Previous studies of arsenic-exposed populations in Bangladesh, Mexico, and Taiwan established *AS3MT*, located on chromosome 10, as the main gene involved in arsenic metabolism in humans (*PLOS Genet*, 8:e1002522, 2012; *J Appl Toxicol*, 30:260-70, 2010; *Cancer Causes Control*, 20:1653-61, 2009). *AS3MT* encodes an enzyme that methylates inorganic arsenic to form mono- and dimethylated forms, which are less toxic than the naked element. The ratio of methylated to total arsenic in a urine sample indicates an individual's ability to metabolize the element and, for researchers, is a key measure of arsenic tolerance.

Broberg and her colleagues hypothesized that the remarkable arsenic tolerance

TOXIC SELECTION: Arsenic exposure can be deadly, but high in the Andes, drinking water laced with the chemical may have driven genetic adaptation in local populations.

of SAC residents might be due to particular variants of *AS3MT* that confer better arsenic metabolism. They wondered, further, if thousands of years of arsenic exposure had given a survival advantage to individuals with these metabolism-driving alleles and had increased the frequencies of these genetic variants.

At altitudes of 3,800 meters, the team traversed unpaved roads collecting samples of blood and urine from study participants. They faced many challenges preserving and transporting the samples and shipping them, safely frozen, all the way to Sweden. But the hassles and high-altitude adventures paid off with what may prove to be the first persuasive evidence in humans of natural selection driven by chronic exposure to a poison. A 2013 analysis of the data the team collected over many trips to the Andes found that residents of SAC had higher levels of *AS3MT* alleles linked to efficient arsenic metabolism than other Native American and Asian populations, suggesting that exposure to arsenic in the environment might have driven the selection for arsenic-protective alleles (*Environ Health Perspect*, 121:53-58, 2013).

To test the selection hypothesis, the group homed in on a subset of 124 women from their earlier study who all had similar levels of arsenic exposure, in order to minimize the variable of intake and focus on genetic factors. The researchers analyzed single-nucleotide polymorphisms using DNA from the blood samples, measured the levels of methylated arsenic in the subjects' urine, and performed a genome-wide association analysis to look for genes influencing arsenic metabolism. As they had predicted, there was a strong association between an individual's *AS3MT* allele and urinary levels of methylated arsenic.

In order to rule out the possibility that something other than selection, such as genetic drift among South American populations, led to the increased frequencies of these protective *AS3MT* variants, the researchers examined publicly available genomic data from people in Peru and Columbia—countries with much lower arsenic exposure. If frequencies of *AS3MT* variants differed markedly between the Argentine and comparison populations, it was likely that arsenic was exerting a selective pressure on the SAC population.

Overall, Broberg says, their genetic analyses revealed that the SAC and comparison populations were genetically similar. The SAC and Peruvian populations were about as alike as, say, the French and the Italians, says evolutionary biologist Rasmus Nielsen of the University of California, Berkeley, who was not involved in the study. The Columbian population, which also had more European ancestry, was a bit more divergent. In the area around *AS3MT*, however, the SAC population differed dramatically from both comparison populations. Not only did the SAC women have higher levels of protective *AS3MT* alleles, but these alleles also had longer stretches of homozygosity—a telltale sign of selection (*Mol Bio Evol*, doi:10.1093/molbev/msv046, 2015).

"There is an extremely strong difference in allele frequency," says Nielsen. "This pattern is almost certainly caused by selection. This is a very convincing study that provides a new and interesting example of human adaptation to the local environment."

The University of Chicago's Habibul Ahsan, who led the first genome-wide association study of arsenic metabolism, says that Broberg and her colleagues "are showing some evidence" for selection of *AS3MT* protective variants in the SAC population, but he notes that the study was limited by its small sample size and by the fact that the comparison groups differ from the Argentine populations in important ways—such as nationality, ethnicity, and culture—that go beyond historical arsenic exposure.

University of Arizona researcher Walter Klimecki, who has studied factors affecting arsenic susceptibility, also urged caution about Broberg's conclusions regarding selection, but admitted that the study could spark important insights into human evolution. "If these findings are confirmed to the level where there's general acceptance that this represents an instance of a toxic compound acting as a selecting agent in natural selection in a human population," he says, "this would be the first report [of that] in a human population, and so there's obvious interest from that standpoint." —Ashley P. Taylor

Improving Crops with RNAi

RNA interference is proving to be a valuable tool for agriculture, allowing researchers to develop pathogen-resistant and more-nutritious crops.

BY NARENDER NEHRA AND NIGEL TAYLOR

RNA interference (RNAi)—the process by which small interfering RNAs (siRNAs) bind to and cleave complementary mRNA sequences, inhibiting their translation into proteins—is not new to agriculture. In fact, as a naturally occurring biological process, RNAi was mediating plant metabolism, growth, and pathogen defense long before humans began cultivating crops for their own benefit. But in the last 15 years, RNAi's role in agriculture has grown as researchers have developed greater understanding of the mechanisms underlying the phenomenon and employed it to improve pathogen resistance, nutrition, and yield of crop plants. RNAi-enhanced crops have been approved for cultivation by regulatory agencies in the United States, Europe, Canada, Australia, New Zealand, and Brazil, and some of these crops—for example, papaya—have already reached our plates.

RNAi is a particularly potent tool for fighting common crop pathogens. By simply integrating virus- or bacteria-derived DNA sequences into the plant genome, pathogen-targeting siRNAs can be produced, triggering the endogenous RNAi mechanisms to target and degrade homologous sequences produced by invading pathogens. Commercial cultivation of virus-resistant papaya and extensive field testing of virus-resistant plum (under high disease pressure) since 1996 have shown that the pathogen-derived RNAi technology can deliver very effective and durable resistance. More recently, this strategy has produced virus-resistant common beans, fungal-resistant bananas, nematode-resistant soybeans, and insect-resistant corn. To date, RNAi has proven more cost-effective and environmentally friendly than the use of pesticides to control pathogens, and RNAi-fortified crops have the potential to impact food security and economic development. Recent regulatory approval in Brazil for cultivation of a common bean modified by RNAi for resistance to bean golden mosaic virus is cause for optimism. In Africa researchers are employing RNAi to combat viral diseases of the tropical root crop cassava (*Manihot esculenta*), a staple of African diets.

Agricultural researchers are also using RNAi-based technology to develop nutritionally enhanced crops. For example, RNAi was used to downregulate the omega-6 fatty acid desaturase gene, resulting in increased levels of monounsaturated (oleic) fatty acids in soybean seeds. The high–oleic oil soybeans are beneficial for human health and industrial oil production. Other nutritionally enhanced products in development include tomatoes with increased carotenoids, high-amylose and reduced-gluten wheat, and oranges with high levels of beta-carotene.

Despite widespread debate on public acceptance of genetically modified organisms, several unique features support the safety profile of RNAi-enhanced products, including the ubiquitous nature of siRNAs in plants; the history of safe use and consumption of naturally occurring and transgene-derived RNAi crops; high species specificity that minimizes off-target effects; and lack of toxicity and allergenicity, resulting from the fact that no transgenic protein is produced by such plants. Clearly, RNAi holds tremendous potential for producing healthier crop plants with enhanced nutritional value. ■

Narender Nehra is the director of regulatory affairs at the Donald Danforth Plant Science Center's Institute for International Crop Improvement (IICI), where Nigel Taylor is a senior research scientist. Mark Halsey, director of product development; Titus Alicai, program leader for root crops research in Uganda; and Douglas Miano, regulatory lead in Kenya, also contributed to this article.

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Courtney Noah, PhD, Senior Marketing Manager, Enzo Life Sciences
Brian McWilliams, PhD, Scientific Support Specialist, Bethyl Laboratories Inc.
Devin Scannell, PhD, Engagement Manager, McKinsey & Co.

PANEL 2: SCIENTIFIC COMMUNICATION | 2:00 – 3:00pm Eastern Time

Erin Cadwalader, PhD, Government Relations Associate, Lewis-Burke Associates LLC
Mark Nichols, PhD, Scientist Administrator, University of Pittsburgh
Kristine Roy, PhD, Medical Writing Fellow, Boston Scientific
Ruth Williams, PhD, Science Writer

PANEL 3: CLINICAL AND INDUSTRY RESEARCH | 3:00 – 4:00pm Eastern Time

Mike Baldwin, PhD, Research Scientist, Theravance Biopharma
Samir Koirala, PhD, Senior Scientist, Amgen
Holly Heaslet Soutter, PhD, Principal Research Scientist, X-Chem

PANEL 4: LAW AND REGULATORY AFFAIRS | 4:00 – 5:00pm Eastern Time

Mary Ellen Cosenza, PhD, President, MEC Regulatory & Toxicology Consulting LLC
Garth M. Dahlen, PhD, Patent Attorney, Mendelsohn, Drucker, & Dunleavy, P.C.
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Turning Data into Discovery

To make the most of the current data deluge, we must reward interdisciplinary researchers who identify and apply the most appropriate analysis methods.

BY VICKI CHANDLER

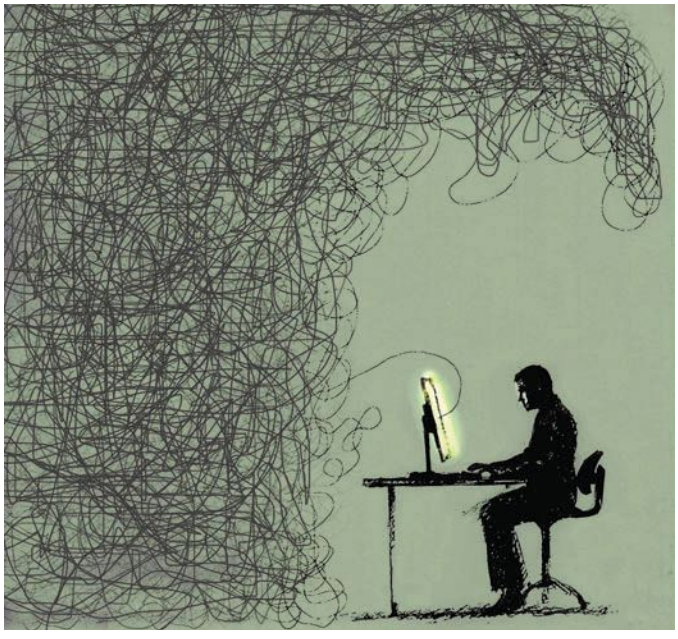
As recently as five years ago, nearly all my genetics data could fit on my personal computer and could be analyzed using basic spreadsheet software. Today, my data require sophisticated analysis tools and larger storage solutions, and I am not alone. Scientists across nearly every field—from genetics to neuroscience, physics to ecology—are generating unprecedented volumes of data at speeds that would have seemed like science fiction just a few years ago. For the first time in history, researchers routinely gather more information than they can analyze in a meaningful way. As a result, science is now data-rich but discovery-poor.

The solution to this modern paradox lies in developing technologies to extract meaning from all this information. But new technologies are not sufficient; researchers must also know which technologies and methods are best equipped to address the important questions for their area of study. There simply aren't enough academic researchers who are capable of harnessing their data deluge.

How can we attract and train the experts needed to transform data into discovery across many scientific fields? This is a problem my colleague Chris Mentzel, program director of the Gordon and Betty Moore Foundation's Data-Driven Discovery Initiative, and I have been thinking about for several years. One of the challenges facing scientific progress is that career advancement has traditionally been fueled by specialization, individual discovery, and publication in high-impact journals. People capable of solving today's—and tomorrow's—data problems don't fit that traditional model. They are truly interdisciplinary and work at the intersection of computer science, statistics, mathematics, and their discipline of interest. As such, they are often overlooked in the traditional academic hierarchy.

We believe there is an urgent need to cultivate this new type of data-driven researcher by recognizing and rewarding those with the necessary skill set. To this end, in October 2014 the foundation announced 14 recipients of the Moore Investigator in Data-Driven Discovery Awards. In addition to advancing data-driven science, we hope these five-year awards will strengthen incentives at research institutions to support more data-driven researchers by highlighting the value of these types of scientists in academia.

Fostering these interdisciplinary researchers also requires creating supportive and collaborative environments within academic institutions. Last year, the Data-Driven Discovery Initiative announced a five-year, $37.8 million partnership with the Sloan Foundation and three universities (the University of California, Berkeley; the University of Washington; and New York University) to build environments that will create homes for academic data scientists across campuses. Many other universities are also starting to invest in data-science centers with similar goals.



Measuring the success of such programs depends on the definition of academic achievement. Traditional metrics, such as the quality and quantity of papers published, are insufficient. For people working to turn data into discovery, success often means developing and sharing new tools, methodologies, and practices that can enable answering research questions at a scale not previously possible. These research outputs can have substantial scientific impact, but are often not rewarded within our current academic culture.

We live in an era of amazing technologies capable of gathering and analyzing astounding amounts of information. We can now sequence more than five whole genomes in a single day. Our telescopes capture ultrahigh-resolution images of the stars every 10 seconds, streaming hundreds of terabytes of data daily. New sensors are capable of capturing vastly complex information that may help predict changes to Earth's ecosystems. If we invest even a fraction as much in the development of data scientists as we do in the development of new technologies and generation of data, science will one day be as rich in discovery as it is in data. ■

Vicki Chandler is the chief program officer in science at the Gordon and Betty Moore Foundation.

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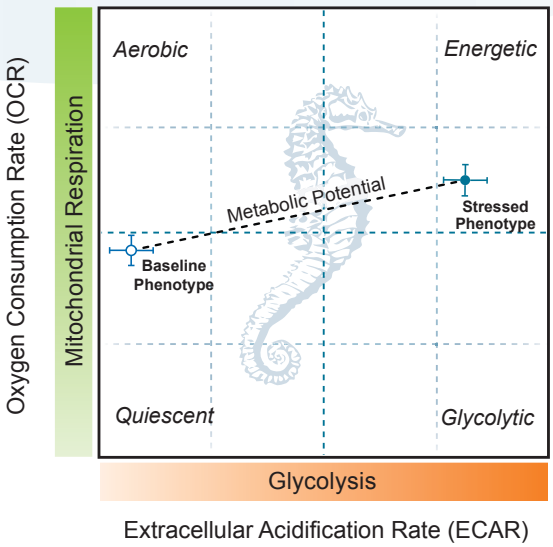


Seahorse Bioscience

RNA Stucturomics

A new high-throughput, transcriptome-wide assay determines RNA structures in vivo.

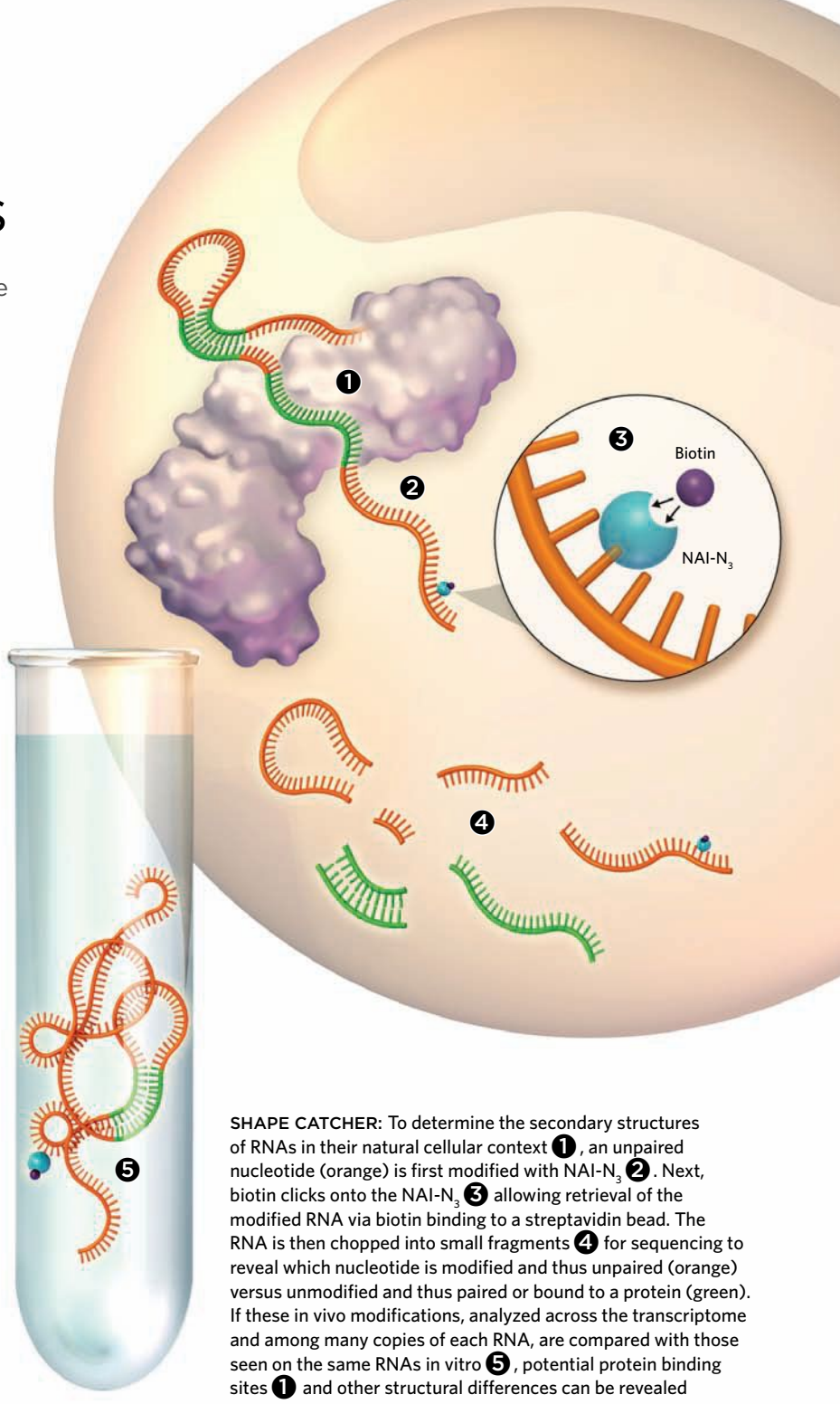
BY RUTH WILLIAMS

Like pieces of sticky tape, single-stranded RNA molecules can fold over and bind to themselves, via base pairing. Determining the complicated three-dimensional structures such folding creates can reveal the RNAs' functions, but classic techniques for investigating molecular structure, such as X-ray crystallography, are "very laborious" in that they "can only be used on one RNA at a time," says Howard Chang of Stanford University.

To study the secondary structures of multiple RNAs simultaneously, researchers have come up with chemical modification techniques that detect unpaired nucleotides in a pool of RNA molecules. Subsequent sequencing of the RNAs reveals the patterns of modified and unmodified bases (their unpaired or paired statuses) and thus indicates how each RNA molecule folds. One of these transcriptome-wide techniques, however, only targets two of the four nucleotides. And another targets all four nucleotides, but only works in vitro—where natural chemicals and proteins affecting structure are absent.

Chang and colleagues have now built upon the all-nucleotide modification technique—called selective 2'-hydroxyl acylation and profiling experiment (SHAPE)—to make it compatible with in vivo RNA analysis, and to allow for the enrichment of modified RNAs, thereby improving the signal-to-noise ratio. All in all, it means "we can now measure structural properties of RNAs at high resolution and very high throughput in their natural cellular context," says Julius Lucks of Cornell University.

Additionally, by comparing how the modifications differ between RNAs inside cells and the same RNAs in vitro, Chang has been able to identify nucleotide sequences that are likely protein-binding sites. (*Nature*, 519:486-90, 2015)



SHAPE CATCHER: To determine the secondary structures of RNAs in their natural cellular context ❶, an unpaired nucleotide (orange) is first modified with NAI-N_3 ❷. Next, biotin clicks onto the NAI-N_3 ❸ allowing retrieval of the modified RNA via biotin binding to a streptavidin bead. The RNA is then chopped into small fragments ❹ for sequencing to reveal which nucleotide is modified and thus unpaired (orange) versus unmodified and thus paired or bound to a protein (green). If these in vivo modifications, analyzed across the transcriptome and among many copies of each RNA, are compared with those seen on the same RNAs in vitro ❺, potential protein binding sites ❶ and other structural differences can be revealed because the probe will bind to different bases.

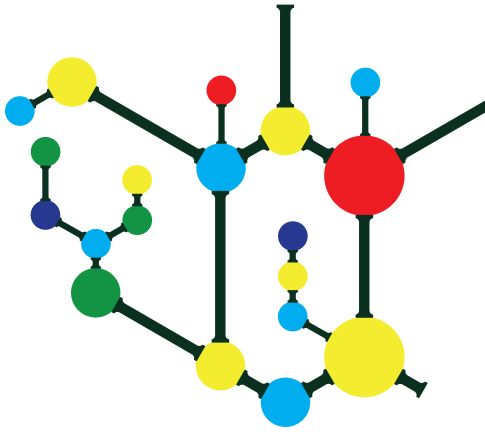
AT A GLANCE

TECHNIQUE	CHEMICAL MODIFICATION	ENRICHMENT OF MODIFIED RNAS?	IN VIVO ANALYSIS	PROTEIN-BINDING ANALYSIS
DMS-seq	Dimethylsulphate modification of adenosines and cytosines only, with an almost 3:1 bias favoring adenosines	No	Yes	Possible in principle, but has not been done
In vivo click (ic) SHAPE	2-(azidomethyl)nicotinic acid acyl imidazole (NAI-N_3) modifies all four nucleotides equally. The NAI-N_3 is then biotinylated.	Yes, using streptavidin beads that bind biotin	Yes	Yes

The Living Set

Mathematical and computational approaches are making strides in understanding how life might have emerged and organized itself from the basic chemistry of early Earth.

BY WIM HORDIJK



Life is a self-sustaining network of chemical reactions. A living system produces its own components from basic food sources in such a way that these components maintain and regulate the very chemical network that produced them.

Based on this notion of life, several models of minimal living systems were developed during the 1970s. While these models captured an essential aspect of the organization of living things, however, they could not directly explain how such systems emerged from a primordial soup of basic chemicals.

Over the past several years, one of these early models—that of autocatalytic sets—has been explored in more detail, both mathematically and with computer simulations. Autocatalytic sets are self-sustaining networks of chemical reactions that create and are catalyzed by components of the system itself. Recent research has overturned early criticisms regarding the plausibility of the spontaneous origin of such networks, and scientists have even applied the theoretical concepts to real chemical and biological systems, yielding important insights regarding the possible emergence, structure, and evolution of such systems. While many studying the origin of life are still focused on finding a self-replicating RNA molecule that could have served as the basis for modern life (see "RNA World 2.0," *The Scientist*, March 2014), some now consider autocatalytic sets necessary conditions for its start.

The organization principle

Put some *E. coli* in a dish with appropriate nutrients, and after a few days the dish will be teeming with new bacterial offspring. But break down those same *E. coli* into their constituent molecules, add that molecular cocktail to a dish of nutrients, and nothing will happen. On the other hand, dried fertilized eggs from the common brine shrimp (*Artemia*) can be frozen in liquid helium at 2 °K (near absolute zero), and then slowly warmed back to life. As biophysicists Arthur Skoultchi and Harold Morowitz demonstrated 50 years ago, eggs that returned to room temperature hatched and grew into healthy adults, which mated and laid viable eggs.

What's the difference between these two scenarios? In Skoultchi and Morowitz's experiment, even though the storage conditions were extreme, the brine shrimp's organized network of chemical pathways remained intact, allowing it to be revived. Life is clearly more than the sum of its parts.

The importance of organization to a system's function supports the idea that a living system can be defined as a *functionally closed* and *self-sustaining* chemical reaction network. Functionally closed means that the system's own components are sufficient to fully implement and regulate its functionality—whatever it does to "make a living." A functionally closed system is thus a self-regulating system. Self-sustaining means that the system's own functionality is, in turn, sufficient to construct and repair its components from a basic food set available from the environment. This can include both naturally occurring substances—such as water, air, and minerals—and compounds that are reliably produced by other nearby systems.

A bacterium, such as *E. coli*, is both functionally closed and self-sustaining. Through the chemical reactions that make up its metabolic network, it can produce its own components from a mixture of glucose and various salts containing phosphate, potassium, and ammonium. These self-constructed components in turn determine and regulate its metabolic network. For example, one particular component, the cell membrane, encloses all the other components (molecules and organelles) in a confined space so that the required metabolism can occur. A bacterium is, by anyone's definition, a living system.

A virus, on the other hand, is not functionally closed when it comes to reproduction. For that, it needs to "borrow" some of the functionality of a host cell. Most scientists do not consider a virus, by itself, to be alive.

Models of minimal life

In the early 1970s, several researchers used this notion of life as a functionally closed and self-sustaining system to develop formal models of what might constitute a minimal living system. These models include the hypercycle (by Nobel laureate Manfred Eigen in Germany), autopoietic systems (by Francisco Varela and Humberto Maturana in Chile), the chemoton model (by Tibor Gánti in Hungary), and autocatalytic sets (by Stuart Kauffman in the U.S.).

Although the mathematical properties of hypercycles—collections of self-replicating molecules in which each molecule "helps" the next one replicate, with the last one helping the first one—have been worked out in detail, to date no example of a real hypercycle exists in a living organism or in one constructed in the laboratory. Similarly, the notion of autopoiesis, a self-sufficient system based on cellular life as we know it today, including a membrane and a metabolism, has remained mostly at a conceptual level. Chemotons add to autopoiesis the idea of a simple genetic system, and while they are mathematically well understood, they represent a rather complex system that most scientists would not consider a suitable model for the origin of life.

Autocatalytic sets are, in a way, somewhere in between simple hypercycles on the one hand and the more complex autopoiesis and chemotons on the other. As the name implies, the notion of catalysis plays a central role in this model. Catalysts—molecules that speed the rate at which chemical reactions happen, without being used up in the reaction—are essential in determining and regulating the functionality of the chemical networks that give rise to and sustain life.

An autocatalytic set is defined as a collection of chemical reactions and their molecules in which: (1) each reaction in the set is catalyzed by at least one of the molecules from the set, and (2) each molecule in the set can be produced from a basic food source through a series of reactions performed within the autocatalytic set. This definition formally captures the organization of life as a functionally closed and self-sustaining chemical reaction network. But can it also say something about the origin of life?

Autocatalytic sets in theory and practice

To explain his idea of autocatalytic sets more clearly, Stuart Kauffman developed a simple model of chemical reaction systems, known as the binary polymer model. In this model, molecules are represented by sequences of zeros and ones, or bit strings. Two possible chemical reactions can occur: ligation, the joining of two bit strings together into a longer one, such as 00 + 111 → 00111, and cleavage, or the cutting of a bit string into two shorter ones, such as 010101 → 0101 + 01. Furthermore, each bit string is able to catalyze some of the ligation and cleavage reactions, but these catalytic abilities are assigned randomly. In this way, many different iterations of the model can be constructed, where each gives rise to a different reaction network. Kauffman then asked what the probability is that a (random) instance of his model contains a subset of reactions and molecules that together form a self-sustaining autocatalytic set.

Autocatalytic sets can be studied mathematically and with computer simulations, but they also show up in experimental systems of nucleic acids and in the metabolic network of living organisms.

Using his binary polymer model, Kauffman constructed a mathematical argument to show that the formation of autocatalytic sets is an expected emergent property of sufficiently complex collections of molecules. In other words, if a chemical reaction network has a large enough diversity of molecules, and if these molecules catalyze a large enough number of reactions, then we can expect there to exist a subset of molecules and reactions that together form a functionally closed and self-sustaining network.[1,2]

Clearly, this would have important implications for the origin of life. According to Kauffman, in a diverse enough collection of molecules and reactions, life will almost certainly emerge. For example, Kauffman explained his ideas explicitly in terms of autocatalytic sets of proteins.[1] We have known since the famous experiments performed by Stanley Miller during the 1950s that various amino acids form spontaneously in a "primordial broth" subjected to electrical sparks, perhaps lightning. Amino acids form peptides and small proteins quite easily, and peptides are known to have catalytic capabilities. So, it is not unthinkable that autocatalytic sets of peptides and small proteins may have formed spontaneously on early Earth.

But these ideas have faced their share of criticism. For example, Kauffman's mathematical argument may require a level of catalysis that is chemically unrealistic. Many molecules can catalyze more than one reaction. However, with increasing system sizes, Kauffman's argument may require each molecule to catalyze dozens or even hundreds of reactions.

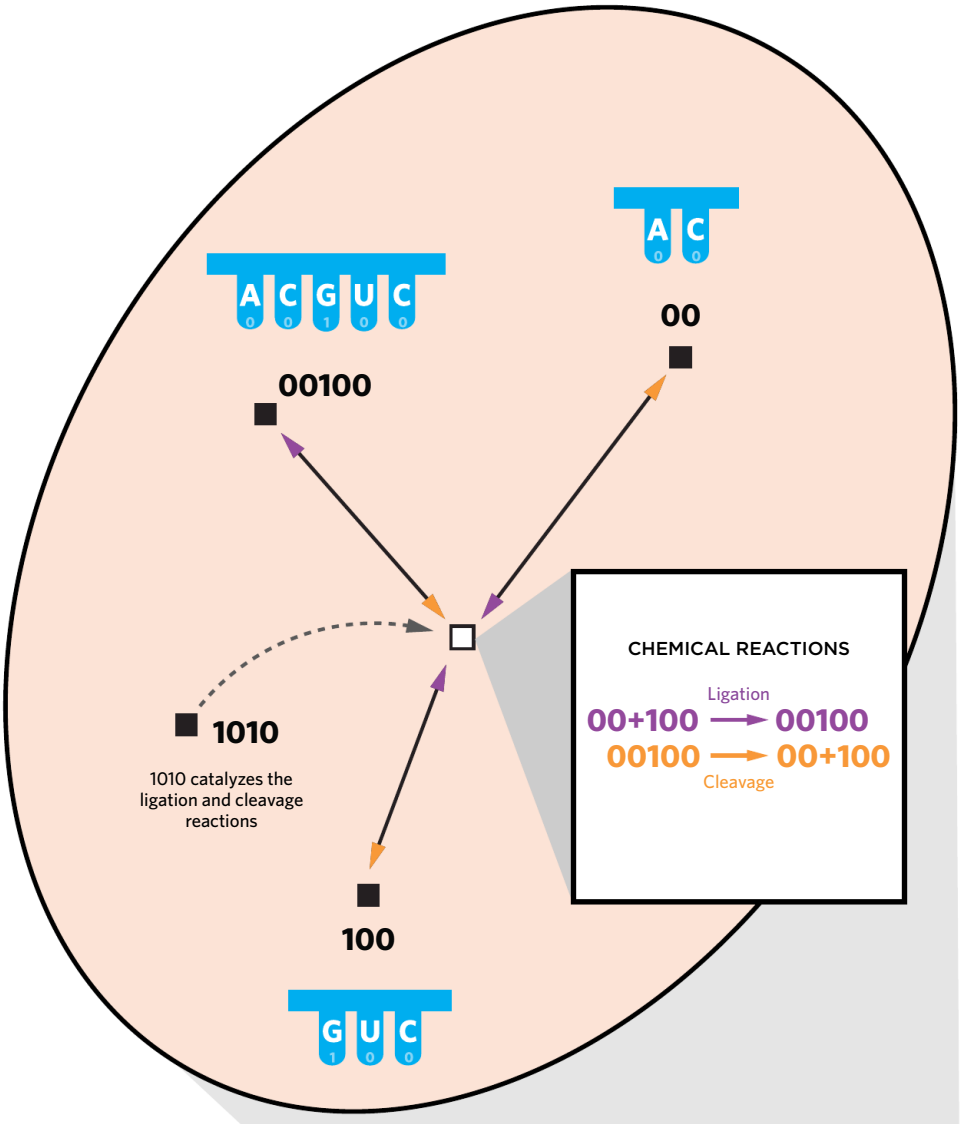
Moreover, autocatalytic sets have been criticized for lacking the ability to evolve. In Kauffman's argument, autocatalytic sets show up as "giant connected components," incorporating almost the entire reaction network. This leaves little room for change and adaptation. Autocatalytic sets, if they do in fact emerge, are thus not able to evolve into more-complex forms, making them biologically uninteresting.

Also, the binary polymer model has been accused of being too simplistic, with abstract bit strings representing real molecules, and even the smallest molecules (such as monomers and dimers) being able to catalyze arbitrary reactions. Because Kauffman's argument is entirely based on this simple model, it is unclear whether his results generalize to real chemical reaction networks.

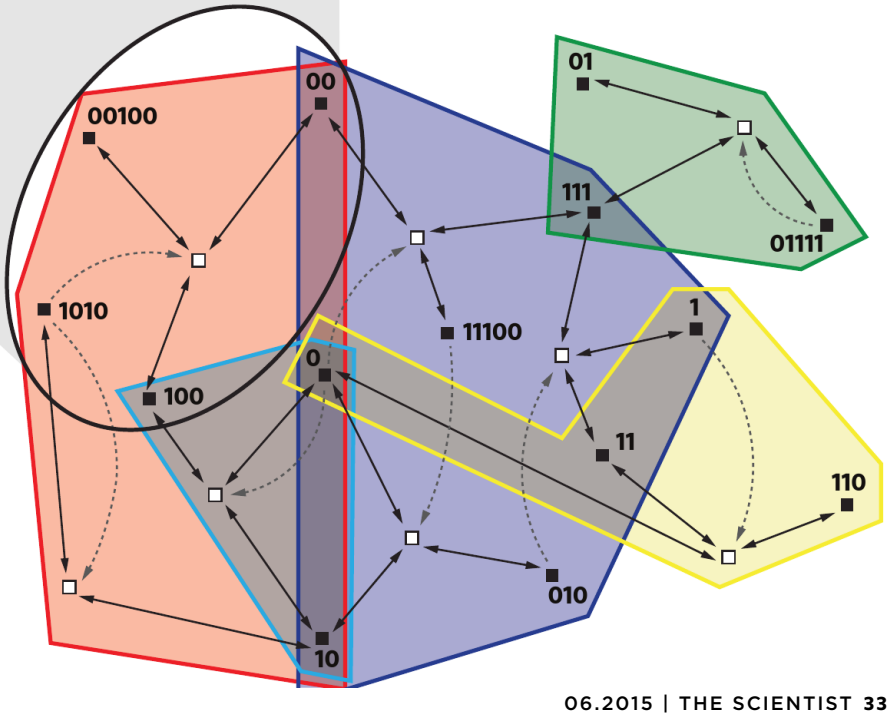
But despite these criticisms, examples of real chemical autocatalytic sets do exist. Günter von Kiedrowski, now at the Ruhr-Universität in Bochum, Germany, and his colleagues constructed the first artificial autocatalytic set in 1994, using two short nucleic acid sequences that mutually catalyze each other's formation from even shorter fragments.[3] Gerald Joyce, of the Scripps Research Institute in La Jolla, California, obtained similar results in 2009.[5] In this case, pairs of slightly longer nucleic acids were artificially evolved to mutually catalyze each other's formation with increasing efficiency.

SELF-SUSTAINING REACTION NETWORKS

The underlying tenet of autocatalytic sets is that a living system is functionally closed and self-sustaining in the presence of a food source. On early Earth, the initial basic ingredients, known as a food set, would have included molecules and elements such as water, hydrogen, nitrogen, carbon dioxide, and iron. These may have spontaneously formed more-complex molecules like the monomers of nucleic acids and proteins. And in more-complex systems—for example, a modern bacterium such as *E. coli*—the food set becomes even larger. In each case, the food set is all that is needed for the system to run: it provides the basic building blocks for the system's component parts. The notion of catalysis plays a central role in this model. Catalysts, which speed chemical reactions but are not themselves used up, are critical to the chemical networks that give rise to and sustain life.



AUTOCATALYTIC SETS AND THE ORIGIN OF LIFE: Spontaneous emergence of a basic food set of the four RNA nucleotides—adenine (A), cytosine (C), guanine (G), and uracil (U)—can give rise to longer RNA molecules that can then catalyze ligation and cleavage reactions among other RNA molecules. Such a scenario may be central to the idea of the RNA world, in which evolving, replicating RNA molecules represent the first rudimentary life on Earth.



SUBSETS WORKING TOGETHER: To computationally envision the behavior of autocatalytic sets, researchers can use a binary polymer model of bit strings (sequences of zeros and ones) to represent real-life polymers. The members of a food set are represented by, for example, one- and two-digit-long bit strings: 0, 1, 01, 10, 00, and 11. From this basic food set, longer polymers can be formed. The network's components catalyze (dashed gray arrows) reactions (white boxes). There are two types of reactions possible: ligation involves the joining of two bit strings together into a longer one, whereas cleavage is the cutting of a bit string into two shorter ones. Within the autocatalytic set shown at right, there are subsets (colored) of the whole that also constitute self-sustaining systems, in which the products all serve to catalyze their own formation.

BIOSCIENCE, 63: 877-81, 2013. REDRAWN WITH PERMISSION.

More-complex autocatalytic sets have also been assembled. In 2004, Joyce's colleague at Scripps, Reza Ghadiri, and other collaborators created an autocatalytic set of nine peptides.[6] And in 2012, Niles Lehman of Portland State University built an autocatalytic set of long nucleic acids formed spontaneously from shorter fragments.[7] In Lehman's system, a larger and larger autocatalytic set emerged over time, until eventually it contained all 16 possible molecule types.

These experiments demonstrate the plausibility of autocatalytic sets, but whether or not such self-sustaining systems served as the basis of the origin of life on Earth remains a matter of debate.

The emergence and structure of autocatalytic sets

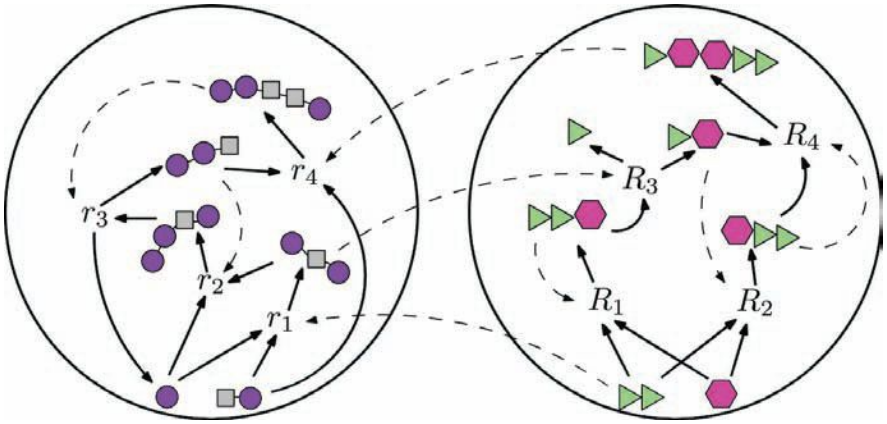
Over the past several years, my colleague Mike Steel of the University of Canterbury in New Zealand and I have studied autocatalytic sets in more detail, both mathematically and with computer simulations. This has led to new insights into the emergence, structure, and possible further evolution of autocatalytic sets. Moreover, these new results counter many earlier criticisms.[8]

We defined Kauffman's original notion of autocatalytic sets in a mathematically more rigorous way. Then we developed an efficient computer algorithm to detect autocatalytic sets in arbitrary chemical reaction networks. We applied this algorithm to random instances of the binary polymer model and derived various mathematical theorems about autocatalytic sets.

After we had run our simulations on a large computer cluster for several weeks, a clear picture emerged. Autocatalytic sets do indeed have a high probability of existence, even for very moderate levels of catalysis. In fact, in instances of the binary polymer model with bit strings up to 20 characters long, each bit string only needs to catalyze one to two reactions, on average, for autocatalytic sets to exist, making this a highly plausible chemical scenario. Additionally, the required level of catalysis increases only very slowly for larger chemical reaction networks, an observation that was subsequently confirmed theoretically. For example, even for bit strings up to 50 characters long, no more than two reactions need to be catalyzed per molecule, on average.

MODELING MULTIPLE MOLECULES

The starting ingredients for life may have included not only RNA nucleotides (purple circles and gray squares) and molecules, but also amino acids (green triangles and pink hexagons) and proteins. In this model, each molecule type can react with like molecules, but RNA molecules cannot react with proteins. Either molecule type can, however, catalyze reactions of the other type.



We also looked at the structure of the autocatalytic sets our algorithm identified. Contrary to Kauffman's original argument that autocatalytic sets emerge as "giant connected components," it turns out that autocatalytic sets can often be decomposed into smaller subsets, which themselves are autocatalytic. (See illustration on preceding page.) In fact, there often exists an entire hierarchy of smaller and smaller autocatalytic subsets. The smallest autocatalytic sets, which cannot be decomposed any further, are called irreducible autocatalytic sets.

In recent groundbreaking work, a group of researchers including Kauffman and Hungarian theoretical evolutionary biologist Eörs Szathmáry convincingly showed that autocatalytic sets composed of multiple small, irreducible subsets can, in fact, evolve.[9] The main idea is that these autocatalytic subsets can exist in different combinations within a compartment (a protocell), thus giving rise to different types of protocells, and, consequently, to competition and selection. This, combined with our own results that one can indeed expect many such irreducible autocatalytic subsets to exist within a reaction network, suggests that autocatalytic sets are likely to arise from sufficiently complex chemical reaction networks and go on to evolve into larger and more complex systems.

We also studied several variations of the standard binary polymer model, incorporating more-realistic assumptions regarding the system's chemistry. For example, rather than assigning catalysts completely randomly, we imposed the constraint that a bit string needs to match a certain number of bits around the ligation or cleavage site in a reaction to be able to catalyze that reaction, simulating catalysis by temporary base pair

J.I. SMITH ET AL., "AUTOCATALYTIC SETS IN A PARTITIONED BIOCHEMICAL NETWORK," *JOURNAL OF SYSTEMS CHEMISTRY*, 5:2, 2014.

formation. We also considered a version of the model in which molecules are partitioned into two separate sets (such as nucleic acids and amino acids), where reactions can only happen between molecules of the same set, but catalysis can happen both within and between sets. (See illustration on opposite page.) Overall, the main results hold up under all of these assumptions.

In order to move away entirely from abstract models, we also successfully applied our formal autocatalytic sets framework to real chemical and biological networks, such as the 16-member autocatalytic set that was constructed in the Lehman lab.[10] Not only were we able to reproduce most of their experimental results, we also derived new insights and predictions that would have been very difficult to obtain from chemical experiments alone. For example, our simulations show that with each repetition of the experiment, a different pattern of larger and larger autocatalytic sets emerges, revealing the rich hierarchical structure of autocatalytic subsets that exists within this system. Furthermore, the model provides a clear explanation for the experimental observation that a "cooperative" system (autocatalytic set) often outcompetes an equivalent but "selfish" system in which each molecule only catalyzes its own production. It also shows why and how such a cooperative system is more robust against environmental perturbations.

With molecular biologists Bill Martin and Filipa Sousa of the Heinrich-Heine-Universität in Düsseldorf, Germany, we analyzed the entire metabolic network of *E. coli* with our formal framework.[11] Not surprisingly, we found that 98 percent of the reactions in this metabolic network together form an autocatalytic set. The resulting set is quite robust to environmental variations, such as different food sources or the removal of random reactions or molecules. This reflects the redundancy that is known to exist in *E. coli*'s metabolism, and the bacterium's ability to live on different food stocks or to produce certain important molecules through multiple alternative chemical pathways. Our results also show a modularity in the network that corresponds well with known functional categories of metabolic reactions. In particular, the computational results underscore the crucial role of cofactors, such as various metals and molecules including ATP, as prime mediators of metabolism. Some of these cofactors may very well have been responsible for generating the very first autocatalytic sets at the origin of life.

If a living system is indeed an autocatalytic set, then the next question to ask is whether an ecosystem, a network of interdependent organisms, can be considered an autocatalytic superset of autocatalytic subsets.

In light of all these results, it seems that the main criticisms against the plausibility and evolvability of autocatalytic sets have now been largely resolved. It took more than 40 years, and the efforts of many scientists, to get from the initial ideas to the latest findings. But the gap between theory and experiments is finally closing.

Autocatalytic sets beyond chemistry

As this recent research has shown, autocatalytic sets capture essential aspects of the organization of living organisms, and their high probability of emergence and potential to evolve have important implications for the origin of life. Autocatalytic sets can be studied mathematically and with computer simulations, but they also show up in experimental systems of nucleic acids and in the metabolic network of living organisms. They truly seem to represent a fundamental property of life.

If a living system is indeed an autocatalytic set, then the next question to ask is whether an ecosystem, a network of interdependent organisms, can be considered an autocatalytic superset of autocatalytic subsets. Or, to take it even further, what about social systems such as the economy? Economic production—converting raw materials into consumer goods—can be compared to chemical reactions, with produced goods from hammers to conveyor belts to the Internet serving as the catalysts to facilitate yet other economic productions.

The possibilities are exciting and seemingly endless. And we now have the mathematical and computational tools to study and develop these ideas more formally and extensively. Indeed, several ecologists, economists, and cognitive and social scientists are interested in applying these ideas and tools in their own areas of research. The journey of investigations into autocatalytic sets, and the origin and organization of life itself, continues today as an international and interdisciplinary scientific collaboration. ■

Wim Hordijk is an independent computer scientist working in the areas of computational biology and origin of life. More information about his collaborative research on autocatalytic sets can be found on the website www.coolscience.club.

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What's Old Is New Again

Revolutionary new methods for extracting, purifying, and sequencing ever-more-ancient DNA have opened an unprecedented window into the history of life on Earth.

BY BOB GRANT

Two researchers sit hunched in front of a fume hood dressed head-to-toe in stark white Tyvek suits, though the yellow-tinted window I'm viewing them through lends the entire scene a sulfurous hue. One of the scientists, a research associate named Hongjie Li, pipettes tiny volumes of solutions containing decades-old DNA into centrifuge tubes, while the other, PhD student Lu Yao, types information into a laptop. Airlock doors and a sensitive ventilation system minimize the incursion of outside air and the myriad bits of contaminating DNA it carries. Yao, reaching a point when she can take a break, looks up from her work and waves, a smile spreading beneath her face mask and crinkling the corners of her eyes.

This is the ancient-DNA lab at the University of Illinois, Urbana-Champaign, tucked in a corner of the basement at the Carl R. Woese Institute for Genomic Biology. Yao has spent hours in this space. Working under the guidance of molecular anthropologist Ripan Malhi, she hopes to answer questions about phylogeny, biogeography, and island dwarfism among long-tailed macaques (*Macaca fascicularis*) in Southeast Asia by sequencing decades- and even century-old mitochondrial DNA collected from the dried skulls of monkeys in museum collections. And thanks to recent methodological, computational, and conceptual advances in the study of ancient DNA, Yao, Li—who studies ancient DNA from native Californians—and other researchers are succeeding, compiling sequences at an unprecedented rate.

In just a few decades, the study of ancient DNA has gone from a scientific curiosity to an extremely powerful method for reconstructing past biological phenomena. Malhi recalls that in his own PhD research, which he finished in 2001, he devoted an entire dissertation chapter and a year of lab work to the genetic analysis of 40 ancient samples from Native Americans, zeroing in on a 300-base-pair-long fragment of mitochondrial DNA. "Now, that's something that one of my students can do in a month," he says. "It's pretty amazing."

In addition to greatly condensing the amount of time it takes to extract and sequence old DNA, new techniques are allowing researchers to pluck sequenceable fragments from ever-more-ancient samples, providing genetic blueprints from long-forgotten epochs of evolution, migration, and ancestry. In 2014 alone, scientists successfully sequenced the mitochondrial genome of a hominin that lived more than 400,000 years ago,[1] exomes from the bones of two Neanderthal individuals more than 40,000 years old,[2] and a nearly complete nuclear genome from a 45,000-year-old modern human fossil,[3] to name but a few. In 2013, an international team of researchers led by scientists at the University of Copenhagen published the full genome sequence of an ancestral horse species that roamed the Middle Pleistocene permafrost of North America more than 700,000 years ago—the oldest complete genome sequenced thus far.[4]

For ancient-DNA researchers, these truly are heady times. "The last two or three years have been amazing," says Mattias Jakobsson, a population geneticist at Uppsala University in Sweden who studies ancient DNA as a way to understand human evolutionary history. And the coming years only promise more sequences from more and older specimens, he adds. "We're certainly heading to much more data. There's going to be many more studies of many more individuals."

VINTAGE DNA: Hominin skulls more that 400,000 years old, discovered at the Sima de los Huesos (Pit of Bones) site in Atapuerca, Spain. From a similarly aged femur excavated at the site, researchers extracted and sequenced a full mitochondrial genome.

Roots and shoots

The seeds of ancient DNA research sprouted in 1984, even before polymerase chain reaction (PCR) became the ubiquitous technique that it is today. Researchers at the University of California, Berkeley, successfully cloned and sequenced two fragments of mitochondrial DNA from a 140-year-old museum specimen of a quagga, an extinct relative of zebras, demonstrating that genetic material could survive and be recovered from the remains of long-dead animals.[5]

The quagga paper and similar reports of ancient DNA recovery from China and Germany ignited excitement among geneticists, and the race was on to comb ever-older specimens for sequenceable DNA. In 1985, Svante Pääbo, then a young PhD student at Uppsala University, published in *Nature* that he had cloned nuclear DNA from a mummified Egyptian child who was laid to rest 2,400 years ago.[6] Just a few years later, however, as PCR burst onto the scene, Pääbo learned that the DNA he'd recovered was at least in part modern human DNA, likely from the archaeologists or museum staff who had handled the specimen.

Soon thereafter, other claims of ancient DNA recovery were determined to be the result of contamination and/or faulty methodology, rather than a glimpse into prehistory. The recovery of supposed chloroplast DNA from 20-million-year-old magnolia leaves,[7] for example, could not be repeated; those who tried turned up only the genetic sequences of contaminating bacteria, mistakenly amplified by PCR. And pieces of mitochondrial DNA supposedly collected from 80-million-year-old dinosaur bone fragments[8] were, in fact, of modern human origin. "In the middle of the '90s you have a lot of people realizing that a lot of things were wrong," says Ludovic Orlando, a leading ancient-DNA researcher at the University of Copenhagen.

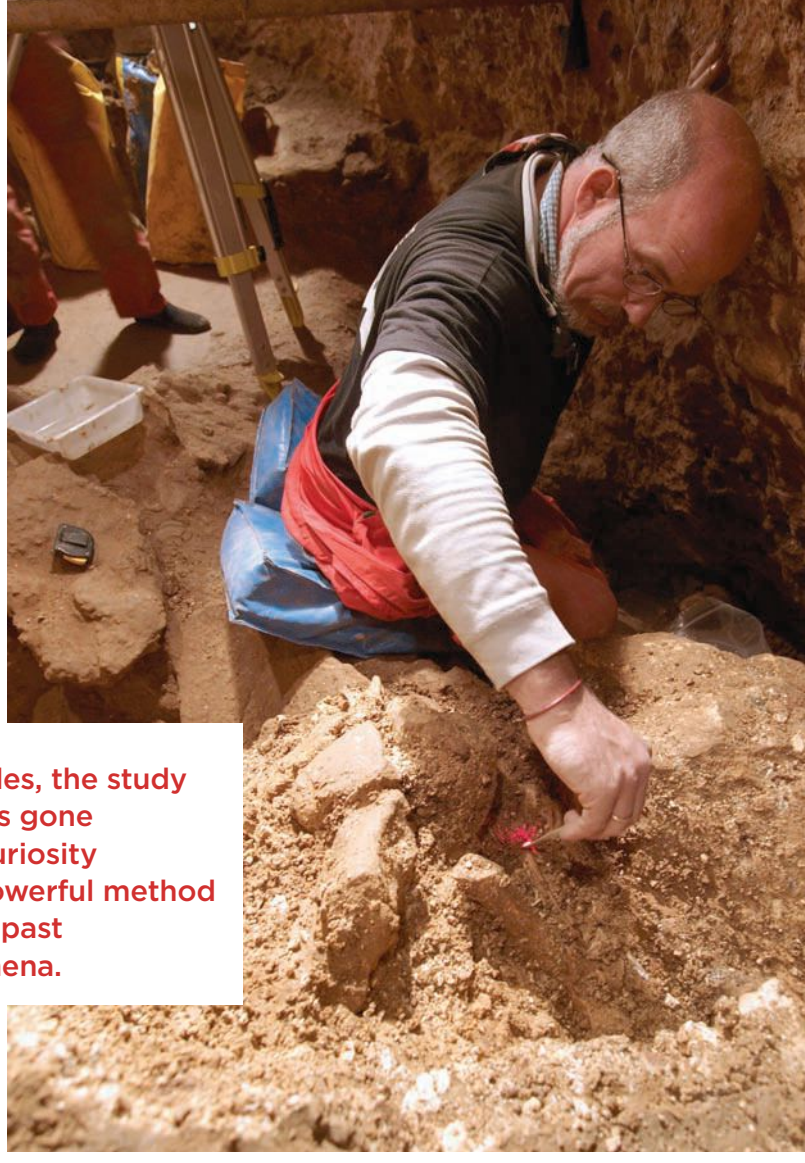
The mistakes made by ancient-DNA pioneers were not wholly uninformative, though. Problems with contamination led researchers to adopt carefully designed protocols for unearthing, cataloging, handling, and studying ancient samples. And realizing the inherent difficulty in working with highly degraded and aged bits of DNA sparked creative strategies for recovering short fragments of truly ancient genetic material and for differentiating them from modern DNA. Complement these protocols with new technologies developed in the past few years to mine, extract, isolate, and sequence genetic material from fossilized specimens, and there has been nothing short of a revolution in ancient DNA research.

Better tools blossom

The first rule of paleogenomics is: the older the sample, the more fragmented the DNA. Exogenous and endogenous nucleases get to work as soon as an organism dies, degrading its tissues and genetic material. Water and oxygen take their toll as well, leaving ancient DNA with characteristic double- and single-strand breaks, crosslinks, and telltale patterns of molecular modification. All told, DNA has an average half-life of only about 521 years in bone,[9] meaning that most fossilized samples contain only trace amounts of endogenous genetic material. It's no wonder early studies of ancient DNA led to so many cases of mistaken identity.

The first problem was that 1980s cloning techniques relied on enzymes that would actually repair damaged DNA, and not always correctly, introducing errors into resultant sequences. The adoption of PCR sidestepped this problem by eliminating the need to clone DNA samples before sequencing, but traditional PCR only amplifies fragments that are at least 90 base pairs in length, longer than the highly degraded fragments found in specimens thousands of years old. As a result, PCR is far more likely to amplify contaminating modern DNA than genetic material originating in a fossil sample. It's also extremely labor- and time-intensive to assemble an entire genome using traditional PCR, because the method only amplifies one specific stretch of DNA at a time.

Enter next-generation sequencing. The technology, which uses faster and simpler library preparation to ready DNA for massively parallel sequencing, came into wide use in the mid-2000s, allowing researchers to read all the DNA molecules in



In just a few decades, the study of ancient DNA has gone from a scientific curiosity to an extremely powerful method for reconstructing past biological phenomena.

IMAGES 1,2,4,5: © JAVIER TRUEBA/MSF/SCIENCE SOURCE; IMAGE 3: © MPI EVA

SEQUENCING INTO THE PAST: (Clockwise from top left) Excavation at the Sima de los Huesos site in Spain has been ongoing since 1997, unearthing more than 5,500 human bones that date to the Middle Pleistocene. Researchers used a thigh bone from of 400,000-year-old hominin from Sima de los Huesos to generate a complete mitochondrial genome sequence; Matthias Meyer analyzes ancient samples in a clean lab at the Max Planck Institute for Evolutionary Anthropology; Meyer (left) and Juan Luis Arsuaga of the Universidad Complutense de Madrid discuss the unique characteristics of the hominin fossils found at Sima de los Huesos; Bones are ground into a fine powder before isolating and extracting the DNA in the sample.

a given sample, not just a target sequence. "You recover all the genetic information that is in your library and in your extract," says Matthias Meyer, a researcher at the Max Planck Institute for Evolutionary Anthropology who has pioneered recent methodological improvements to working with ancient DNA. "With the same amount of DNA extract, you can now get thousands or tens of thousands of times more information. This is really what has made ancient DNA sequencing on a larger scale possible."

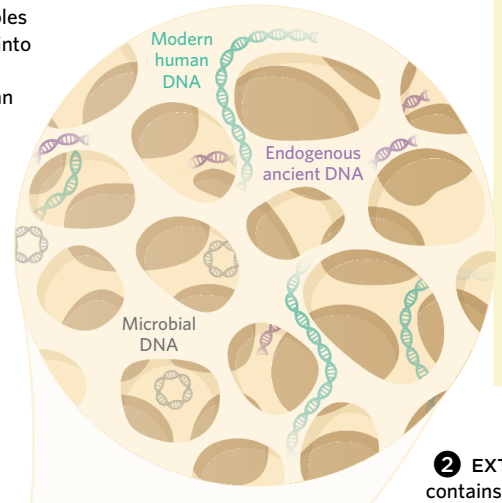
Next-gen sequencing is particularly useful for analyzing highly fragmented DNA, adds Eske Willerslev, a geneticist at the University of Copenhagen's Natural History Museum of Denmark, because it can capture the sequences of exceedingly short stretches of nucleotides. "The ability to go down and take 30 or 35 base pairs makes a huge difference," he says. "Those technological improvements that come with next-generation sequencing have made the biggest difference."

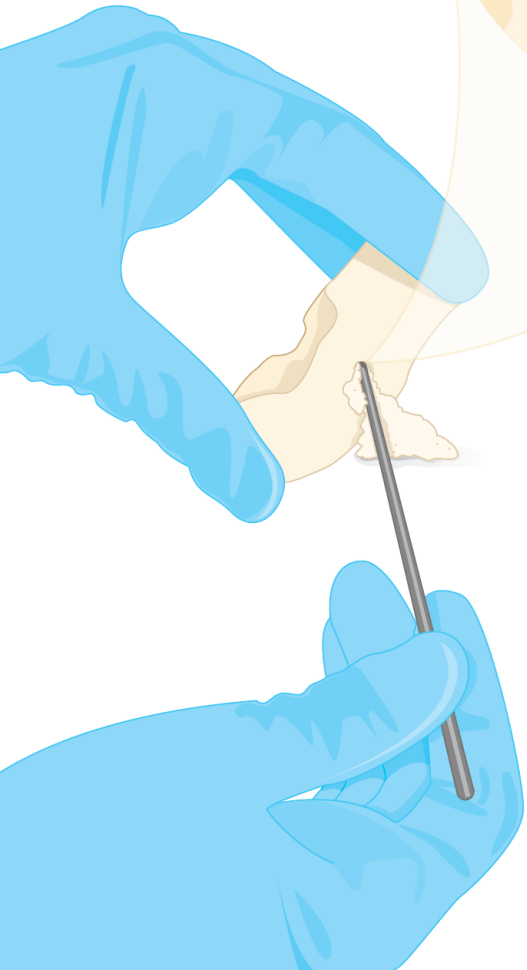
Improvements in the processing of ancient genetic material prior to sequencing have also helped researchers in the quest to retrieve older and shorter fragments of DNA. In ancient bone, DNA is nestled among diverse organic and inorganic molecules,

DIGGING FOR DNA

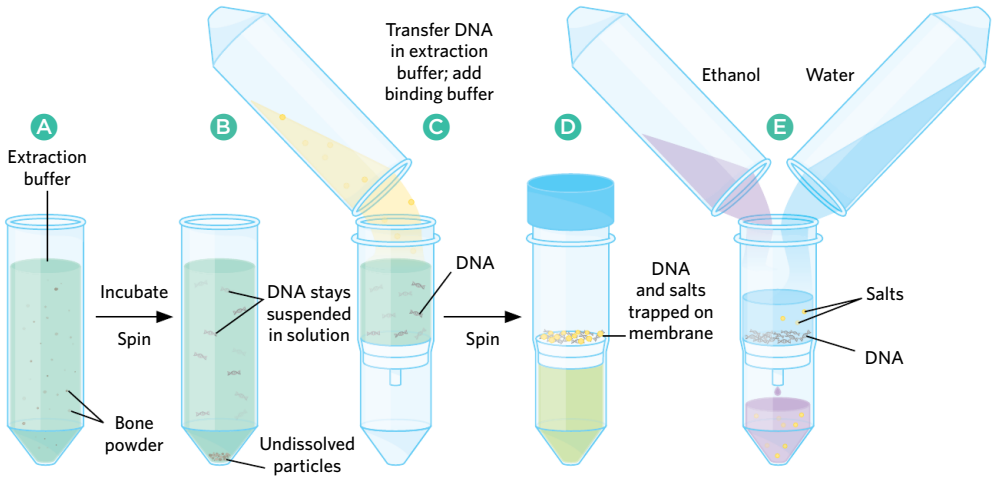
Ancient-DNA studies have come a long way in a few short years. Recent technological advances in extracting, isolating, preparing, sequencing, and assembling degraded and fragmentary DNA have helped researchers turn back the clock in terms of recovering genome sequences from millennia-old biological remains: last year, a 45,000-year-old leg bone became the oldest *Homo sapiens* DNA sequenced; researchers assembled a nearly complete mitochondrial genome sequence from a hominin

1 PROCESS BONE: Bone samples taken from the femur are ground into a powder. Ancient hominin DNA, bacterial DNA, and modern human DNA intermingle in the intricate matrix of a fossilized bone. Over time, ancient DNA is fragmented and undergoes cytosine deamination, a process in which Cs in the genome are replaced with Us (for uracil, a base that usually occurs in RNA).





2 EXTRACT DNA: Researchers add an extraction buffer that contains ethylenediaminetetraacetic acid (EDTA) and proteinase K to the powdered bone, and incubate the solution overnight. The extraction buffer dissolves most of the major inorganic (hydroxyapatite) and organic (collagen) components of bone, freeing DNA molecules A. After centrifuging to separate DNA from larger debris particles B, researchers add a binding buffer containing the salt guanidine hydrochloride, which promotes binding of DNA to silica, and isopropanol, which aids in the capture of very short DNA fragments C. This solution is loaded into a new centrifuge tube that contains a silica membrane to trap the salt-bound DNA D. The silica membrane helps capture smaller fragments of DNA, which is key to harvesting more of the degraded ancient DNA. After centrifuging, salts are washed from the silica membrane with ethanol, and DNA is eluted by adding water E.



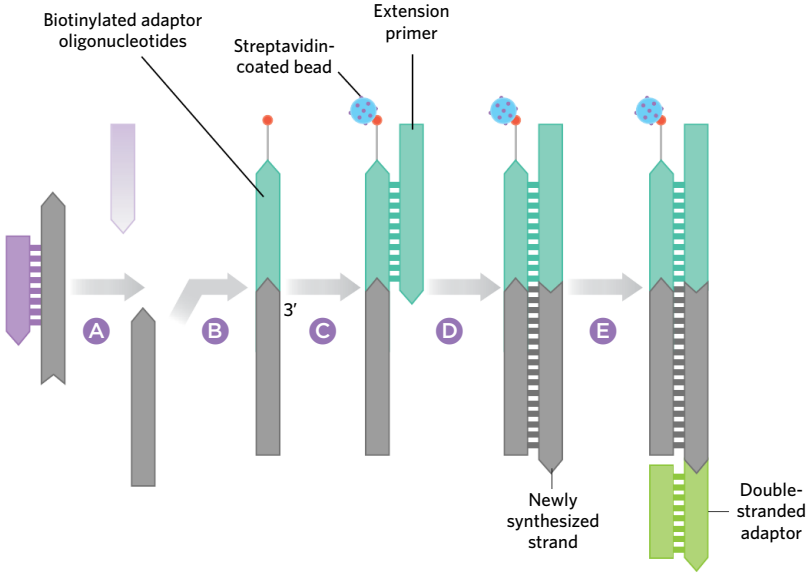
including collagen and a mineral form of calcium called hydroxyapatite, which must be dissolved away to extract sequenceable genetic material. "The challenge of DNA extraction is to purify the DNA," Meyer says, "to wash out the substances that are interfering with your downstream analysis." Meyer's lab used an extraction buffer to address this issue, employing the chelating agent ethylenediaminetetraacetic acid (EDTA) to dissolve hydroxyapatite and the enzyme proteinase K to dissolve collagen.

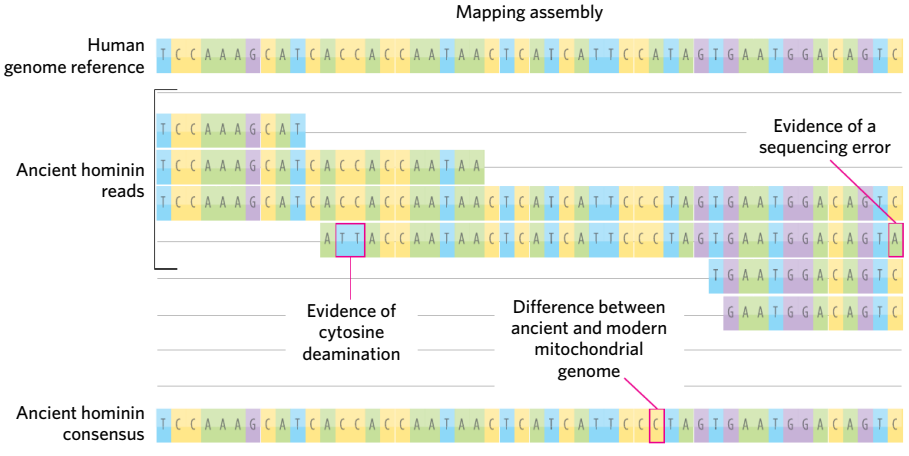
Meyer's lab also optimized a formula of binding buffers that contains isopropanol, which aids in the capture of very short DNA fragments, and the salt guanidine hydrochloride, to help attach DNA to special silica filters nestled inside centrifuge tubes. (See illustration below.) In a 2012 *Science* paper, Meyer led an international team of researchers that obtained a high-quality genome sequence from the finger bone of a Siberian Denisovan, an extinct relative of Neanderthals, by capturing, sequencing, and stitching together DNA fragments that were as small as 35 base pairs long. (That bone is estimated to be somewhere between 30,000 and 80,000 years old, but because it is just the tip of a finger, it does not contain enough carbon for dating, and its true age is still debated.)

fossil that is more than 400,000 years old; and in 2013, a 700,000-year-old bone from a Pleistocene horse yielded the oldest complete genome yet. The advent of next-generation sequencing has had the biggest impact on the field, but a few subtler improvements to the extraction and processing of ancient DNA have aided in expanding the temporal limits of sequenceability. Here is an overview of the steps involved in sequencing ancient DNA from the 400,000-year-old hominin bone.

4 SEQUENCE: The library molecules are then amplified via PCR and fed into a next-generation sequencer.

3 PREP A SINGLE-STRANDED LIBRARY: Double-stranded DNA molecules are heat-denatured A, and biotinylated adaptor oligonucleotides are attached to the 3′ ends of each resulting single strand (only one strand shown here) B. Streptavidin-coated beads immobilize the adaptors. Researchers then add an extension primer to the adaptor C to copy the DNA strand D. One strand of a double-stranded adaptor is ligated to the newly synthesized strand of DNA E.





5 ANALYZE: The resulting fragmentary sequences are then reconstructed by matching them to a reference human genome. Ancient DNA stretches are differentiated from modern human DNA in the bone sample by measuring frequencies of cytosine deamination (which appear as Ts in resultant sequences). Those that map to the reference genome are retained and, after passing all the fragmentary sequences over the reference genome, a nearly complete ancient mitochondrial genome is stitched together. Fragments that don't map to the reference genome, for example those from microbial DNA, are discarded.

ANCIENT-DNA TECHNOLOGY MOVES INTO THE CRIME LAB

Sitting locked in bone for millennia is just one way that DNA can become fragmented and degraded. Although not very old, genetic material in biological fluids or tissues left at crime scenes can be similarly damaged and sparse. As a result, some in the forensic science field are calling for the use of the latest paleogenomic techniques in the crime lab. "It makes sense that forensic scientists adopt the protocols that are most effective," says University of Illinois molecular anthropologist Ripan Malhi, "because we're dealing with the same types of issues, which are degraded DNA in low concentrations that is subject to contamination and damage."

Two separate companies, Parabon Nanolabs and Identitas, now offer modern genomic techniques to forensic investigators looking to generate leads in cold cases or instances of missing persons. Both firms utilize microarray genotyping to pinpoint hundreds of thousands of single nucleotide polymorphisms (SNPs) that indicate phenotypic traits such as eye color, hair color, and freckling as well as geographic ancestry. "What our system does is really generate new information just from DNA," says Parabon's director of bioinformatics, Ellen Greytak. "It's like if there were an eyewitness that was telling [investigators], 'This is what that person looked like.'"

Such techniques are still rarely used in state, local, federal, or international crime labs, but they could eventually supplant the standard forensic genetic method—matching DNA to known samples using short tandem repeats (STRs) that serve as a genetic fingerprint to identify suspects. SNPs, because they can be pulled out of shorter and more degraded fragments of DNA, could prove extremely informative to criminal investigators. "I hope that . . . the standards in the forensics space will be informed by innovations in the academic labs," says Cris Hughes, a University of Illinois forensic anthropologist who also works as deputy forensic anthropologist at the Champaign County Coroner's Office.

Because forensic scientists around the world have already compiled STR databases that contain information about thousands of individuals, and because the work can be done relatively cheaply, the move to newer genomic technologies at working crime labs may happen very slowly. Laurence Rubin, Identitas CEO and a practicing rheumatologist at St. Michael's Hospital in Toronto, says that his company is now shifting gears from demonstrating the utility of SNP-based microarrays for analyzing forensic samples to into more of an advocacy role, introducing law enforcement agencies to the power of these genomic technologies.

"Until there's a willingness to adopt this on a larger scale, we and others in the field will be faced with doing exploratory efforts," he says, such as a 2013 study of their proprietary chip that predicted eye color, hair color, and biparental ancestry—with anywhere from 48 percent to 94 percent accuracy—among more than 3,000 blinded DNA samples (*Int J Legal Med*, 127: 559-72, 2013). "The problem right now is that we need to advance the technology with the agencies and groups that can use it the most."

In addition to the DNA extraction and isolation improvements, Meyer and his colleagues accomplished the unprecedented feat by using a homegrown single-stranded library preparation method to ready the DNA for sequencing. Instead of using double-stranded DNA to make genetic libraries that can be fed into a next-gen sequencer, as was standard practice at the time, Meyer and his team first separated the double helix, then prepared the sequence library using each of the single strands, doubling the amount of fragments the group had to sequence. This also had the benefit of circumventing a purification step that leads to the loss of some genetic material in the sample, further increasing the number of precious ancient DNA fragments that could be recovered from such a small and degraded specimen. "One thing that we noticed when we generated the first high-quality DNA sequence is that not only did we get more DNA, but we got much shorter sequences, which had always been lost before during library preparation," Meyer says. "This technique allowed us to achieve a level of resolution that has not been achievable before."

But being able to recover tiny fragments of DNA doesn't change the fact that any sample will contain genetic sequences from more than just the organism of interest. Even with the widespread adoption of handling procedures to minimize the risk of contamination, modern DNA fragments—from humans, plants, microbes, or other organisms—far outnumber the bits of ancient DNA in any centuries- or millennia-old biological specimen. To overcome this dilemma, researchers have turned to clever analytical methods that allow them to differentiate ancient and modern sequences based on characteristic patterns of molecular modification to which degrading DNA is subjected.

Chief among these telltale DNA alteration patterns is cytosine deamination, in which cytosine (C) bases are replaced with uracil (U), a base that normally occurs in RNA. Researchers have established a clear correlation between the postmortem age of a biological sample, the preservation conditions in which the sample was found, and cytosine deamination rates. These patterns of cytosine deamination can help ancient-DNA hunters to dis-

tinguish modern human DNA from Neanderthal sequences, for example, which are otherwise genetically very similar.[11] "[DNA] damage makes your life more difficult, but it also gives you a lot of power," says Orlando.

The fruits of technology

A couple of years ago, as Meyer and his colleagues continued to tinker with ways to recover shorter fragments of DNA, they got their hands on some 400,000-year-old bear bones from the Sima de los Huesos (Pit of Bones) site in Atapuerca, Spain. Applying their new techniques to the samples, the team was able to recover DNA fragments, 95 percent of which were shorter than 50 base pairs, Meyer says. Then, encouraged by their success using the single-stranded library preparation method on the Denisovan fossil, the Meyer lab turned its efforts to another fossil from the same Spanish cave—that of a 400,000-year-old hominin femur—and succeeded in generating a high-coverage mitochondrial genome sequence.[1]

"It's very exciting that you can look directly into the past with ancient DNA," says Rasmus Nielsen, a University of California, Berkeley, computational biologist who studies population genetics using modern and ancient DNA. "We've gotten some big surprises from ancient DNA."

One of the biggest of these surprises involves the spread of lactase persistence, the ability to metabolize the milk sugar lactose, through human populations in Europe. Lactase persistence in Europeans is strongly associated with well-described genetic polymorphisms that confer the production of the lactose-digesting enzyme lactase into adulthood. (Distinct polymorphisms conferring the trait became established independently in parts of Africa, an example of convergent evolution.) In the distant past, humans, like all other mammals today, only produced lactase as young feeding on their mothers' milk; adults were lactose intolerant. But at some point in the species's evolution the 13,910*T allele arose in certain European populations, likely conferring lactase persistence.

Genome analysis of SNP and microsatellite variation among modern European genomes led researchers to propose that the 13,910*T allele swept through European populations sometime between about 7,000 and 10,000 years ago. But in 2011, researchers in Hungary sequenced DNA collected from 23 ancient bone samples—European commoners and Asian conquerors who lived in the 10th and 11th centuries. The DNA sequences revealed that the 13,910*T allele was relatively rare among commoners and completely absent among invaders from Asia, where the modern human population still has a relatively low prevalence of the allele.[12] Then, in 2014, another European research team reported that lactase-persistence alleles were completely absent from DNA they had pulled from the bones of Europeans inhabiting the Great Hungarian Plain between 5,700 BC and 800 BC.[13] Those ancient DNA analyses suggest that the 13,910*T allele swept across Europe much more recently—probably between 3,000 and 4,000 years ago—than researchers had surmised by studying modern DNA and archaeology.

With the sequencing and analysis of ancient DNA, "you have the way here to really make the difference between the two evolutionary scenarios," says Orlando.

Improved analysis of ancient DNA has also led to important revisions in models of early human migration. Last year, Willerslev's group at the Natural History Museum of Denmark sequenced the genome of a 12,600-year-old ancient Native American Clovis boy who lived in what is now Montana. The sequence revealed that roughly 80 percent of all modern Native Americans are direct descendants of the boy's family.[14] It also confirmed that North America was first populated by individuals from Northeast Asia, not Western Europe, as proposed by one contrarian hypothesis. Other recent analyses of ancient DNA samples have led to revisions of hypotheses about the peopling of Europe, the Arctic, and Australia.

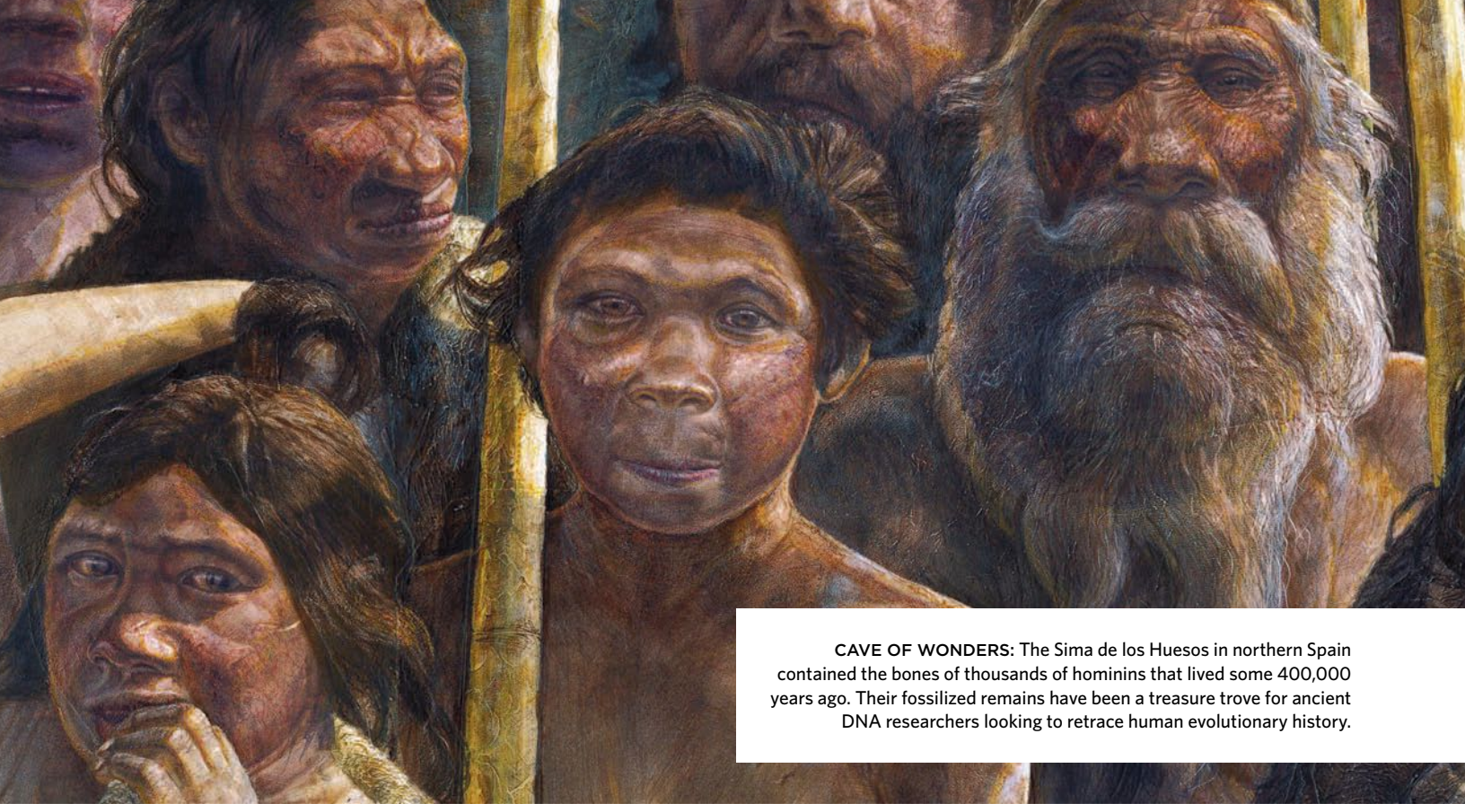
> It's very exciting that you can look directly into the past with ancient DNA. We've gotten some big surprises.
>
> —Rasmus Nielsen, University of California, Berkeley

Going further back in hominin evolution, ancient DNA work has helped to uncover human ancestors that were completely unknown to science. That same finger bone from which Meyer's group generated a high-quality Denisovan genome sequence was first used in 2010 to generate a draft genome sequence of the hominin, which had never before been described.[15] "They discovered something that we think is a new species of humans that anthropology had overlooked for hundreds of years," says Orlando. "You can see how those techniques have big surprises and they can really discover things that are unexpected."

What's next (gen)?

While the past few years have seen a profusion of new and interesting uses of ancient DNA spurred by the rapid improvement of research methods, scientists working in the field agree that the coming years hold even more in store. For one thing, sequencing technologies continue to develop at a breakneck pace, and, according to Willerslev, as third-generation technologies such as nanopore sequencing are applied to ancient-DNA work, researchers will be able to probe even deeper into the biological past. Using a combination of next-gen sequencing and a third-generation, single-molecule sequencer from Helicos Biosciences that can sequence DNA directly without the need for an amplification step, his group sequenced the 700,000-year-old horse DNA that still holds the record as the oldest genome yet sequenced.[4] "This type of technology will be the future," he says.

Researchers are also beginning to apply their newfound skills in dealing with ancient materials to branch out beyond simply sequencing genomes. In 2012, for example, Willerslev's lab published an analysis of proteins, which are generally longer lived



CAVE OF WONDERS: The Sima de los Huesos in northern Spain contained the bones of thousands of hominins that lived some 400,000 years ago. Their fossilized remains have been a treasure trove for ancient DNA researchers looking to retrace human evolutionary history.

postmortem than genetic material, of 43,000-year-old woolly mammoth bones.[16] And last year, Willerslev, Orlando, and colleagues published a genome-wide nucleosome map and survey of cytosine methylation levels in the DNA they pulled from the 4,000-year-old hair shafts of a Paleo-Eskimo, effectively launching the field of ancient epigenetics.[17] Also last year, Pääbo's group at the Max Planck Institute for Evolutionary Anthropology published the first full DNA methylation maps of the Neanderthal and Denisovan genomes.[18] "For the first time we'll be able to address what is the role of epigenomics and epigenetics in evolution," Willerslev says.

But just how far back into biological history will ancient DNA researchers be able to reach? Most scientists feel that recovering sequenceable DNA isn't likely in samples more than 1,000 millennia in age. "I would bet all my money that 1 million is the limit," says Meyer. But some are confident that further improvements to DNA isolation and sequencing techniques could take us even further back. "It would not surprise me if we were able to sequence DNA older than 1 million years given appropriate environmental conditions," Malhi says. Willerslev agrees, speculating that researchers may eventually be able to sequence DNA collected from samples dating to 2 million years ago. "I wouldn't be surprised at all." ■

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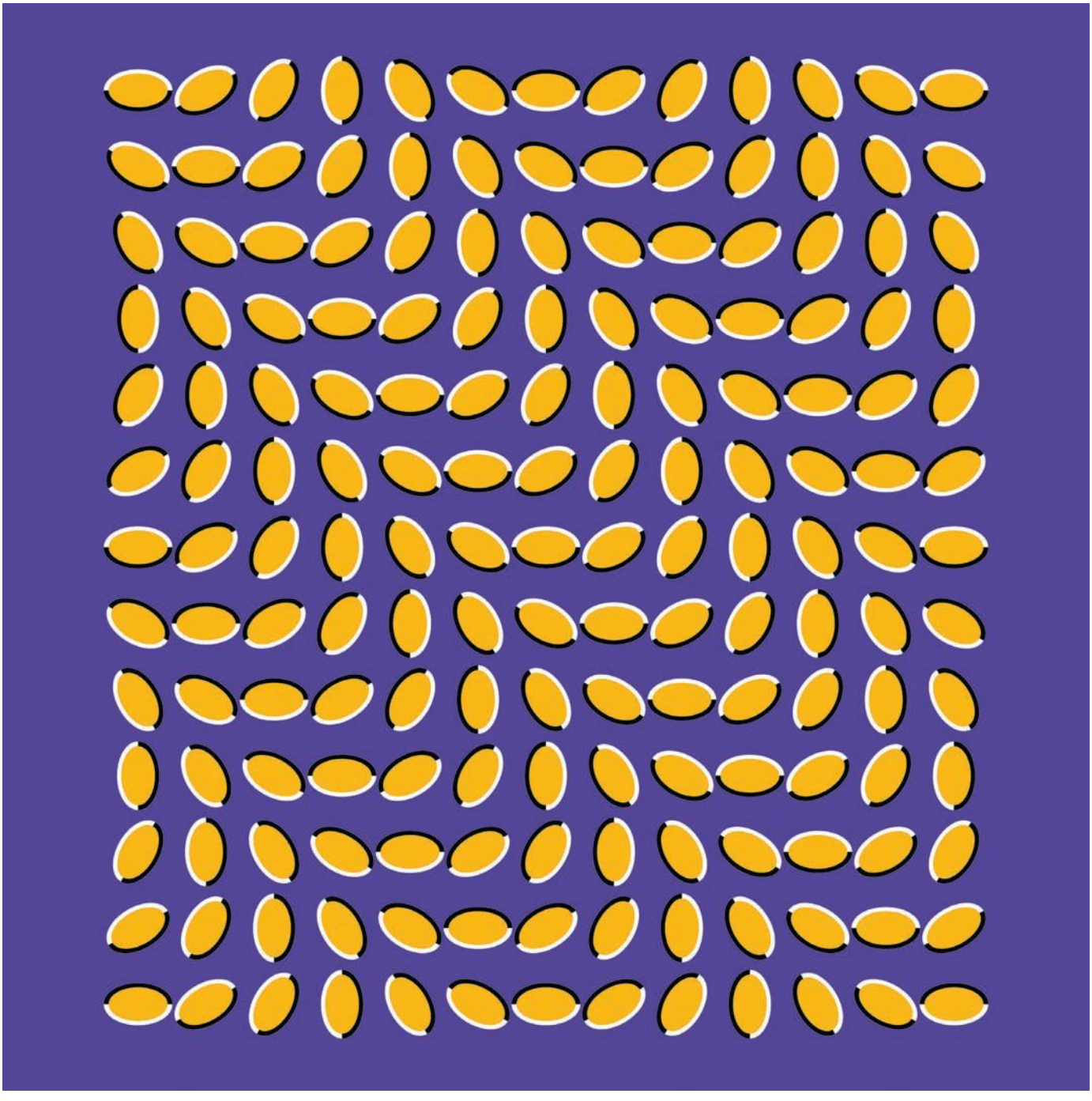
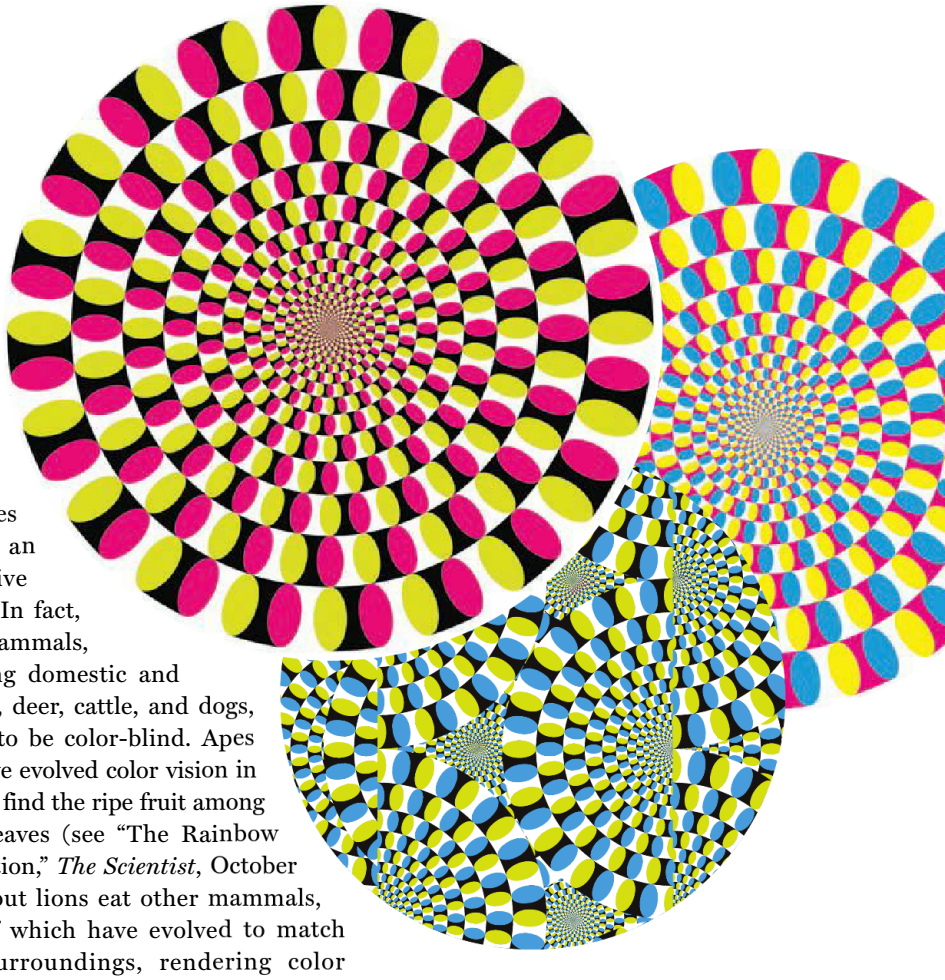
SEEING ISN'T BELIEVING

How motion illusions trick the visual system, and what they can teach us about how our eyes and brains evolved

BY STUART ANSTIS





Animal vision has not evolved as one might think. In contrast to the invention of photography and film—which began with the first black-and-white daguerreotypes in 1839, then added color in 1861, and finally motion in 1891—motion perception in animals appears to have evolved long before color vision. Indeed, as vision researcher Gordon Walls declared in 1942, perceiving motion is one of the most ancient and primitive forms of vision.

Even the humble housefly, which can only distinguish four to six different colors, is remarkably good at seeing motion. Try to swat a fly with your hand, and it will be gone long before you even get close. (The best way is to clap your hands above it so that it flies up between your hands. Wear gloves.) Oddly, however, while a fly is quick to register these fast movements, it cannot recognize slow movement at all. Move your hand very, very slowly toward a fly, and you can tap its back before it knows that you are there.

Much of the early research on motion perception was performed on insects,[1] but similar results have been found for a huge range of species, from fishes to birds to mammals. Frogs, which eat insects, respond to small, rapidly moving prey, as well as to overall dimming or darkening that likely signals an approaching predator, but they often ignore stationary objects, perhaps because they cannot see them.[2]

Mammals are likewise tuned in to motion. Although many people believe that it is the bright red color of the matador's cape that enrages the bull, the popular TV program *Mythbusters* found that the color made no difference; it was the motion of the cape's fabric that mattered. Red, blue, and white capes got equal, halfhearted attacks when they were motionless, but waving the capes elicited an aggressive charge. In fact, most mammals, including domestic and big cats, deer, cattle, and dogs, appear to be color-blind. Apes may have evolved color vision in order to find the ripe fruit among green leaves (see "The Rainbow Connection," *The Scientist*, October 2014), but lions eat other mammals, most of which have evolved to match their surroundings, rendering color vision useless in finding prey. When a gazelle runs away, however, it becomes a strong stimulus for the lion's keen motion vision. It's no wonder that young deer will often freeze when they sense danger. Correspondingly, prey animals would find color vision of little use, but they are extremely good at seeing the motion of an approaching predator.

As good as animals are at detecting motion, they can also be fooled. Seeing the errors that a system makes can help us to understand how that system works normally.

But as good as animals are at detecting motion, they can also be fooled. I study visual illusions of motion because seeing the errors that a system makes can help us to understand how that system works normally. Visual perception goes far beyond our retinal images, which provide only partial sensory information. We use our knowledge and expectations of the world to fill in the gaps, for instance, when an object is partly hidden. Ambiguous illusions that can be interpreted in two different ways, but not both ways at the same time, can also shed light on how we perceive the world around us.

Illusions of movement

Visual movement can be thought of as a change in brightness, or luminance, over space and time. A white spot that glides across a black screen shows real movement. If the same spot jumps back and forth between two positions, or makes a series of intermittent forward jumps, the brain can still perceive movement. Small, fast jumps give the smoothest impression of movement, but even large, slow jumps give a strong impression that the spot is, in fact, moving across the screen.

Why does the visual system treat this jumping dot as a single object in motion, instead of seeing one spot disappear while an unrelated spot appears nearby at the same instant? First, the brain usually treats "suspicious coincidences" as being more

than coincidences: it is more likely that this is a single spot in motion rather than two separate events. Second, the visual system is tolerant of brief gaps in stimuli, filling in those gaps when necessary. This perception of apparent motion is, of course, the basis of the entire movie and TV industries, as viewers see a smooth motion picture when in reality they are simply watching a series of stationary stills.

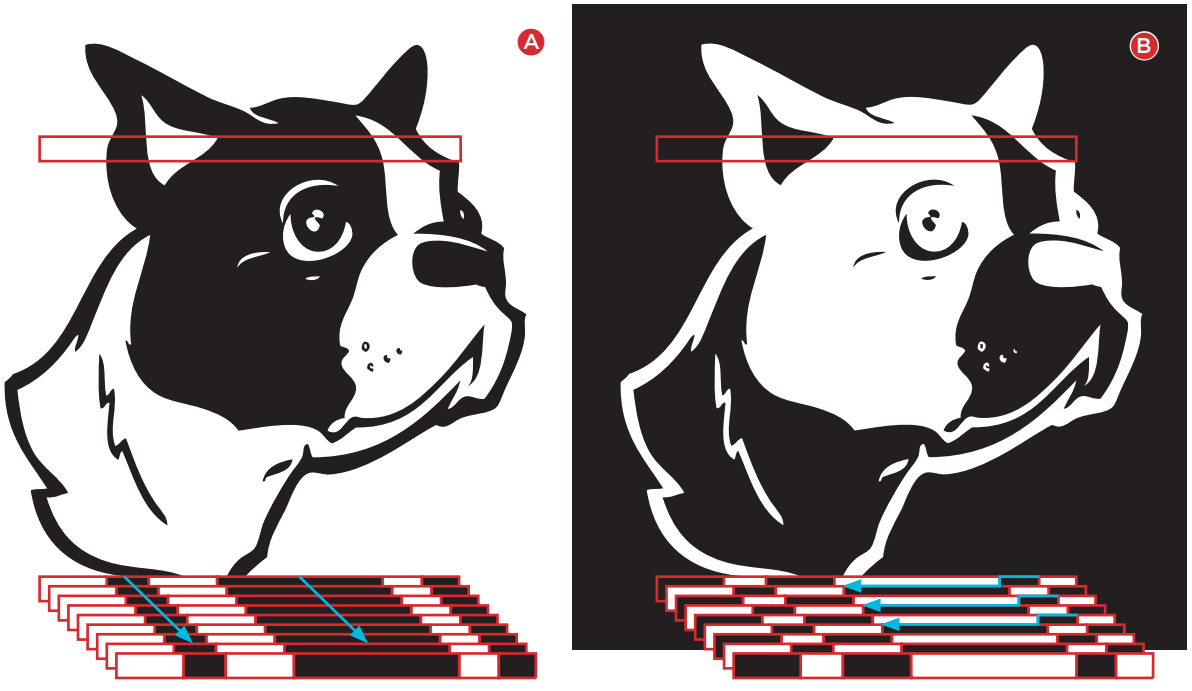
We can pose a riddle to the visual system by presenting two apparent motions in opposite directions simultaneously. For example, an image of a white horse and an image of a black horse suddenly exchange positions. But you do not see each horse independently changing in color. Rather, you see the horses jumping from one location to the other. The coincidence is too great, and, instead of two independent events, the visual system economically infers a single event: the jumping horse. (Watch a video of this visual illusion and many others on the-scientist.com.)

But which horse jumps? The answer depends on the context. On a dark background, the white horse appears to jump back and forth; on a light background, the black horse appears to move. In other words, the horse with the higher contrast wins. This is because the strength of a motion signal in the brain of the observer is equal to the product of the contrast of each horse against the background color, a measure called motion energy.[3] Interestingly, if contrast is held constant, the color of the horses makes no difference because color has little or no input into the motion pathways of the brain.[4]

Contrast can also explain why a black or white object on a background of the opposite color seems to move faster than a gray object on a gray background, and why cars appear to move more slowly in the fog. Indeed, we tend to judge motion not in absolute terms, but relative to the background: the perceived strength and speed of motion depend on the contrast of the moving object against its surroundings.[5] In fact, a driver partly judges his own speed by the rate at which landmarks such as trees flash past him. In the fog, the trees appear slowed down, so he underestimates the speed of all cars, including his own, with potentially disastrous consequences.[6]

Combining movement and changes in contrast results in an even more complex outcome. Suppose that a black spot on a medium-gray background makes a small jump to the right—a total distance much smaller than the diameter of the spot itself—and, at the same time, instantaneously changes to white. Instead of seeing a slight motion to the right, one sees something quite unexpected: the spot appears to move to the *left*, toward the starting position and opposite to the physical displacement.

This effect, known as reverse phi, is particularly strong in peripheral vision: if someone fixes his gaze on a small stationary cross and observes the moving spot out of the corner of his eye, the backwards leap will be even more pronounced.[7,8] Once again, this phenomenon is consistent with the idea that perceived motion depends on motion energy, or the product of the contrasts of moving objects.[3] If the spot makes a long series of jumps to the right, changing between black and white on each jump,

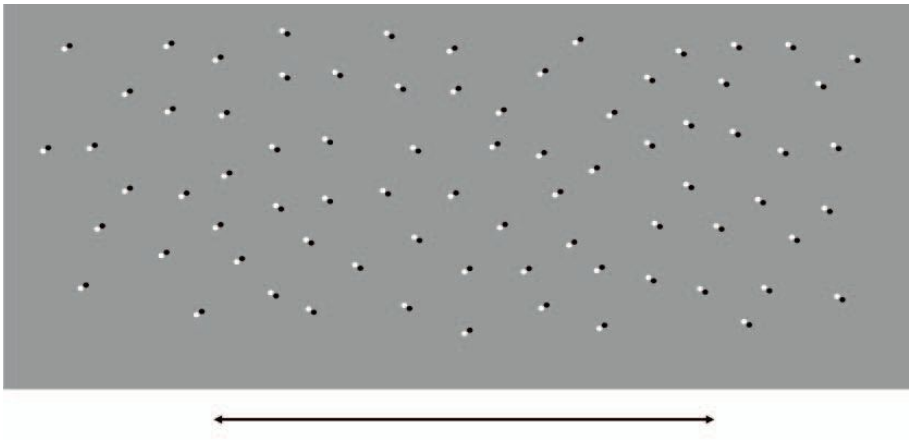


RIGHT OR LEFT: Figure A shows a horizontal slice through a black and white picture, as it moves to the right in successive movie frames. When contrast is held constant, an observer accurately sees movement to the right. But in B, the even numbered movie frames are photographic negative of the odd numbered frames. As a result of the reversing contrast, an observer experiences a visual phenomenon known as reverse phi, and incorrectly sees movement to the left. (Experience a reverse phi illusion in motion at the-scientist.com.)

one still sees steady motion to the left, but after a while the observer will recognize that, paradoxically, the spot is now farther to the right, demonstrating that position and motion are signaled independently.

Why we are fooled

The phenomena described above are "low-level" illusions that are probably based on "bottom-up" sensory signals from brain cells in the visual system that are specialized to detect motion. Normally, sensory information agrees. If a cat is partly hidden behind a tree, for example, all the cues of color, shadows, and texture tell the same story—that the hidden part of the cat exists out of view behind the tree. The brain acts like a judge, confirming the same story as told by independent witnesses. The brain also strengthens this verdict with "top-down" information based upon prior learning: if the cat's whiskers stick out on one side of the tree, and its tail on the other, the brain automatically "fills in" that there is a continuous cat partly hidden by the tree, not two unrelated cat bits. This interpolation process, called visual amodal completion, starts from a representation of the visible features of the stimulus in early visual cortex, probably an area called V1, and ends with a completed representation of the stimulus in the inferior temporal cortex.[9] Jay Hegdé of the University of Minnesota and colleagues even found two regions in the object-processing pathways of the brain that actually responded more strongly to partly hidden objects than to complete ones.[10]

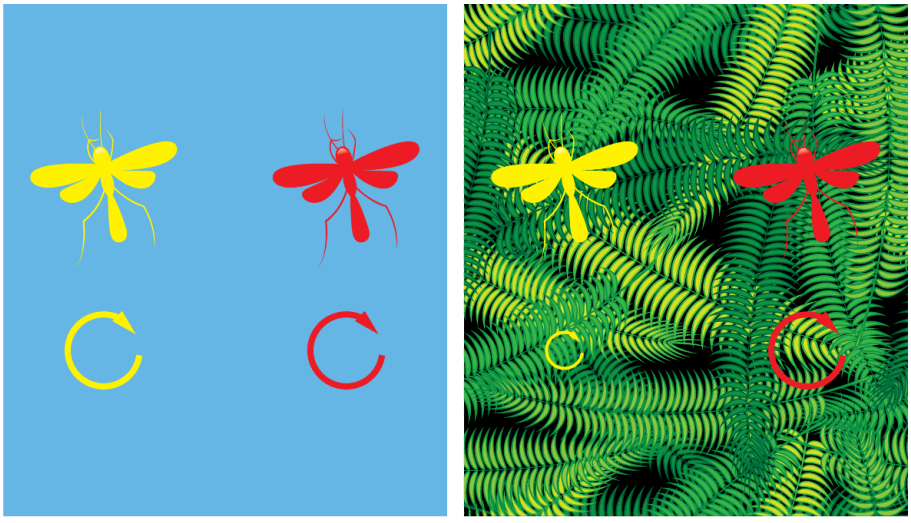


MINIMAL MOTION: The static oblique pairs of dots look random, but in fact are a minimum stimulus for seeing motion. Run your finger from left to right across the arrow at the bottom, following your finger with your eyes, and the dots will almost magically segregate and move up and down.

Visual object recognition thus involves two stages: a bottom-up inputting of perceptual information, and a top-down memory stage in which perceptual information is matched with an object's stored representation. Tomoya Taminato of Tohoku University School of Medicine in Japan and colleagues last year presented volunteers with blurry pictures that gradually became sharper. Observers responded once when they could guess the identity of the object in the image, representing the perception stage, and a second time when they were certain of the identity, the memory stage. Their results attributed the perception stage to the right medial occipitotemporal region of the brain, and the memory stage to the posterior part of the rostral medial frontal cortex.[11]

If a cat's whiskers stick out on one side of the tree, and its tail on the other, the brain automatically "fills in" that there is a continuous cat partly hidden by the tree, not two unrelated cat bits.

Visualizing motion is similarly subject to both bottom-up and top-down processes. Reverse phi, in which an object that changes contrast as it travels is viewed as moving in the reverse direction, is a bottom-up illusion that happens early in the brain's visual processing pathway. Researchers have tracked the origin of this illusion to V1 cells, which in awake monkeys respond to the reverse phi illusion in the same way they respond



MOTION IN CONTEXT: A yellow bug and a red bug both fly around in perfect clockwise circles of the same size, though the red bug moves much more rapidly. When a background is added that also circles clockwise, the yellow bug's orbit, which syncs up with the motion of the background, seems to shrink to about half the size of the red bug's orbit. (Experience this illusion in motion at the-scientist.com.)

to backwards-moving objects.[12] Meanwhile, top-down processes predict what objects these signals probably represent, based upon memory and previous learning. Object parsing, for example, is a process that guides perception by deciding what objects are likely to be present based upon prior knowledge of the world.[13]

Consider the closing blades of a pair of scissors. The intersection itself is not an object; only the blades are. This distinction is not lost on the visual system. Observers make 10 times the tracking errors—their eyes deviating from the target—when they attempt to follow a sliding rather than a rigid intersection.[14] Although you can sense the movement of a sliding intersection, you do not interpret it as an object.

This phenomenon stems from the fact that smooth eye movements require a smoothly moving target. Move your thumb from side to side in front of you and ask a friend to follow your thumb with his eyes. Watch his eyes and you will see them move smoothly from side to side. Now hold up both your thumbs a yard apart and ask him to move his eyes smoothly from one stationary thumb to the other. He cannot do it! You will see his eyes moving in a series of jerky eye movements called saccades. This shows that a moving object is necessary to drive smooth-pursuit eye movements.

Visual signals flow forward from the visual cortex at the back of the brain, then travel along the ventral stream for the decision about what objects are present, and also up along the dorsal stream to the medial temporal area, which analyzes motion. Finally, the nerve signals travel forward to the frontal eye fields that control eye movements. A sliding intersection is not parsed as a real object, and it cannot support smooth eye movements.

The visual system can also flip between local and global motions, but it cannot see both at once. The brain considers incompatible interpretations—Are there many small groups, or a few large groups?—and adopts them in alternation, but never both at the same time. The shape and spacing of spots on a screen, the duration and position of your fixations, and other factors can all influence which percept you see.

Motion can shift an object's perceived position. If an image of an upright cross flashes briefly on a textured wheel that is rotating clockwise, the cross itself will appear to be tilted clockwise, and it sometimes even looks distorted. Notably, only the motion of the background that occurs after the flash can drag the cross along: motion beforehand has no effect.[15]

In sum, illusions teach us that perception goes far beyond the information picked up by our senses. Perception is an indirect, interpretive top-down process that is not driven simply by stimulus patterns, but is instead a dynamic, active search for the best interpretation of the available sensory data. ■

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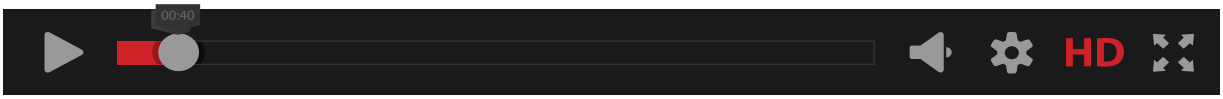

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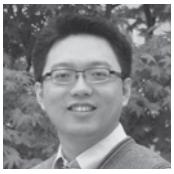

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Bioengineering and Chemical and Systems Biology
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ONDEMAND | Protein Analysis Made Simple

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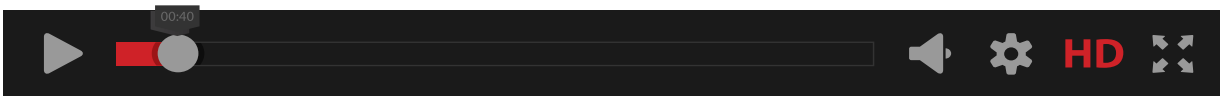

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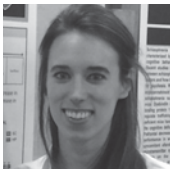

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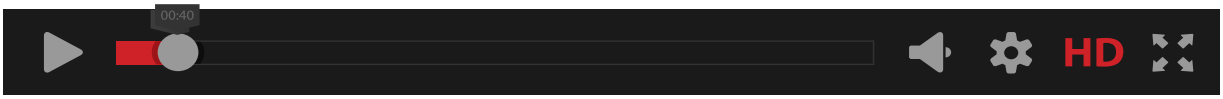
ONDEMAND | Large-Scale Detection of Oncogenic Mutations for Molecular Oncology

Massively parallel sequencing of tumors enables the identification of oncogenomic mutations that correlate with response and resistance to targeted therapies. This webinar presents the path followed by Memorial Sloan Kettering Cancer Center (MSKCC) to develop a hybridization-based capture panel encompassing more than 300 key cancer-associated genes, which they have applied to characterize several thousand tumors. Their translational research efforts are discussed, including examples in which the speaker's group has identified genomic biomarkers predictive of drug response in a variety of tumor types.



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MICHAEL BERGER, PhD
Associate Director, Marie-Josée and Henry R. Kravis Center for Molecular Oncology
Assistant Attending Geneticist, Department of Pathology
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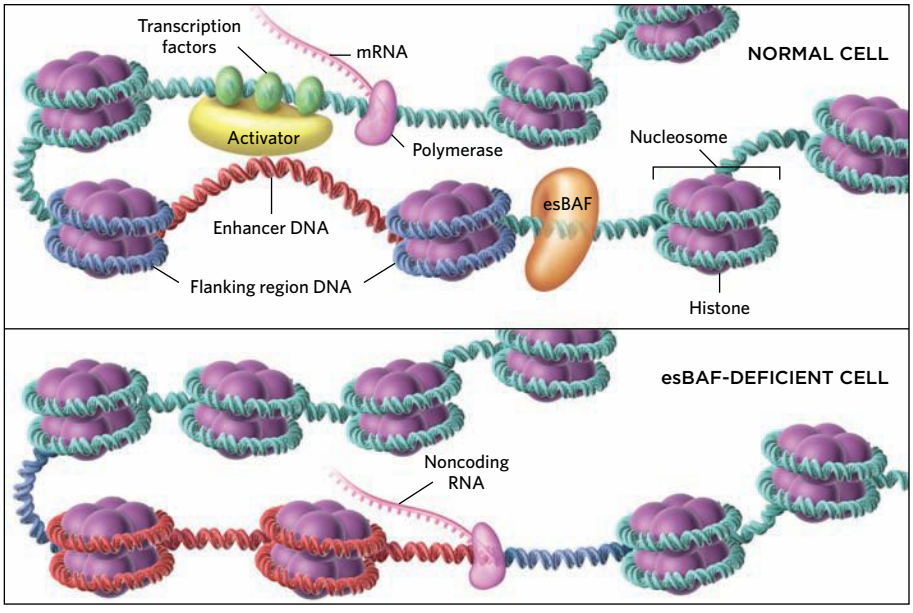
The Literature

GENETICS & GENOMICS

Silencing Surprise

THE PAPER

S.J. Hainer et al., "Suppression of pervasive noncoding transcription in embryonic stem cells by esBAF," *Genes and Development*, 29:362-78, 2015.



BAF BLOCKER: In normal embryonic stem cells (top panel), the chromatin remodeling complex esBAF moves nucleosomes into enhancer-flanking DNA regions (blue) to keep enhancers (red) free of nucleosomes so they can promote transcription of protein-coding genes. When esBAF is absent (lower panel), nucleosomes move out of the flanking regions and into the enhancer, freeing up noncoding RNA transcription that begins in the enhancer and continues into the flanking DNA.

To stuff more than six feet of DNA into the nucleus of each cell in our body, the genome winds tightly around a core of eight histone proteins, creating structures called nucleosomes that, in turn, are densely packed into chromatin. To transcribe genetic information from DNA to RNA, nucleosomes must be shifted out of the way, a task performed in some cases by the embryonic stem cell chromatin remodeling complex esBAF. This process opens up not just the region of DNA to be transcribed, but promoters and enhancers that encourage transcription.

Recent genome-sequencing work has revealed that enhancer regions not only promote the transcription of protein-coding genes, but can also contain the script for noncoding RNAs. Although noncoding RNAs are a hot topic, how they are regulated isn't understood, says Toshio Tsukiyama of the University of Washington.

Thomas Fazzio, a former graduate student of Tsukiyama who is now an assistant professor at the University of Massachusetts Medical School, wanted to understand how the positioning of nucleosomes near enhancers might regulate noncoding RNA transcribed from the enhancer region. "We thought it was almost obvious that if BAF is important for keeping the chromatin structure of lots of enhancers open," it would support the transcription of noncoding RNAs from those genomic stretches as well, he says. Yet when Fazzio and his colleagues sequenced the RNA from esBAF knockdown mouse embryonic stem cells, they found that noncoding RNAs from approximately 57,000 genomic regions were expressed at higher levels than in the control. The effect was not seen in more-differentiated mouse embryonic fibroblasts.

To further explore the unexpected finding, the researchers treated the DNA with an enzyme that only cuts in nucleosome-free regions and sequenced the fragments to determine the nucleosomes' locations. Enhancer regions of esBAF knockout cells were covered by nucleosomes, but there were far fewer nucleosomes in flanking regions, suggesting that esBAF increases the nucleosome occupancy there. Adding DNA sequences that lock nucleosomes in place at both sides of the enhancer repressed the noncoding transcripts, even without esBAF. "I was really impressed that using mammalian cells they actually . . . directly show that it is really the nucleosome occupancy that matters for noncoding RNA regulation," says Tsukiyama, who did not participate in the experiments.

The results point "to the intricacies of how [chromatin remodeling] complexes are used by the cell," says Swaminathan Venkatesh of the Stowers Institute for Medical Research in Kansas City, Missouri, who was not involved in the study. "A complex that has traditionally been viewed to be involved in activating transcription is actually repressing noncoding transcripts."

It's not clear how important the repression of noncoding transcripts is for pluripotency, Fazzio says. There are many long noncoding RNAs implicated in differentiation, says Venkatesh, and "you can imagine that suppressing them in embryonic stem cells is preventing differentiation from taking place." —Jenny Rood

MISNOMER: A peptide (pink, with its predicted binding to a muscle protein) is translated from an RNA thought to have been noncoding.

D.M. ANDERSON ET AL., *CELL*, 160:595-606, 2015; ZHENYU CHENG

CELL & MOLECULAR BIOLOGY

Not So Noncoding

THE PAPER

D.M. Anderson et al., "A micropeptide encoded by a putative long noncoding RNA regulates muscle performance," *Cell*, 160:595-606, 2015.

THE TRANSCRIPT

Eric Olson and his colleagues at the University of Texas Southwestern Medical Center were combing through muscle-specific long noncoding RNAs (lncRNAs) to understand their function when they found one expressed exclusively in skeletal muscle. Although the RNA had previously been categorized as noncoding, its sequence contained a short stretch that looked suspiciously like a coding region.

THE MICROPEPTIDE

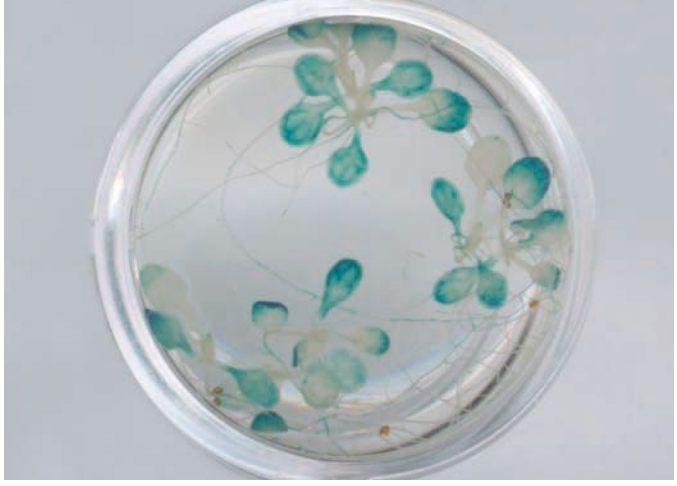
The researchers verified that the RNA encoded a 46-amino-acid micropeptide expressed in vivo that they named myoregulin. Myoregulin forms a membrane-spanning alpha helix similar in structure to two other small proteins expressed in cardiac and slow skeletal muscle, phospholamban (PLN) and sarcolipin (SLN).

THE REGULATOR

PLN and SLN inhibit the activity of SERCA, a membrane protein that pumps calcium out of the cytoplasm and into muscle-cell compartments to help muscles relax. Myoregulin also inhibits SERCA, providing a "dimmer switch" for muscle response to calcium in tissues where PLN and SLN aren't normally expressed, Olson says. Removing the regulator can enhance athletic ability; myoregulin knockout mice ran 31 percent longer than wild-type animals.

THE HUNT

Not all lncRNAs encode small proteins, but "there will undoubtedly be a very large number of tiny peptides encoded by misannotated lncRNAs," Olson says. Already, several hundred of them have been found, and myoregulin's functional similarity to PLN and SLN—despite limited conserved residues—is "a wake-up call," says Antonio Giraldez of Yale University School of Medicine. "[W]e really need to be more aware of looking for conserved regions or looking for translated regions within these noncoding RNAs." —Jenny Rood



RACK ATTACK: *Arabidopsis* leaves respond to a bacterial protease through an immune pathway involving the scaffolding protein RACK1.

IMMUNOLOGY

New Immunity

THE PAPER

Z. Cheng et al., "Pathogen-secreted proteases activate a novel plant immune pathway," *Nature*, doi:10.1038/nature14243, 2015.

THE QUESTION

In his quest to uncover the ways organisms ward off pathogens, Fred Ausubel at Massachusetts General Hospital and his lab had been looking for molecules (referred to by those in the field as MAMPs—microbe-associated molecular patterns) present in the pathogenic bacterium *Pseudomonas aeruginosa* that could spark a response in *Arabidopsis*.

THE ANSWER

What the team turned up was unusual: a *P. aeruginosa*–secreted protease. Other MAMPs known to trigger an innate immune response in plants don't exhibit enzymatic activity. "It was kind of surprising," says postdoc Zhenyu Cheng, who led the study. "So we tried to identify the components involved in detecting this protease."

THE NEW PATHWAY

Again, their findings were unexpected. None of the receptor-like kinases that usually initiate a MAMP response acted as the receptor. Instead, the protease—through an as-yet-unknown event—triggered mitogen-activated protein kinases (MAPKs) via G proteins at the cell membrane. "The involvement of G proteins which appear to be functioning upstream of the MAPK cascade was unique because that's not how it works in the canonical MAMPs pathway," Ausubel says.

What united these signaling components, they figured out, was the scaffolding protein called receptor for activated C kinase 1 (RACK1). The G protein-RACK1-MAPK pathway represents an immune response in plants that had been completely overlooked before, says Ausubel.

RACKING UP ROLES

RACK1 is a widely conserved protein involved in numerous cellular activities. Howard University's Hemayet Ullah, who studies RACK1 signaling in plants, says it's not entirely surprising the protein is involved in plant immunity too. "How this protein remarkably coordinates so many functions is a mystery." —Kerry Grens

PROFILE

Resistance Fighter

Stuart Levy has spent a lifetime studying mechanisms of antibiotic resistance and crusading to abolish the use of antibiotics in animal feed.

BY ANNA AZVOLINSKY

As a visiting research fellow at the Pasteur Institute in 1962, on leave from medical school, Stuart Levy met a Japanese scientist who introduced him to an exciting recent breakthrough by researchers from his country. "The Japanese had discovered that resistance to antibiotics could be transferred from one bacterium to another," Levy says—even across species. "This was unheard of previously. It was the beginning of studies on transferrable drug-resistance genes and infectious drug resistance." Inspired, Levy traveled to Tokyo's Keio University in 1964 and spent several months in Tsutomu Watanabe's laboratory, working on the so-called R (resistance) factors. Watanabe is credited with bringing the topic to a wide scientific audience with the publication of a 1963 review in English, highlighting the results of Japanese research on what he called the "infective heredity" of multidrug resistance.

Levy published several papers with Watanabe, including a description of episomal resistance factors of Enterobacteriaceae and an investigation of methods for inhibiting their transfer. "We didn't know at the time about the mechanism, but we knew it was an exciting moment in the history of antibiotics and resistance," says Levy. "Later, transfer was linked to small pieces of DNA—plasmids—that bore different resistances to antibiotics."

Here, Levy talks about the prank he and his twin brother (Jay Levy, who was among the first to discover the HIV virus) executed that earned them a brief spot in the limelight; how science allowed him to travel the world—and befriend Samuel Beckett; and an urgent call to a castle in Prague about chicken eggs.

LEVY LEARNS

Sunday mornings. As young kids growing up in Wilmington, Delaware, Levy and his identical twin brother Jay used to accompany their father, a physician, on Sunday house calls. "House calls were not that common then, but not as rare as they are now," says Levy. His father, who came from a poor immigrant family, would visit patients, many of whom could only pay him with food grown in their gardens or with services. "He would see the Italian gardener who would exchange Dad's expertise for his fruit. He was brought up under that kind of understanding, and the patients respected and loved him. He would sometimes discuss with us patients he was seeing; that is probably how my interest in medicine began."

All in the family. "My twin brother, sister, and I were all interested in biology. We lived in the country near a farm and spent a lot of time outdoors with the animals. All three of us went to medical school, but, unlike my father, we stayed in academic circles rather than going into private practice. My brother, Jay Levy, and my sister, Ellen Koenig, both do HIV research."

Foray into football. Both Levy and his twin brother wanted to play high school football, but their parents refused, fearing injury. So the head coach took on both boys as coaches. "We got to be near the team and did things like measurements and statistics of the plays. My parents didn't know then what we know now about concussions, because no one talked about that, but they knew it was a potentially dangerous sport," says Levy.

Playing both sides. At Williams College, Levy majored in English. "My brother knew by sophomore year that he wanted to go to medical school; I only made the decision my junior year. But I had lots of interests, namely literature and arts. I loved the fact that I could keep these interests and still go to medical school. When I could do something and not give up another I loved, I was happy," Levy says.

Mistaken identity. After exchanging identities for a day in high school, the Levy brothers took the prank even further in college. As sophomores, the twins swapped identities for an entire week and each wrote an essay about the experience. Stuart lived life as Jay at Wesleyan University and Jay as Stuart at Williams. "This was our first taste of being in the limelight." The brothers had received permission from the presidents of their respective colleges to switch spots, but then played a prank on those authorities—telling them the wrong week for the intended swap. "We wanted to see if we could even fool the presidents and had a few friends help us play along. When classmates suspected a change, we answered by stating our wish to be considered individuals and not a single entity."

LEVY LAUNCHES

Medical school travels. Stuart Levy started medical school at the University of Pennsylvania in 1960. His brother, in medical school at Columbia University, received a Fulbright scholarship and studied at the Sorbonne Institute in Paris. "Our relationship was such that we wanted the other to have what we had, so when my brother was successful in getting a position in Paris at the Sorbonne, he told me, 'You have to do this, it's fantastic to be here on your own! There is never anyone directing you,'" says Levy. The following year, he followed Jay to Europe, first as a research scholar in Milan and then at the Pasteur Institute in Paris. There Levy worked on a model of viral resistance in a mammalian cell line in Raymond

ALONSO NICHOLS/TUFTS UNIVERSITY

STUART LEVY

Professor of Medicine, Molecular Biology and Microbiology, Public Health and Community Medicine
Tufts University School of Medicine
Director, Center for Adaptation Genetics and Drug Resistance
Boston, Massachusetts

Greatest Hits

- Discovered the first active efflux pump involved in tetracycline resistance in Enterobacteriaceae
- Identified the *mar* operon, a bacterial regulatory locus that results in multidrug resistance to different antibiotics as well as to disinfectants
- Provided some of the first evidence that feeding animals low doses of antibiotics leads to high levels of resistant bacterial strains that can spread to other animals, people, and the environment
- Established the international Alliance for the Prudent Use of Antibiotics
- In 1993, published *The Antibiotic Paradox: How Miracle Drugs Are Destroying the Miracle*
- Served as an advisor on antibiotic resistance to multiple organizations, including the National Institutes of Health, the World Health Organization, the FDA, and the Environmental Protection Agency

Latarjet's laboratory. "To tell you the truth, I didn't care what I was doing, I just wanted to have a new experience, and Latarjet was a wonderful mentor. He loved golf, which is what I was raised on. We had wonderful times golfing together at dusk," Levy recalls.

For the love of literature. "When my brother was first in Paris, he met Samuel Beckett. Jay had written his thesis at Wesleyan on Beckett and sent it to Sam's address, which everyone said you could not get through to. Beckett liked what [Jay] had written. Beckett was not a snob, he was shy. So Jay introduced me to Beckett when I was in Paris and every four to six weeks we would have lunch together in the Latin Quarter. I'd tell him what we were doing in the laboratory, and he would share with me accounts about the production of his new play. It was such a unique opportunity."

Tangents. Levy did his residency in medicine at Mount Sinai Hospital in New York City. While there, he spent much of his free time working in the laboratory of Charlotte Friend, a microbiologist who had discovered a virus that caused a leukemia-like disorder in mice. "She took me under her wing—I was always looking for something else to do other than look at pathology slides. Jay did the same. We weren't interested in the status quo. We did what was needed to get the degree, but also pursued our own interests," says Levy. Although his clinical focus was officially hematology, Levy continued to pursue his interest in antibiotic resistance. "I was so interested in infectious diseases that I used to go on the rounds with the infectious-diseases group in addition to my regular clinical duties."

A system to call his own. Levy became a staff scientist at the National Institutes of Health (NIH) in 1967, working for two years in Loretta Leive's lab on synthesis of the lipopolysaccharide that populates the outer membrane of *E. coli*. As an independent researcher on R plasmids and chromosomeless minicells, Levy developed a way to purify large amounts of these *E. coli* minicells, which form from an aberrant cell division site and possess no bacterial chromosome—what he calls the plasmid-in-minicell system. "The NIH brought me together with senior scientists in the field, but no one was interested in tetracycline resistance. They wanted to understand enzymatic resistance," says Levy. In 1970, Levy demonstrated that the tetracycline resistance gene is found on plasmids that are transferred to minicells.

Mechanism of resistance. In 1971, Levy moved to the Tufts University School of Medicine as an assistant professor of medi-

cine and of molecular biology and microbiology; he has remained ever since. There, his lab went on to show that an R plasmid encoded a protein associated with tetracycline resistance and that no other positive regulation was required for the bacterium to synthesize this protein. "There could be several hundred genes on the plasmid, and in the 1970s, we were not that sophisticated yet to identify the specific gene," says Levy. In 1978, his lab determined that the plasmid-derived resistance to tetracycline involved a novel transport system for tetracyclines. Levy's lab then discovered the first active efflux mechanism, showing that *E. coli* resistant to tetracycline actively pumped the drug out of the cell and that this mechanism of resistance was encoded by a single R-plasmid gene. Levy also showed that a nonefflux mechanism was present as well. Others subsequently demonstrated that this second mechanism for tetracycline resistance involved a ribosome protection protein. "The use of minicells and the discovery of the mechanism of tetracycline resistance is what really put me on a clear path to a successful career," says Levy.

LEVY LEADS

Ahead of his time. The Animal Health Institute of New York asked Levy to study growth-promoting antibiotics in farm animals. "They were looking for scientists who had not spoken negatively about this use of antibiotics," says Levy. Still a young investigator, Levy fit the bill. His lab found a farm outside of Boston that was willing to have scientists come in and raise chickens. Levy's students raised 150 control and 150 experimental chickens fed regular and tetracycline-spiked feed, respectively. "There is a funny story about me at a castle in Prague and not remembering that I had placed an order for 300 eggs, one-half male and one-half female. Someone was looking for me all over the castle so that I would confirm [over the phone] that we should order the eggs anyway. There is no way to identify if eggs are male or female!" The study, published in 1976 in the *New England Journal of Medicine*, showed the ecological effects of feeding farm animals low-dose antibiotics: not only did the antibiotic-resistant bacteria replace the microbiota in the animals' intestines, they also altered the gut microbiome of the humans who lived and worked on the farm. Through contact with the chickens and their tetracycline-laced feed, resistance was in turn transferred to the microbiome of the animal handlers. Levy's lab also demonstrated that animals can transfer antibiotic-resistance plasmids to humans and other animals. "That low-dose antibiotics given as growth promotion will lead to high levels of resistance was a surprise," says Levy. "No one has tried to replicate that study to this day."

Multidrug-resistance find. Levy's laboratory also identified a chromosomal operon found in different bacterial species that results in drug resistance to different classes of antibiotics, including tetracyclines, penicillins, and fluoroquinolones. "[The discovery] was serendipitous. I was trying to get a chromosomal mutant to tetracycline, and when the bacteria grew, they were multidrug resistant from the start, which meant there was a single locus that controlled multidrug resistance and that emerged with selection from a single drug exposure," says Levy.

"That low-dose antibiotics given as growth promotion will lead to high levels of resistance [in humans] was a surprise. No one has tried to replicate that study to this day."

Antibiotics and politics. Levy has testified many times before Congress on the subject of antibiotic resistance. "Our study from 1976 was [and still is] the only prospective U.S. study on this, and industry didn't want more studies. They were upset that our data showed them to be wrong. This was highly political." Levy says he is now more optimistic about prudent antibiotic use, as this issue has garnered more and more attention, especially since this past March when the White House announced a national action plan, allocating $1.2 billion to combat antibiotic-resistant bacteria. "I think the moment has come for new antibiotics and better use of antibiotics so that people are not as subject to resistance emerging through animal use of other drugs. We won't see real change until there is a genuine commitment to improve antibiotic use, and I think it's coming."

Banding together. In 1981, Levy founded the Alliance for the Prudent Use of Antibiotics (APUA), an international nonprofit with chapters in 65 countries. The idea started at a meeting in the Dominican Republic in the early 1980s because of concern about rising antibiotic resistance in the developing world. The organization provides funding for countries in the developing world to study antibiotic resistance. "I've learned a lot from being part of the APUA. The science is one thing, but you need to package the science with good politics to get what you want," says Levy.

Up to the challenge. "We did the first study that took a patient-by-patient analysis of resistance in a [single] Chicago hospital and what the cost was," says Levy. The analysis showed a cost of about $21,000 per antimicrobial-resistant infection patient, producing a cost to the hospital of about $4 million and a total societal cost of as high as $15 million including the loss of productivity. "[The study] came from a challenge that Ted Kennedy gave me. He said that if you are not going to save money you won't get much interest in [antibiotic resistance], and we took him up on the challenge."

Dream experiment. If money were no object, Levy says, he would design an experiment that would definitively and quantitatively demonstrate the link between subtherapeutic use in animals and the emergence of antibiotic-resistant infections in people.

Influential mentor. "I think the biggest training I received was with Watanabe, and that was just for a summer! He was patient, methodical, and a master." ■

William Greenleaf: Born for Biophysics

Assistant Professor, Department of Genetics, Stanford University. Age: 35

BY ANNA AZVOLINSKY

William Greenleaf has been at the forefront of life-science tool design since grad school, building a new type of DNA sequencer and pushing genomics technologies to unprecedented limits.

"Biology is becoming more of a playground for cross-disciplinary work, which is very exciting, because in the end, human biology is incredibly complicated," says Greenleaf. "Now we finally have the tools to match some of that complexity."

As a Harvard University physics major, Greenleaf applied his training to biological questions. During the summers, he worked in the laboratory of Julio Fernandez, then at the Mayo Clinic in Rochester, Minnesota, using atomic force microscopy to detect structural changes in proteins at the single-atom level.

Greenleaf went on to do a PhD at Stanford University in biophysicist Steven Block's lab. There, the young grad student was able to measure, at the single-molecule level, the energy and forces required for RNA polymerase transcription termination.[1]

Using an optical trapping system they developed, Greenleaf and fellow graduate student Elio Abbondanzieri also measured the movement of RNA polymerase along DNA, from one base to the next, to within one diameter of a hydrogen atom.[2] The work "is arguably the world's record holder for the most-sensitive displacement measurement ever made on a single protein," says Block.

This technique led Greenleaf and Block to demonstrate that base pair–resolution measurements can be used to sequence DNA directly.[3]

"Never before had a graduate student in my lab managed the perfect trifecta—publication of a paper in *Nature, Science,* and *Cell;* a quadfecta, actually, since he also published a paper in the top physics journal, *Physical Review Letters,*" says Block. "For a biophysics researcher, that is as good as it gets."

Greenleaf joined Sunney Xie's chemical biology laboratory at Harvard as a postdoc. Along with graduate student Peter Sims, Greenleaf built a prototype of a novel DNA sequencer that combined two previous technologies—terminal phosphate-labeled nucleotides and resealable microreactors.

Now in his own lab back at Stanford, Greenleaf is taking apart old DNA sequencers to rebuild them for new high-throughput genomic assays and studying chromatin at the single-cell level. Greenleaf's lab has developed a relatively quick method to identify open chromatin regions genome-wide using hundreds- to thousands-fold fewer cells than required by current technologies.[4] The method, says Greenleaf, opens the door to epigenomic analyses on clinical timescales: just one day.

Greenleaf is showing that single-molecule methods can be scaled to measure thousands of molecules at a time, says Block. Howard Chang, a genomics researcher at Stanford University who collaborated with Greenleaf on the open chromatin assay, agrees. "What makes him unique is his strong physics bent—he always brings quantitative thinking to scientific problems and has a good intuitive sense of the single-molecule level and how that scales," says Chang.

"I look forward to something extraordinary from him in the future," adds Xie. ■

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- time-resolved fluorescence
- temperature control

detect and identify

Flow Cytometry On-a-Chip

Novel microfluidic devices give researchers new ways to count and sort single cells.

BY JEFFREY M. PERKEL

Originally an immunology tool and a fixture of cell biology research for decades, flow cytometry allows users to catalog a dozen or more molecular and physical features in a single cell. A related, more sophisticated instrument, the fluorescence-activated cell sorter (FACS), enables researchers to isolate particular cells from heterogeneous populations.

These instruments have had a profound impact on biological research. Yet there is much they cannot do. For instance, with some exceptions, they can only count or sort cells based on their protein content. Thus, researchers cannot easily use the technique to identify cells that contain, say, specific mutations. Researchers also have to know in advance what molecules they are looking for, and have fluorescently labeled antibodies available to target them. And because the method depends on antibody binding to surface receptors, there's always the possibility that sorted cell populations will be activated or altered by the process itself.

"For the most part, flow cytometry is used when you have a good affinity assay—an antibody or oligomer probe that can specifically label your cell type," says Adam Abate, an assistant professor of bioengineering and therapeutic sciences at the University of California, San Francisco. "And that is a very small set of all possible assays you may want to do."

These days, researchers are developing novel strategies to explore these possibilities. But they aren't modifying traditional flow sorters to do so; they are downsizing their assays onto novel microfluidic devices. These miniaturized labs-on-a-chip enable investigators to enumerate and sort cells based on a wider range of physical and molecular properties than ever before, often without antibodies. *The Scientist* spoke with four researchers about the strategies they use for microfluidic cell sorting and evaluation.

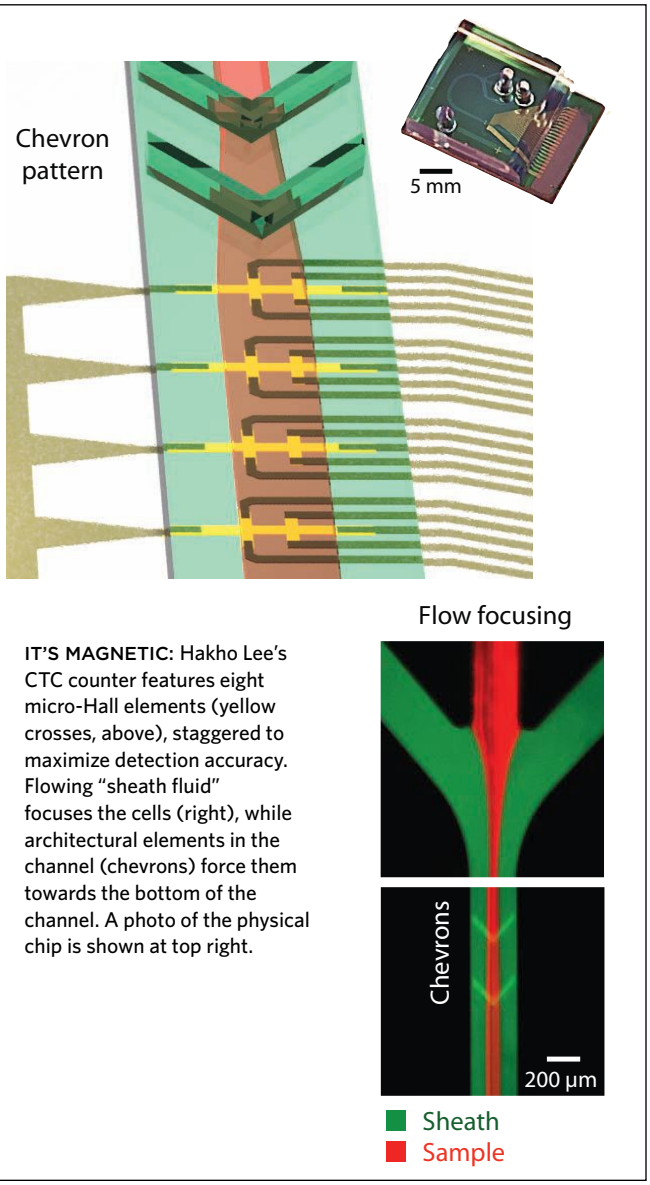
MAGNETIC COUNTING

RESEARCHER: Hakho Lee, Assistant Professor of Radiology, Harvard Medical School

PROJECT: Enumerating and characterizing circulating tumor cells

PROBLEM: Circulating tumor cells (CTCs) are incredibly rare, with just a handful per milliliter of human blood.

SOLUTION: Lee's group fabricated a hybrid microfluidic device out of polydimethylsiloxane (PDMS) that can count CTCs in real time using tiny sensors called micro-Hall detectors. (*Sci Transl Med*, 4:141ra92, 2012)



IT'S MAGNETIC: Hakho Lee's CTC counter features eight micro-Hall elements (yellow crosses, above), staggered to maximize detection accuracy. Flowing "sheath fluid" focuses the cells (right), while architectural elements in the channel (chevrons) force them towards the bottom of the channel. A photo of the physical chip is shown at top right.

A micro-Hall detector, Lee explains, records the magnetic field strength of an object—in this case, a cell studded with manganese-doped ferrite nanoparticles—based on the electrical current it induces. The device comprises a flow channel lined with eight micro-Hall detectors and structural features that force the cells towards the channel bottom (i.e., near the sensors). As the tagged

D. ISSADORE ET AL., *SCIENCE TRANSL MED*, 4:141RA92, 2012.

cells flow down this channel, these sensors "ping," Lee says, and counting those pings measures the CTC population. Because the strength of each ping "is proportional to the number of nanoparticles per cell," the signal also indicates antigen abundance, the equivalent of measuring fluorescence intensity in flow cytometry.

But while fluorescence-based flow cytometry can detect multiple colors simultaneously, magnetic sensors are "gray-scale;" they can measure only one marker at a time. Lee's team circumvented that problem by using nanoparticles of different sizes, each of which produces a distinct signal and in this way quantified three CTC biomarkers simultaneously. When applied to 20 blood samples from ovarian cancer patients, the system achieved a diagnostic accuracy of 94 percent.

SENSORS 2.0: Lee's team has developed a separate device for CTC isolation that is based on cell size rather than on magnetism (*Adv Healthcare Mater*, 1:432-36, 2012). For cell counting, though, the team is migrating away from microfluidics and towards solid-state microchips, he says. "Controlling the cells such that they can fly over the [micro-Hall] sensors is very hard," he explains. Lee envisions a two-dimensional sensor array that applies the micro-Hall principle but without the flowing liquid, like a digital camera for magnetic fields. "We can increase the number of sensors," he says, "and the data-acquisition part could be automated."

PCR-ACTIVATED SORTING

RESEARCHER: Adam Abate, Assistant Professor of Bioengineering and Therapeutic Sciences, University of California, San Francisco

PROJECT: Analysis of rare, uncultivable microbes

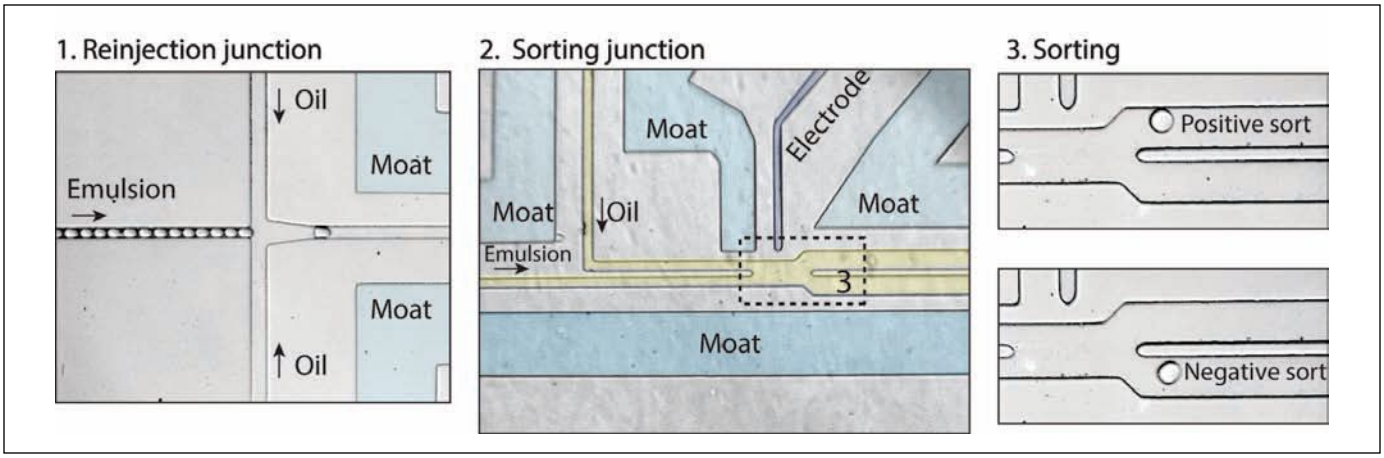
PROBLEM: Developing specific antibodies for bacteria that cannot be cultured

SOLUTION: As a postdoc in the Harvard University lab of droplet-based microfluidics pioneer David Weitz, Abate codeveloped a device that could sort droplets according to their fluorescence intensity. Unlike traditional microfluidics, in which molecules and cells flow naked through channels, droplet-based devices encapsulate molecules or cells in uniform, picoliter-scale aqueous reaction chambers encased in oil.

To solve his uncultivable microbes problem, Abate adapted that design to sort cells based on their genetics—a process his team calls "genomic cytometry." Microbiologists, Abate explains, often want to identify cells with particular skill sets, such as the ability to metabolize perchlorate, an environmental contaminant. But they have no easy way to pick them out. "With conventional FACS, it's almost impossible to sort cells by nucleic acid content," he says. PCR, in contrast, "is trivially scalable; all you have to do is change the primers and probes and you can change out which cells you're targeting."

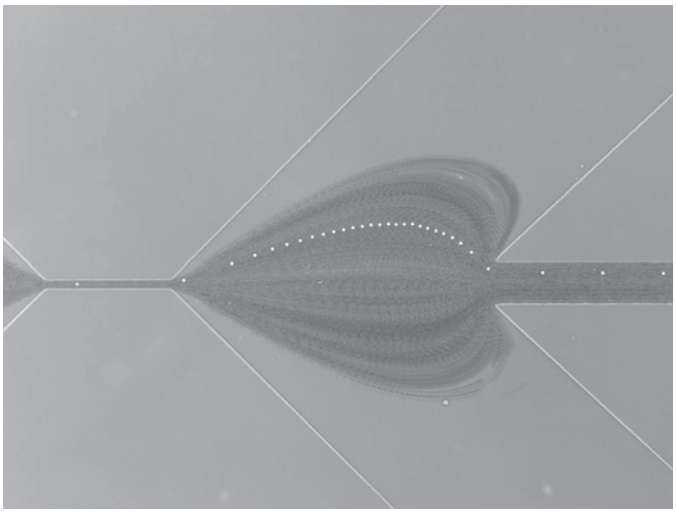
His solution is PCR-activated cell sorting (PACS). Each droplet contains a single cell, PCR reagents, primers targeting the sequence of interest, and a fluorescent TaqMan probe. Successful amplification of the cellular DNA produces a fluorescent signal, which is detected in a microfluidic circuit that uses a laser to interrogate each droplet and then sorts based on the results. In a recent proof-of-principle experiment, Abate's team sorted a heterogeneous population of *E. coli* that differed in the ability to synthesize the protein TolA, achieving 90-fold enrichment. (*PLOS ONE*, 10:e0113549, 2015)

TRY PCR + FACS: Researchers can duplicate his chip design, Abate says, but they'll also need the microscope, software, lasers, and electronics to run it—an investment of at least $40,000, he estimates. An easier approach, he says, is to create the PCR droplet emulsion and sort those droplets in a FACS machine (*Lab Chip*, 13:4563- 72, 2013). "There are commercially available droplet generators," Abate notes. But you'll need one capable of generating double emulsions, such as those from Dolomite Microfluidics. "FACS doesn't work with oil," he explains. "It requires a water droplet inside an oil droplet, suspended in water." To the flow cytometer, "it looks like a cell."



DROP SORT: As tightly packed droplets enter the PACS device, they are mixed with oil to space them out (left) prior to reaching the sorting junction (middle, dashed box). Positive and negative sorting events, controlled by the electrode, are shown at right.

S.W. LIM ET AL., *PLOS ONE*, DOI:10.1371/JOURNAL.PONE.0113549, 2015



GUCK LAB AND STARTUP PROJECT ZELLMECHANIK DRESDEN

SQUISHY CELLS: Blood cells flow at 10 cm/s from right to left through a real-time deformability cytometry (RT-DC) device. Sheath flow from the upper and lower right focuses the cells in the narrowest part of the channel, causing the formation of heart-shaped streamlines as illustrated here by an inverted overlay of multiple frames. Highlighted is the track of the only white blood cell among a thousand red blood cells.

SORTING BY CELL DEFORMABILITY

RESEARCHER: Jochen Guck, Alexander von Humboldt Professor, Technische Universität Dresden

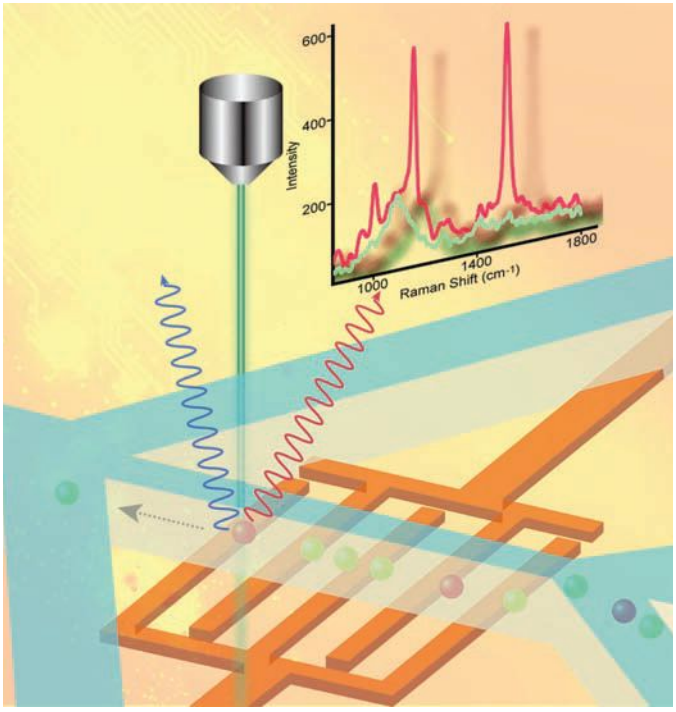
PROJECT: Cancer cell phenotyping

PROBLEM: Not every cell type has a known antigen that defines it. Plus, antibody binding may activate receptors, potentially changing the cell's behavior.

SOLUTION: A physicist by training, Guck used laser beams in his graduate work to study the physical properties of cells. Not all cells are equally squishy, he found: while normal cells are relatively rigid, cancer cells are more pliable. "The more aggressive the cell, the softer it is, and that may be necessary for it to pass into tissues," Guck explains.

He suspected it might be possible to sort otherwise identical cells based on this phenomenon—and to do so without antibodies. Some interesting cell types and cancers have no known unique molecular identifiers, Guck notes. But deformability is an "inherent property" of cells, enabling label-free cell analysis. "The cell cannot help but have that property."

Researchers had already developed one approach called deformability cytometry (DC), in which flowing cells smash head on into a wall of flowing liquid, deforming like crash-test dummies. Using an ultrahigh-speed camera, the method monitors 1,000 cells/second, but only for a few seconds, as the size of the data sets quickly become unwieldy. For some rare cell types, that isn't enough for statistically reliable numbers. And as the analysis isn't in real time, sorting is out of the question.



CATCH & RELEASE: In Raman-activated cell sorting, cells are trapped in an electric field and probed with a laser to determine their Raman signature. The cells are then released, and positive cells are collected downstream using a suction-based mechanism.

Guck's alternative is real-time deformability cytometry (RT-DC), a microfluidic method that is lower throughput, but instantaneous and continuous (*Nat Methods*, 12:199-202, 2015). "As the cell deforms, we analyze that before the next image is recorded," Guck explains. "That means we can continuously analyze samples, [and] just build up data until we have enough." Those cells theoretically could be sorted, he adds, assuming the team added the necessary circuitry.

RT-DC induces deformation by flowing cells into a narrow channel. Although the channel is wider than the cells, they deform because they "feel" the channel walls, Guck says. That causes the cell periphery to slow relative to its center, forming a bullet-like shape. Guck's team quantifies that change by computing the ratio of cell area to cell circumference, a metric called circularity.

Using RT-DC, Guck's team showed that $CD34^+$ cells from peripheral blood and those from bone marrow—which cannot be distinguished using FACS—differ somewhat in deformability (the bone marrow cells are slightly squishier). Similarly, they were able to identify the different cell types in whole blood, no antibodies required.

DIY: Replicating the RT-DC device is possible, but neither straightforward nor trivial, Guck says. "You would need a very skilled postdoc," not to mention the chips, the camera, the algorithm, and the syringe pumps required to assemble and operate the apparatus. He estimates a cost of "probably 100,000 euros, plus a microscope." Members of Guck's team are forming a company (ZellMechanik Dresden) to commercialize the method.

RAMAN-ACTIVATED CELL SORTING

RESEARCHER: Jian Xu, Professor and Director, and Bo Ma, Group Lead of Microfluidics, Single-Cell Center, Qingdao Institute of Bioenergy and Bioprocess Technology, Chinese Academy of Sciences

PROJECT: Microbial biofuels development

PROBLEM: Biofuels R&D requires identifying cells capable of specific carbon chemistries. But as these cells have yet to be cultured and studied, researchers have few if any molecular hooks for identifying and sorting them.

SOLUTION: The team turned to a label-free method of single-cell interrogation known as Raman-activated cell sorting (RACS) (*Anal Chem*, 87:2282-89, 2015).

Raman spectroscopy captures a sample's molecular fingerprint. When a specimen is illuminated with a laser beam of a specific wavelength, most of the light bounces back at that same wavelength, as with a mirror. A very small fraction, though, does not, reflecting the sample's molecular composition. This is called Raman scattering.

"Raman gives you global information about the cell—nucleic acids, proteins, lipids, starch, pigments, everything—all at the same time," Xu says. Such information can help researchers identify which cells in a microbial consortium fix carbon dioxide, degrade cellulose, or produce starch or oil, for instance.

Ma and Xu developed a microfluidic circuit and instrument that captures, holds, and interrogates single cells for anywhere from milliseconds to seconds, however long is necessary to acquire and interpret the Raman signal. "For most cells, it's less than a second," Xu says, and up to four cells per second is possible—a substantial improvement over previous efforts, which required up to three minutes per cell.

Cell signals are compared to a database of Raman signatures to identify positives. That, in turn, activates a suction-based sorting mechanism.

Xu and Ma used the system to sort a heterogeneous yeast population, only some of which produced carotenoids, producing an eight-fold enrichment. Now they are working to apply their system to such problems as identifying antibiotic-resistant bacteria, sorting stem cells, and selecting better biofuel producers.

USER ACCESS: According to Xu, two microfluidic RACS systems have been installed in China, and two more are in the works. A third is being installed at Oxford University. Users will be able to request time on any of these instruments, Xu says. "Any question is welcome. We have funding to support this kind of collaboration." ■

COURTESY OF PEIRAN ZHANG AND MINGMING SUN

An Array of Options

A guide for how and when to transition from the microarray to RNA-seq

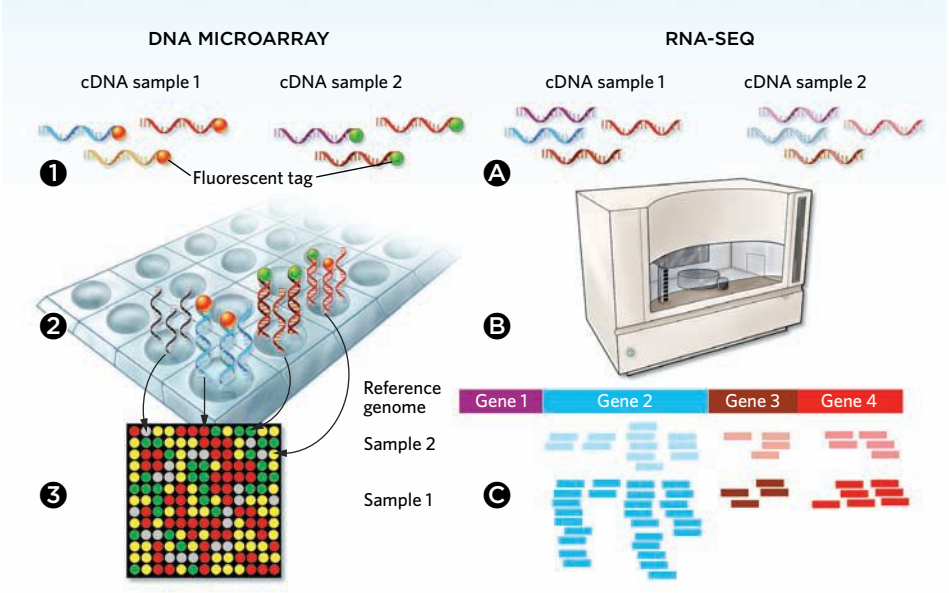
BY KATE YANDELL

For 20 years, DNA microarrays have been the go-to method for revealing patterns of gene expression. But as the price of next-generation sequencing continues to fall, RNA sequencing (RNA-seq) has become an increasingly popular method for assessing transcriptomes.

A DNA microarray consists of a predetermined assortment of nucleic acid probes attached to a surface. To assess gene expression, researchers derive complementary DNA (cDNA) from cellular RNA, label the cDNA with a fluorescent marker, wash labeled cDNA over the array, and use lasers to assess how much cDNA has stuck to each probe. RNA-seq relies on converting RNA into a cDNA library and then directly sequencing the cDNA.

While learning how to deal with the raw data RNA-seq produces can be tricky, RNA-seq has capabilities that microarrays lack. The technique can reveal previously uncharacterized transcripts, gene fusions, and genetic polymorphisms, while microarrays only pull out transcripts that researchers explicitly fish for. With sufficient sequencing depth, RNA-seq can also detect high-abundance and low-abundance transcripts more effectively than microarrays can.

Scientists are voting with their feet. The revenue that Affymetrix, a leading microarray producer, received from the gene expression portion of its business decreased from $104.5 million in 2012 to $73.4 million by 2014, according to the company's 2014 annual report. In 2009, nearly all NIH funding to grants in their first year concerning gene expression went to projects using microarrays, according to an analysis by Dave Delano, senior product manager for gene expression and regulation at Illumina. By 2013, microarrays' share had fallen to approximately one-third of new funding.



MEASURING EXPRESSION: DNA microarrays (above, left) consist of nucleic acid probes affixed to a surface. First, RNA is extracted from samples and converted into complimentary DNA (cDNA), which is labeled with fluorescent tags ❶. Next, labeled cDNA fragments hybridize with the nucleic acids on the array ❷. Scanning the microarray measures the fluorescence level at each spot, revealing levels of gene expression ❸. In RNA-seq, RNA is also extracted from samples, fragmented, and converted into cDNA in preparation for sequencing Ⓐ. Next, the cDNA library is sequenced Ⓑ. The resulting reads are mapped to the genome and gene expression is quantified Ⓒ.

But due to the ease of using microarrays to analyze large numbers of samples rapidly, the technology continues to dominate RNA-seq in terms of sheer numbers of samples analyzed. Weida Tong, director of bioinformatics and biostatistics at the US Food and Drug Administration (FDA) National Center for Toxicological Research in Jefferson, Arkansas, notes that, in 2014, data from more than 54,000 samples analyzed via arrays were deposited into the Gene Expression Omnibus (GEO) database, compared to data from just around 9,000 samples analyzed using RNA-seq (*Genome Biol*, 15:523, 2014).

Eventually, the research community will fully switch to RNA-seq, Tong says. Until then, microarray and RNA-seq data need to be more compatible and the data analysis and storage for RNA-seq must become easier. "This is just giving birth," Tong says. "It's painful, but once the process is finished, the community can enjoy this technology."

Here, *The Scientist* discusses the transition from microarrays to RNA-seq, when researchers should make the switch, and strategies for making the process as painless as possible.

A WHOLE NEW WORLD

For applications such as exploratory work or research using nonmodel organisms, RNA-seq is a clear winner because it reveals transcriptomes without bias, uncovering novel splice junctions, small RNAs, and even novel genes that micro-

arrays simply miss. (See "Transcriptomics for the Animal Kingdom," *The Scientist*, July 2013.)

"Unlike microarray probes, RNA sequencing does not require a priori sequence knowledge of the sample for analysis," Kevin Poon, global product manager of gene regulation at Agilent Technologies in Santa Clara, California, writes in an e-mail to *The Scientist*. "In this way, it is an ideal platform for discovery research; obtaining the absolute sequence of transcripts enables the discovery of mutations and fusion transcripts." Agilent produces both microarrays and RNA-seq tools.

Mariano Alvarez, a graduate student in the lab of Christina Richards at the University of South Florida (USF) in Tampa, studies how the 2010 Gulf oil spill has affected gene expression in the hexaploid salt marsh grass *Spartina alterniflora*. Alvarez and his collaborators started out using microarrays to assess gene expression in oil-exposed versus nonexposed plants. But for a new project surveying gene expression in invasive populations of Japanese knotweed *(Fallopia japonica)*, the researchers are including RNA-seq data, in hopes of better understanding how expression of gene variants and isoforms differ in different habits.

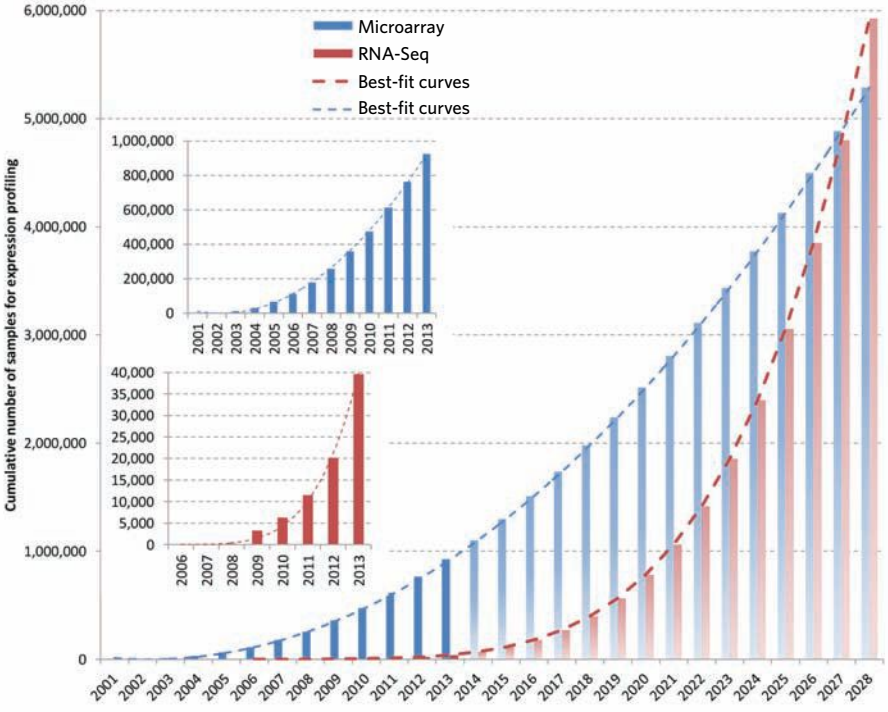
RNA-seq has also been valuable for exploring the uncharted regions of even well-studied species' transcriptomes. For instance, in December the University of Toronto's Benjamin Blencowe and his colleagues used a novel RNA-seq computational method to demonstrate altered transcription patterns of tiny snippets of DNA called microexons in different brain tissues and in people with autism versus controls (*Cell*, 159:1511-23, 2014).

Researchers who switch to RNA-seq are often "seeing dimensions of the biology they just weren't picking up in microarrays," says Anup Parikh, a senior product manager at Thermo Fisher Scientific, which sells RNA-seq tools through its Ion Torrent brand.

IMPRESSIVE RANGE

RNA-seq is also the right choice for researchers hoping to detect transcripts expressed at very low abundance, according to some scientists. Last year, the FDA's Tong and his colleagues used both Illumina's RNA-seq platform and Affymetrix microarrays to assess changes in gene expression in rat liver samples following chemical treatments (*Nature Biotechnol*, 32:926-32, 2014). The researchers found that, for the more abundant half of the differentially expressed genes, the two platforms were in near-complete agreement. For the less-expressed genes, RNA-seq was more accurate. Other studies support this conclusion (*BMC Bioinformatics*, 14:9, 2013; *PLOS ONE*, 9:e78644, 2014).

The main reason for the difference is that when transcript levels are low the fluorescence emitted by those cDNAs bound to a probe in a microarray can be so low that it is outcompeted by background fluorescence. RNA-seq, meanwhile, can detect increasingly lower levels of transcripts the higher the coverage used, with no hard bottom limit. The same is true at the top end of gene expression. For highly expressed genes, microarrays can become saturated.



DATA RACE: While RNA-seq is growing in popularity, the bulk of samples are still analyzed using microarrays. FDA's Weida Tong and his colleagues predict that the cumulative number of samples analyzed using RNA-Seq deposited in the Gene Expression Omnibus Database will overtake the cumulative number of microarray-analyzed samples in 2028.

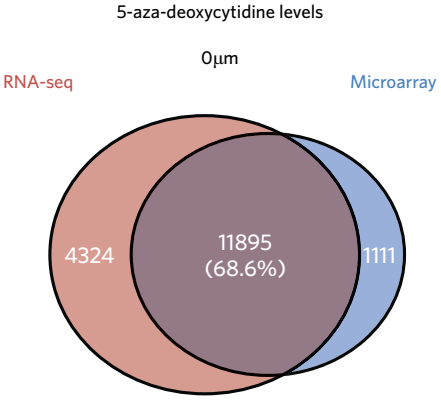


FIGURE S1, *GENOME BIOLOGY*, 15:523, 2014;
FIGURE 1, *BMC BIOINFORMATICS* 14(SUPPL 9):S1, 2013

STAYING POWER

Despite RNA-seq's strong points, many researchers continue to use microarrays, particularly for research involving large numbers of samples. And microarrays shine in clinical studies, as data can be turned around quickly and easily. "Microarrays provide highly consistent data and use well-established analytical pipelines," says Poon. "From analyzing hundreds to thousands of samples, gene and miRNA expression signatures have been developed with clinical diagnostic value."

"I'll always do microarray," says Kirk Mantione, head of molecular biology for the fledgling mitochondrial therapeutics company MitoGenetics based in Farmingdale, New York. "I know how to do it already, and the results are more easily interpreted."

Mantione uses microarrays to assess the effects of drugs he is developing on gene expression in cell lines and in animals. Microarrays can quickly and easily tell him how compounds affect specific genes. However, Mantione also hopes to begin using RNA-seq to study underexplored organisms or to look for previously undetected polymorphisms in the transcripts he studies.

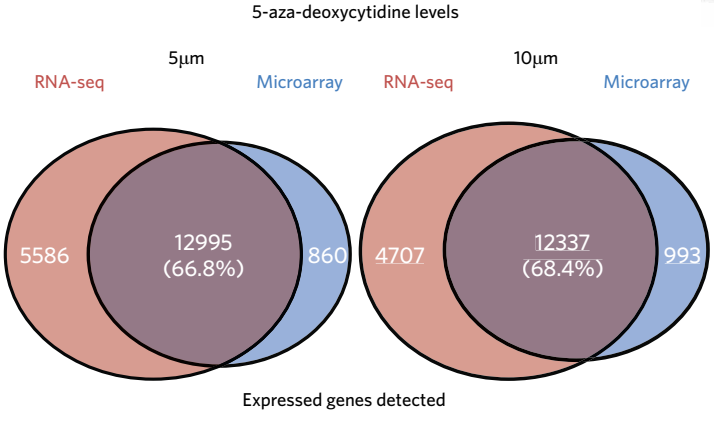
Affymetrix suggests that some researchers may want to use microarrays to quickly screen large numbers of samples and then use the results to guide their RNA-seq projects. Or, microarrays could be used to validate RNA-seq data. And sometimes people simply continue to use microarrays because they want to compare new data with previously gathered data, which is easier if all of the data are produced the same way.

FALLING COSTS

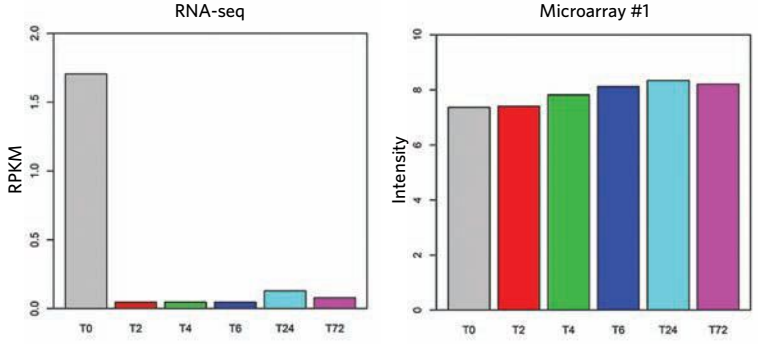
Despite the decreasing costs of sequencing, the expense of microarrays versus RNA-seq remains a factor for some projects. Microarray analyses cost as little as $100 per sample for standard gene-expression analysis and $300 per sample for more-complex analyses involving differentiation between variant splice forms, according to Affymetrix. (This estimate excludes extra fees from service providers.) The cost of RNA-seq is more variable, as it depends on number of reads, read length, sequencing technique, number of samples that fit into a single run, and familiarity with the species being sequenced, among other factors.

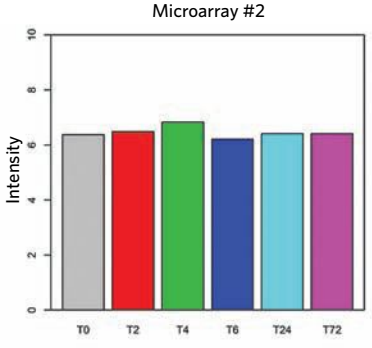
"It's definitely possible to get the lowest cost sequencing to be at least equivalent to the cost of a microarray," says Shawn Baker, chief science officer and cofounder of AllSeq, an online sequencing marketplace where individuals, core facilities, and companies can sell their services to users. "It's just that most people don't do that. Instead of really trying to drive the cost down as much as possible, they try to get more out of the data." They want to look at low-abundance genes, or novel gene fusions or splice junctions, for instance, which drives the cost up.

Baker recently analyzed prices of RNA-seq projects commissioned via the AllSeq marketplace. He found that if he normalized prices to projects with 10 million reads, or DNA fragments sequenced per sample, the average RNA-seq project cost was quoted at approximately $565 per sample. However, many research-



SUBSTANTIAL OVERLAP: Researchers treated colon cancer cells with different quantities of a DNA methylation inhibitor called 5-aza-deoxycytidine in order to induce changes in gene expression. They then analyzed gene expression in the cells using either Affymetrix microarrays or RNA-seq using the Illumina platform. Regardless of treatment, the two methods detected the same subset of expressed genes the majority of the time. But RNA-Seq detected more expressed genes overall than did microarrays.





GET LOW: When researchers profiled the transcriptomes of T cells as they activated, they found that Illumina RNA-seq did better than Affymetrix microarrays at detecting changes in expression of genes expressed at low levels. Here, RNA-seq shows that the levels of the gene *MYCL1* drop 32-fold as T cells are activated (above). Two different microarrays (above right and right) detected no change in the expression of this gene over the same time range.

PLOS ONE, DOI:10.1371/JOURNAL.PONE.0078644, 2014.

ers do more than 10 million reads, which could drive up the cost considerably. Affymetrix estimates that RNA-seq costs anywhere from a few hundred to a few thousand dollars per sample.

Researchers will also need to take into account how time-consuming data analysis and storage will be. According to Affymetrix, microarray data can be translated into a usable format nearly immediately through free software provided by the company, while RNA-seq analysis can take weeks or even months. "This massively reduces the overall costs for each microarray project relative to RNA-seq," Affymetrix says.

But Tong says that, in many types of studies, such as toxicological evaluations, the difference between the price of RNA-seq and microarray analysis has become negligible. Researchers analyzing huge numbers of in vitro samples are more likely to find that price differences add up.

DATA DELUGE

Perhaps the largest obstacle for researchers trying out RNA-seq is the tsunami of data that the process produces. For one study comparing microarrays and RNA-seq, each Affymetrix array produced 5 megabytes of raw data. RNA-seq on the same immune-cell sample using 100 million reads—admittedly extraordinarily high coverage—produced using the Illumina system generated a staggering 23 gigabytes of raw data per sample (*PLOS ONE*, 9:e78644, 2014).

While methods for analyzing microarray data are fully mature and straightforward, there is no consensus on which pipelines—or series of computational steps—to use to analyze RNA-seq data. Riki Kawaguchi, a senior bioinformatics scientist at the University of California, Los Angeles, says he often compares the performance of several pipelines on each data set he analyzes for collaborators. "There's no one best package you can use for every data set," he says.

"Once you have basic programming skills, it's pretty straightforward to use existing pipelines to clean up your data and to get it into the format you need . . . and then either to upload into a cloud service or analyze it using the software you have in the lab," says USF's Alvarez. "I think it is just a time commitment; if you're used to using microarrays, you just sort of have to bite the bullet and dive in."

Illumina's Delano adds that last year the company introduced applications in BaseSpace, a computing environment for sequencing data analysis and management, that should also make RNA-seq easier for the average user. These applications allow researchers to select and apply various popular pipelines to their data.

While methods for analyzing microarray data are fully mature and straightforward, there is no consensus on which pipelines—or series of computational steps—to use to analyze RNA-seq data.

GETTING HELP

One way to ease into the RNA-seq era is to outsource the process. Researchers can pay a core facility or sequencing service provider to carry out library preparation and sequencing. Baker of AllSeq says that researchers should probably only purchase and use their own sequencing machines if they know they will frequently need maximally fast turnaround, if they have enough samples to keep their machine running constantly, or if they're doing something very unusual and experimental with their sequencing technique. Science Exchange and Genohub are other companies that will connect researchers with service providers.

Alternatively, Thermo Fisher recently came out with a service that attempts to make RNA-seq a little more similar to microarrays. For researchers just hoping to sequence known transcripts, the company last August launched AmpliSeq Transcriptome, a library preparation kit and sequencing workflow that reverse transcribes and amplifies 20,000 human RNA transcripts at once using PCR. Researchers then sequence the resulting library using the Ion Proton sequencer and use Thermo Fisher software to convert data to a format that should be recognizable and manageable to researchers familiar with microarrays.

"If what you really care about is gene expression of the known transcripts within your experiment, AmpliSeq Transcriptome will give you exactly that at a much lower cost and complexity than the whole RNA-seq method," Parikh says. The cost of the Ion AmpliSeq Transcriptome Human Gene Expression Kits ranges from $65 to $104 per assay, depending on whether researchers buy the 24-, 96-, or 384-assay kits.

While researchers using AmpliSeq will not gain information about novel transcripts, they will profit from some of RNA-seq's benefits, such as good ability to detect transcripts expressed at especially high or low levels. AmpliSeq is also well-suited for preparing samples with low RNA abundance and quality, such as preserved patient tissues.

Beyond the AmpliSeq Transcriptome kit, Thermo Fisher also offers customized AmpliSeq panels allowing researchers to assess chosen transcripts. Illumina, meanwhile, offers TruSeq Targeted RNA Expression Kits, which similarly allow researchers to assess expression of a subset of genes, isoforms, gene variants, or other features whose expression most interests them. And Agilent SureSelect allows researchers to select targeted regions of the transcriptome for sequencing. Using SureSelect, researchers can detect novel transcripts and polymorphisms in their targeted genomic region.

The benefit of sequencing only a limited portion of the transcriptome is that the resulting data output is less overwhelming than full RNA-seq results. Thermo Fisher's Scott Dewell, a product manager at the company, says for the "everyperson," it is much easier to simply get the spreadsheet with expression values that AmpliSeq produces. "You could e-mail the results to your collaborator," Dewell says. ■

Clinical Matchmaker

Enrolling the right patient population could be key to a successful clinical trial.

BY KATE YANDELL

In late 2011, the outlook for AstraZeneca's cancer drug Lynparza (olaparib) was grim. After an interim analysis revealed disappointing results in a Phase 2 trial, the company ceased development of the ovarian cancer drug. While the oral medication did delay disease progression by a median three and a half months, there was no significant effect on overall survival (*N Engl J Med*, 366:1382-92, 2012), so the company decided not to pursue Phase 3 trials.

But a closer look at a subset of the Phase 2 trial participants convinced AstraZeneca researchers to forge ahead after all: for patients with BRCA mutations, the drug had delayed progression by nearly twice as long as the overall patient population (*Lancet Oncol*, 15:852-61, 2014). Last December, based on further data regarding the drug's efficacy in patients with BRCA mutations who had undergone chemotherapy at least three times, the US Food and Drug Administration (FDA) granted accelerated approval to Lynparza for this subgroup, contingent on the success of continuing Phase 3 trials.

The trials and tribulations of Lynparza's path to market are not unique. Patients suffering from any disease are a heterogeneous group, and traditional clinical trials that simply average the effects of drugs across all participants can muddy the results, sometimes missing positive effects in a subset of patients. But in recent years, improved ability to collect and analyze genetic and proteomic data has researchers increasingly interested in developing targeted therapies only for those patients who are likely to benefit. According to a recent FDA white paper, 45 percent of FDA approvals in 2013 were for targeted therapies requiring genetic or other tests to determine which patients would benefit from them or be able to take them safely, compared with just 5 percent in the early 1990s. In January, the Obama administration announced a $215 million Precision Medicine Initiative that will fund agencies such as the National Institutes of Health and the FDA to develop and evaluate targeted therapies.

Parsing patient heterogeneity and shepherding targeted therapies into the clinic come with unique challenges, however. For example, as biomarkers become more complex and numerous, researchers will have the hard task of deciding how to divide the patient population. "As we move forward, it is going to be increasingly difficult to do trials, because soon every cancer patient will have an orphan disease," says Donald Berry, a professor of biostatistics at the University of Texas MD Anderson Cancer Center in Houston. Greater stratification of the population also means a smaller market, making it hard for companies to recoup the billions of dollars it costs to develop a drug.

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Meanwhile, analyzing drugs' effectiveness in treating precise subpopulations requires new trial designs. Researchers must avoid statistical pitfalls while navigating still-evolving FDA requirements for both demonstrating therapeutic efficacy and validating accompanying diagnostic tests. Below, *The Scientist* examines how clinical researchers are adapting clinical trial design in this new era of precision medicine.

Rethinking recruitment

Drug companies have historically conducted clinical trials out of a few major medical centers, recruiting a new set of patients for each therapy under investigation. But because specific biomarkers are often present in just a small percentage of the population, recruiting participants from a limited area is not a sustainable model for companies pursuing precision medicines.

Instead, investigators testing targeted therapies are increasingly turning to collaborative efforts to recruit patients nationwide, or even internationally.

The US National Cancer Institute (NCI) last year established the National Clinical Trials Network, composed of a group of hospitals and medical centers in North America that will serve as trial sites. Not only will the network make it easier to recruit specialized subsets of patients to cancer therapy trials, it will also expand the potential patient pool to rural areas of the country. "We have no option but to be more collaborative," says Lisa McShane, a biostatistician at the NCI. "It's good for everybody." Later this year, the NCI will use the new network to launch its NCI-MATCH (Molecular Analysis for Therapy Choice) trial, which will sequence patients' lymphomas and advanced solid tumors and place participants in appropriate trials.

In late 2010, the nonprofit Cancer Research UK founded a similar initiative, called the Stratified Medicine Programme (SMP), which takes advantage of a network of 18 cancer centers across the United Kingdom. The SMP's first trial, called the National Lung Matrix Trial, began in March 2015 and will assess the efficacy of eight drugs in development at Pfizer and AstraZeneca for treating non–small cell lung cancer. The researchers will target the therapies to 18 different genetic abnormalities determined by a gene panel developed by Illumina. "The Matrix Trial is . . . an umbrella program which basically screens large numbers of patients to generate the numbers necessary to go into what are often quite small cohorts," explains Gary Middleton, head of the new trial and a professor of medical oncology at the University of Birmingham.

The European Prevention of Alzheimer's Dementia (EPAD) consortium will apply the same wide-reaching strategy to construct a 24,000-patient registry of people from across Europe judged to be at risk for dementia. From this registry, EPAD researchers will select 6,000 people for closer monitoring, based on factors including as-yet-undetermined biomarkers that put them at the highest risk of dementia progression, then funnel 1,500 at a time into a clinical trial, projected to launch in 2016. By targeting preclinical patients and designing trials that will test multiple therapies for different dementia subtypes, the EPAD aims to overcome over a decade of high-profile dementia drug failures, says EPAD coordinator Craig Ritchie, who studies the psychiatry of aging at the University of Edinburgh. "EPAD is an attempt to rip up the rule book completely."

Each of these large-scale trial networks, while resource intensive to set up, could cut down on the legwork required to launch clinical studies in the future, seamlessly feeding patients into trials testing novel therapies and identifying new biomarkers. "The whole project is to create a needed infrastructure in order to be able to have this perpetual trial," José Luis Molinuevo, a national lead of EPAD based at the Barcelonaβeta Brain Research Center in Spain, says of the Alzheimer's initiative.

Creative trial design

In some cases, there is such strong evidence that a treatment will only benefit a subset of patients that it would arguably be unethical—never mind a waste of time and resources—to test it on biomarker-negative patients. Other times, however, researchers do choose to trial targeted therapies on relatively broad patient populations to confirm that a treatment works in the way that preclinical research has suggested. And in some cases, the therapies can have surprising effects in nontarget patient populations, says NCI's McShane. "If you lock in too early it's very difficult to go back."

Genentech's Herceptin (trastuzumab), for instance, is a monoclonal antibody that binds to HER2 growth receptors on cancerous cells, preventing them from undergoing uncontrolled proliferation. Assuming the therapy would help the 20 percent of breast cancer cases where tumor growth is enhanced by an overabundance of HER2 receptors, researchers primarily tested it in these patients. But after the drug had been approved, researchers uncovered some results that made them wonder if Herceptin might be useful in a broader range of patients than previously thought.

The original trials of Herceptin as a treatment for metastatic breast cancer tested HER2 levels in a central laboratory. But a later trial investigating the drug's use as a secondary therapy to prevent recurrence relied on multiple local labs to measure HER2 levels. In a reanalysis of samples after the later trial had concluded, the researchers found that 10 percent of participants identified as having elevated HER2 by the local labs had been misclassified. The more rigorous test revealed that, while HER2 was in some cases overexpressed, the receptor levels were below the cutoff to be considered HER2-positive in these patients. Nevertheless, many of these patients had benefited from trastuzumab therapy. Researchers are now testing the efficacy of trastuzumab combined with chemotherapy in a Phase 3 clinical trial of breast cancer patients whose tumors have only slightly elevated HER2 levels.

As we move forward, it is going to be increasingly difficult to do trials, because soon every cancer patient will have an orphan disease.

—Donald Berry,
University of Texas MD Anderson Cancer Center

To capture all patient populations that might benefit from a therapy, there is a new type of trial, the so-called adaptive trial, that enables researchers to start out testing multiple groups but change course based on interim results. Midway through a study, researchers can increase sample size for subgroups of patients who are doing particularly well on a therapy, for example, or adjust a treatment regimen to more thoroughly investigate doses that appear to be working best, explains MD Anderson's Berry, who in 2000 cofounded Berry Consultants to help companies design adaptive clinical trials. "The usual thing is you do treatment A versus treatment B and close your eyes, and at the end of your study open your eyes and look at the data, and you're surprised almost always with what you see," says Berry. "Sometimes you say, 'Gee, things are happening that I could have taken advantage of.'"

Of course, there's a reason trials have traditionally been blinded: to avoid bias. To maintain that same scientific rigor in adaptive trials, adjustments are predetermined at the beginning of the study as a series of branching if-then situations, and cannot be tweaked based on researchers' whims once the trial is underway.

Currently, Berry is co-principal investigator for the I-SPY 2 trial, a government, academia, and industry collaboration to test combinations of targeted therapies for their ability to shrink breast tumors prior to surgery in patients with 10 different biomarker combinations. Therapy-biomarker pairings that do poorly will eventually be eliminated from the study, while those that appear to be working will trigger the assignment of more patients with the relevant biomarkers to the treatment group. In this way, researchers can screen a wide variety of therapies for efficacy in diverse patients, without spending the time and money they would to run a separate study on each therapy. Therapy–patient group combinations that continue to do well will "graduate" from the study and may be tested independently in Phase 3 trials. One combination of two agents, carboplatin and veliparib, that was evaluated in the study is now being tested in a Phase 3 involving patients with triple-negative breast cancer, i.e., breast cancer negative for elevated estrogen, progesterone, and HER2 receptors.

A number of other trials, including the National Lung Matrix Trial and the EPAD Alzheimer's trial, will also have adaptive designs. But adaptive trials are still new and require caution, says Berry. "The bane of our approach is false positives. We're looking at lots of drugs; we're looking at lots of subsets of patients. When you look at lots of things, you see some things that aren't real."

Making it to market

Researchers seeking to develop targeted therapies need to start thinking early about their communications with regulatory bodies such as the FDA. The agency has in recent years issued guidelines for dealing with adaptive trials, noting that it is particularly important that investigators provide a thorough explanation of how they will ensure that the study will remain blinded even as the trial is adjusted based on intermediate outcomes. Typically, this requires designating people who are largely independent from the primary personnel to assess interim results and make prespecified adjustments.

It is also possible that sponsors of trials involving particularly unfamiliar adaptive designs may need to plan for extra discussion with FDA statisticians to assure that their plan will pass muster. "If a sponsor is considering the use of an adaptive design for a trial intended to support registration, then the sponsor should discuss this plan with [the FDA] early in the protocol development stage," Lisa LaVange, director of the agency's Office of Biostatistics, writes in an e-mail to *The Scientist*.

In addition, it is important to talk to the FDA early about any diagnostic tools that will need to accompany a new therapy. Historically, the FDA has exercised discretion in scrutinizing diagnostics developed in individual labs. "Unlike therapies, which go through a very rigorous regulatory process and get approval by the FDA to be marketed, for laboratory tests the situation has been quite different," McShane says. But last fall, the agency released draft guidance stating that it plans to become stricter about validating even familiar tests. With the rise of targeted therapies, "the [test] result matters," says McShane. "It's going to make a difference in what treatment the patient gets." ■

Reimagining Humanity

As the science of paleoanthropology developed, human evolutionary trees changed as much as the minds that constructed them.

BY IAN TATTERSALL

Before the mid-1900s, human fossils were for the most part studied and pronounced upon by specialists in human anatomy. Based in medical schools, these researchers were exquisitely attuned to variation within *Homo sapiens*, but were largely unconcerned with the riotous diversity of species out there in the living world. Ignorant of taxonomic norms, they branded newly discovered hominin fossils with new Latin names, much as they gave each of their children a separate name. In this way, throughout the first half of the 20th century the rapidly expanding paleoanthropological literature became littered with formal names—even though many of the freshly unearthed fossils actually belonged to species that had already been described.

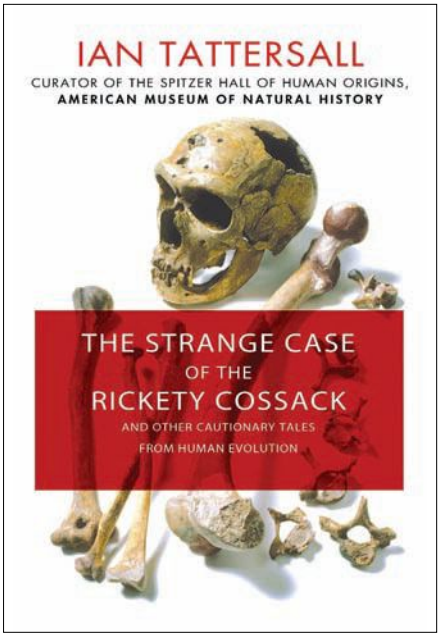
I write about this crucible of discovery and folly in my new book, *The Strange Case of the Rickety Cossack*.

The haphazard application of species names to every new hominin fossil was a practice that could not continue indefinitely. And in 1950 the ornithologist Ernst Mayr, one of the fathers of the Evolutionary Synthesis, took it upon himself to lecture the paleoanthropologists on the error of their ways. The Synthesis was an elaboration of evolutionary theory that saw most evolutionary change as the gradual accumulation, via natural selection, of small genetic innovations within ancestor-descendant sequences. So Mayr depicted human evolution as the slow modification of a single lineage culminating in *Homo sapiens*. Among the fifteen hominin genera then described, Mayr said, there was in reality only one: our own genus, *Homo*. What's more, the *Homo* lineage contained only three species: *Homo transvaalensis* (an early biped) had given rise to *Homo erectus*, which in turn had evolved into *Homo sapiens*. Acutely aware that their nomenclatural proliferation lacked any theoretical justification, the paleoanthropologists capitulated. For half a century thereafter, most of them viewed human evolution as a single-minded progression from primitiveness to sapient perfection driven by natural selection: an idea that fit rather well with the undeniable fact that only one hominin exists in the world today.

From the beginning, though, it was obvious that Mayr's scheme was a huge oversimplification. As fossil discoveries rapidly continued to accumulate, his single lineage began to bulge at the seams. A new image of hominin evolution began to develop.

Extensive additions to the hominin fossil record beginning in the 1960s eventually made it glaringly evident both that many more morphologies are present in that record than could ever be accommodated by Mayr's minimalist taxonomy, and that the saga of human evolution was immensely more complex than the burnishing of a single lineage.

A hominin family tree that I drew up in 1993 already featured 12 species, spanning the period from 4 million years ago to the present, while one of my recent trees contains twice as many species, scattered over the last 7 million years. Either way, at any one point in the past, several different hominin species typically coexisted, revealing human evolution not as a linear affair but as a process of vigorous and continuing experimentation with the hominin adaptive potential. *Homo sapiens* is evidently a huge exception in being the sole hominin species on the planet, and its lonely state cannot be taken as a guide to the past. There is something unprecedented about our species that makes it both intolerant of competition and uniquely able to eliminate it.



Palgrave Macmillan Trade, June 2015

Almost certainly, this novel element lies in the unusual way in which we process information, whereby a vocabulary of mental symbols makes it possible for us to remake the world in our minds. And both the form of our family tree and the archaeological record make it plain that this unique capacity was acquired not only very recently, but also very abruptly in evolutionary terms. Mayr's perspective suggests that we were gradually fine-tuned by natural selection, over the eons, to be the kind of creatures we are. But the diversity of the rapidly expanding hominin fossil record strongly argues otherwise. And if that is the case, we are not condemned by our biology to act in any specific ways. Instead, we are responsible for our own individual behaviors. ■

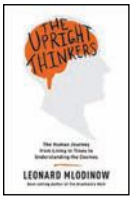
Ian Tattersall is a curator emeritus in the Division of Anthropology of the American Museum of Natural History in New York City. Read an excerpt from The Strange Case of the Rickety Cossack: And Other Cautionary Tales from Human Evolution *at the-scientist.com.*

How to Clone a Mammoth: The Science of De-Extinction
Beth Shapiro
Princeton University Press, April 2015

The majestic mammoth will never again roam the Earth. That is, unless we want it to. As University of California, Santa Cruz, ancient-DNA researcher Beth Shapiro explains in *How to Clone a Mammoth*, the scientific know-how exists to accomplish such goals of "de-extinction."

From her front-row seat as one of the pioneers of ancient-DNA research, Shapiro explains the fieldwork, lab science, and prospective ecology involved with the so-far hypothetical endeavor. As paleogenomic science has progressed to the point that cloning a mammoth has become possible (sort of), such propositions have also attracted controversy. Shapiro calls herself "an enthusiastic realist," writing that while recklessly resurrecting bygone species for Jurassic Park jollies would be "scientifically and ethically unjustified," de-extinction has the potential to become "an important tool for conserving species and [restoring] habitats." Breeding elephants that express some mammoth traits for cold hardiness, for example, might allow the pachyderms to be relocated to Arctic ecosystems to restore lost productivity there and aid in elephant conservation efforts.

The Upright Thinkers: The Human Journey from Living in Trees to Understanding the Cosmos
Leonard Mlodinow
Pantheon, May 2015

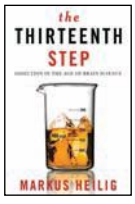


We've come a long way, baby. In the space of a few million years, primates have gone from squabbling in the canopy to walking on the Moon. But what is it about our ancestors and us that has made possible the meteoric rise of *Homo sapiens* to unprecedented cognitive heights? This is the question physicist Leonard Mlodinow asks and answers in his latest book, *The Upright Thinkers*.

Mlodinow traverses evolutionary time from our ratlike mammalian forebears to the emergence of genus *Homo* in Africa, from the dawn of civilization to the birth of quantum physics, in search of answers. He considers the nascent curiosity in our ancestors' brains as the seed of *Homo sapiens*'s success. "While other animals are adept at surviving the harshness of the jungle or the savanna, we seem more suited to sitting in a café, sipping mochas," Mlodinow writes. "That sitting, though, is not to be underestimated. For as we sit, we think, and we question."

As the human mind flowered, so too did culture and civilization. Modern humans continue to harvest the fruits of thinking and questioning by some of the greatest minds in human history. As Mlodinow details, the unquenchable curiosity morphed into science, a way of seeing the world that has in turn shaped our cultures, our bodies, and our concepts of the universe's functioning.

The Thirteenth Step: Addiction in the Age of Brain Science
Markus Heilig
Columbia University Press, May 2015

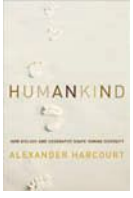


In the past 25 years, modern science has made quantum leaps in understanding the neurological roots of addiction. But because of humanity's long relationship with the disease, many nonscientific approaches have become entrenched in our treatment of addiction. These older methods and newer non–evidence-based therapies, argues NIH addiction researcher Markus Heilig in *The Thirteenth Step*, need to yield to approaches informed by the avalanche of data continually being uncovered by today's scientists.

"The time has come to bring together the everyday realities of addictive disorders and the emerging science and available evidence," Heilig writes.

Through a series of case studies of real patients, the author elucidates the brain structure and function that underlie relapse, drug-seeking behavior, and other addiction-related phenomena. But as complete as the science seems, there are still obstacles to implementing therapies based on its findings. "Prejudice, stigma, and financial interests conspire to prevent science-based treatments from reaching patients who need them," Heilig writes.

Humankind: How Biology and Geography Shape Human Diversity
Alexander H. Harcourt
Pegasus Books, June 2015



Humanity spreads, quilt-like, across the globe, different sizes, shapes, colors, and textures evolving into a diverse patchwork of peoples and customs. UC Davis anthropologist Alexander Harcourt celebrates this diversity and dissects its origins in *Humankind*. Evolution, whether biological or cultural, doesn't occur in a vacuum. There is intimate feedback between the physical environment, climate, and cohabiting plants, animals, and microbes that shapes and prunes the tree of life down to the twig of each species. And humans are no exception.

As species of *Homo* spread out of Africa to encounter new and different ecosystems, they embarked on a slow and steady journey that would mold different phenotypes, genotypes, and cultures from the same starting material. Harcourt retraces this process, bringing the latest science to bear in painting his dynamic portrait of human biogeography.

The author succeeds in drawing parallels between humans and the other species with which we share the planet. "In humanity's distribution around the world, and in the biological explanations for that distribution, humans show many of the same patterns as other animals," Harcourt writes. "All species are unique—they would not be separate species if they were not. But as regards the biology behind our species' global distribution, our biogeography, we humans are often not obviously unique."

—Bob Grant

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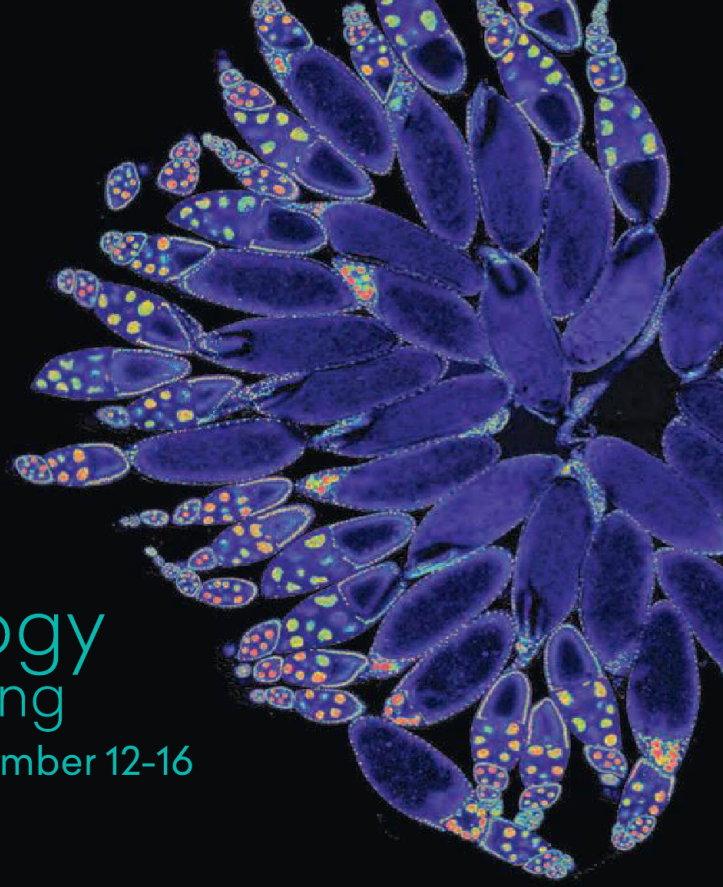

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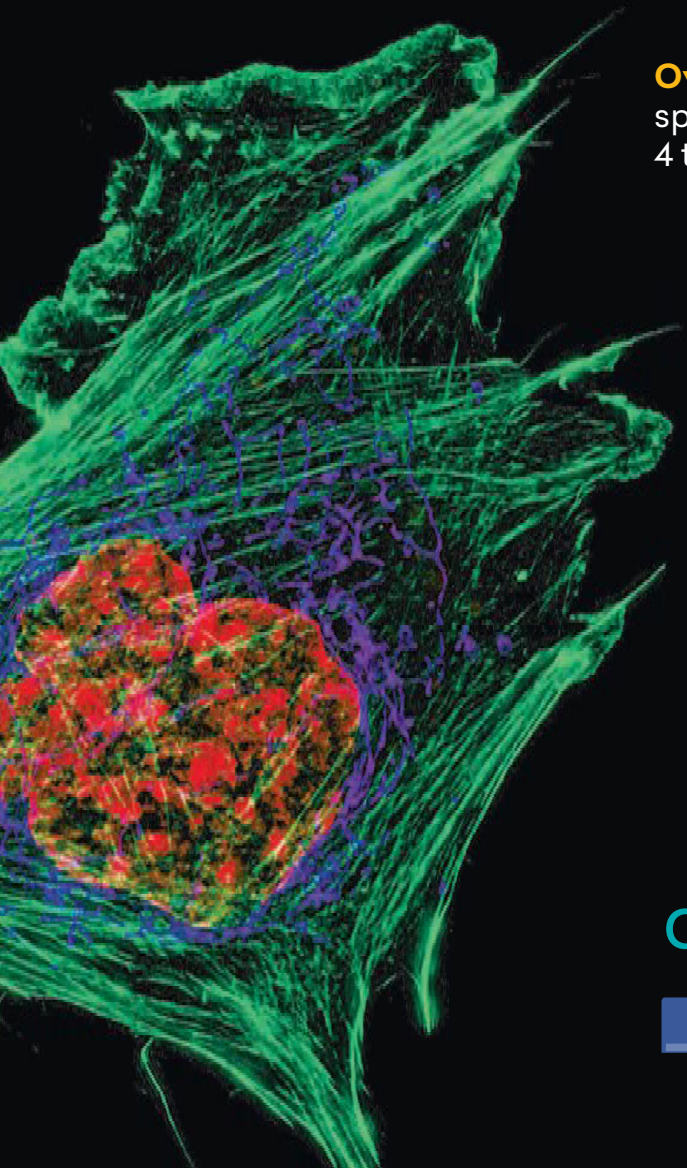

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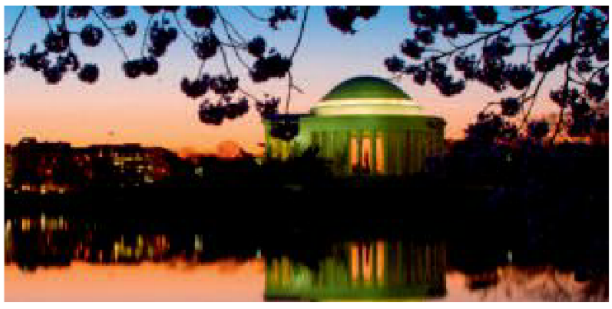

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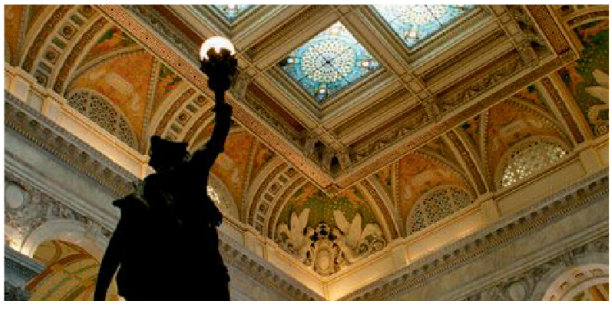

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Scholarship & Discounted Abstract Deadline: June 16, 2015; Abstract Deadline: July 14, 2015; Discounted Registration Deadline: August 13, 2015

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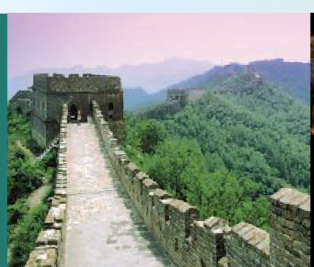

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Water Fleas, 1755

BY JENNY ROOD

Jacob Christian Schäffer, a naturalist, inventor, and pastor, had spent months searching the streams of southern Germany for green freshwater polyps (*Hydra*) before sloshing through swamps in pursuit of the little animals. In 1755, he published a 94-page volume devoted to the 10-millimeter-long critters, but the highlight of the manuscript turned out not to be descriptions of the polyp but rather the intricate images of one of its main food sources, a tiny invertebrate known to Schäffer as the "tailed, pronged water flea" and to contemporary scientists as *Daphnia magna*.

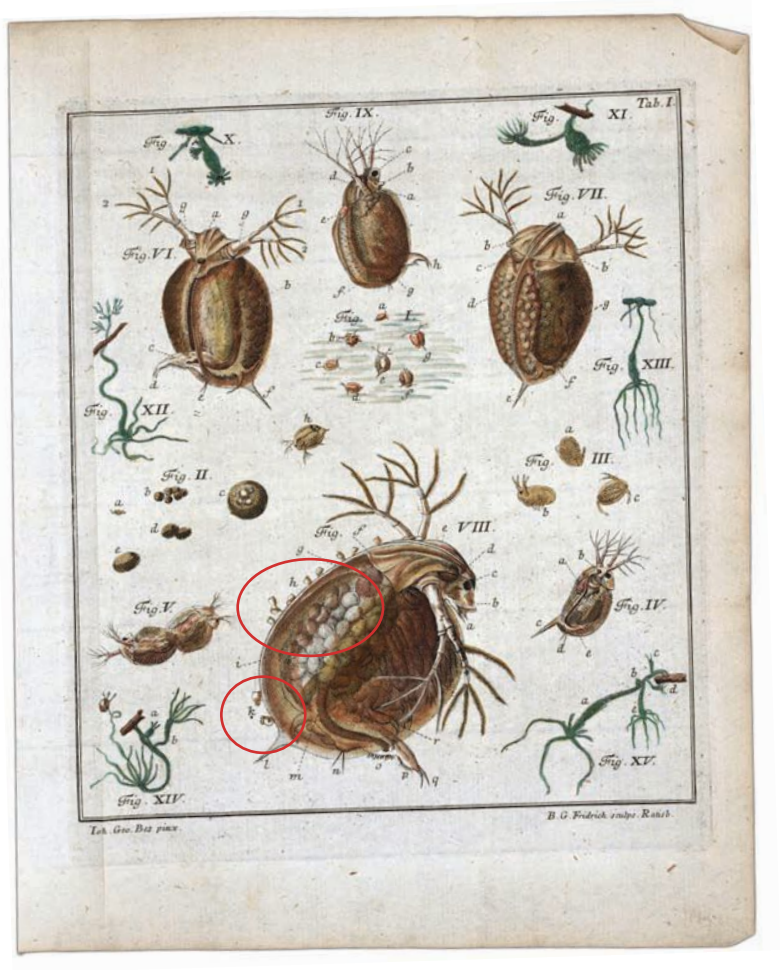
Jan Swammerdam, a pioneering Dutch microscopist, first illustrated the 2- to 5-millimeter-long animals in 1669 as minuscule, birdlike creatures with a pointy beak for eating and antler-like antennae for swimming. Schäffer's work, less than a century later, "is in a different league," says William Ashworth, a history professor at the University of Missouri, Kansas City.

The work of Swammerdam and others was "so incomplete that I was left to make not a few improvements and additions," Schäffer wrote in his treatise (as translated from German by this reporter). "I couldn't imagine that these otherwise sharp-sighted men could have seen this incorrectly." For example, the animals only have one eye, instead of the two that previous scientists expected to see by analogy with macroscopic organisms. "[Schäffer] was able to divorce all his preconceptions and see [*Daphnia*] for the unusual creature that it is," Ashworth says.

In addition to the detailed illustrations accompanying the manuscript, Schäffer's colorful observations of the animal's exterior and behaviors make the creature spring to life. The part of the carapace protecting the head and neck "has the shape of a nun's wimple or a mourning veil," and the antennae are "arboreal with feather barbs." Through observation and experiment, Schäffer was able to disprove Swammerdam's idea that *Daphnia* had beaks by demonstrating that the animal's arms are not mere "rudders on a ship," but create currents to bring food to their mouths.

Schäffer also investigated the crustaceans' innards by dumping them in alcohol to kill them, softening their shells with a 24-hour water soak, and dissecting them with pins and a delicate paintbrush to reveal nerve bundles, circulatory vessels, hidden mandibles, a digestive system, and ovaries harboring up to 40 eggs. "A man with a very crude microscope was able to figure out how [water fleas] functioned . . . without any precedent whatsoever," Ashworth says. "He was able not only to unravel the anatomy of it, but to a large extent an important part of its physiology, which was very unusual in 1755."

Apparently satisfied with his detailed description of the water fleas, Schäffer turned to other scientific pursuits, including similarly incisive work on insects and fungi; investigations of color theory; finding new sources of papermaking material, such as potatoes and peat; and designing a washing machine that remained popular for another 100 years. The water flea study may only form a small part of Schäffer's scientific legacy, but his detailed observations exemplify his keen observational skills. ■



THE LIFE OF A WATER FLEA: The first of three engraved and colored copper plates in Jacob Christian Schäffer's *The green polyps: the tailed and tailless pronged water fleas and a special kind of small water eel* shows various stages of the female *Daphnia magna* life cycle. Figure I, life-size adult animals; Figure II, eggs; Figure III, embryos; Figure IV, juveniles; Figure V, two entangled females that Schäffer mistakenly thought were mating hermaphrodites; Figures VI and VII, magnified adult females from different angles; Figure VIII, a magnified adult female with the right side of the carapace removed to show the inside. The plate also shows to scale the even smaller protozoans and rotifers (h and k, respectively; circled) that attach to the carapace, the first illustrations of epibionts (organisms that live on another creature). Figure IX, a different species, *Simocephalus vetulus*, which Schäffer called the "tailless pronged water flea"; Figures X through XV, magnified images of the green polyps that eat the water fleas.

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