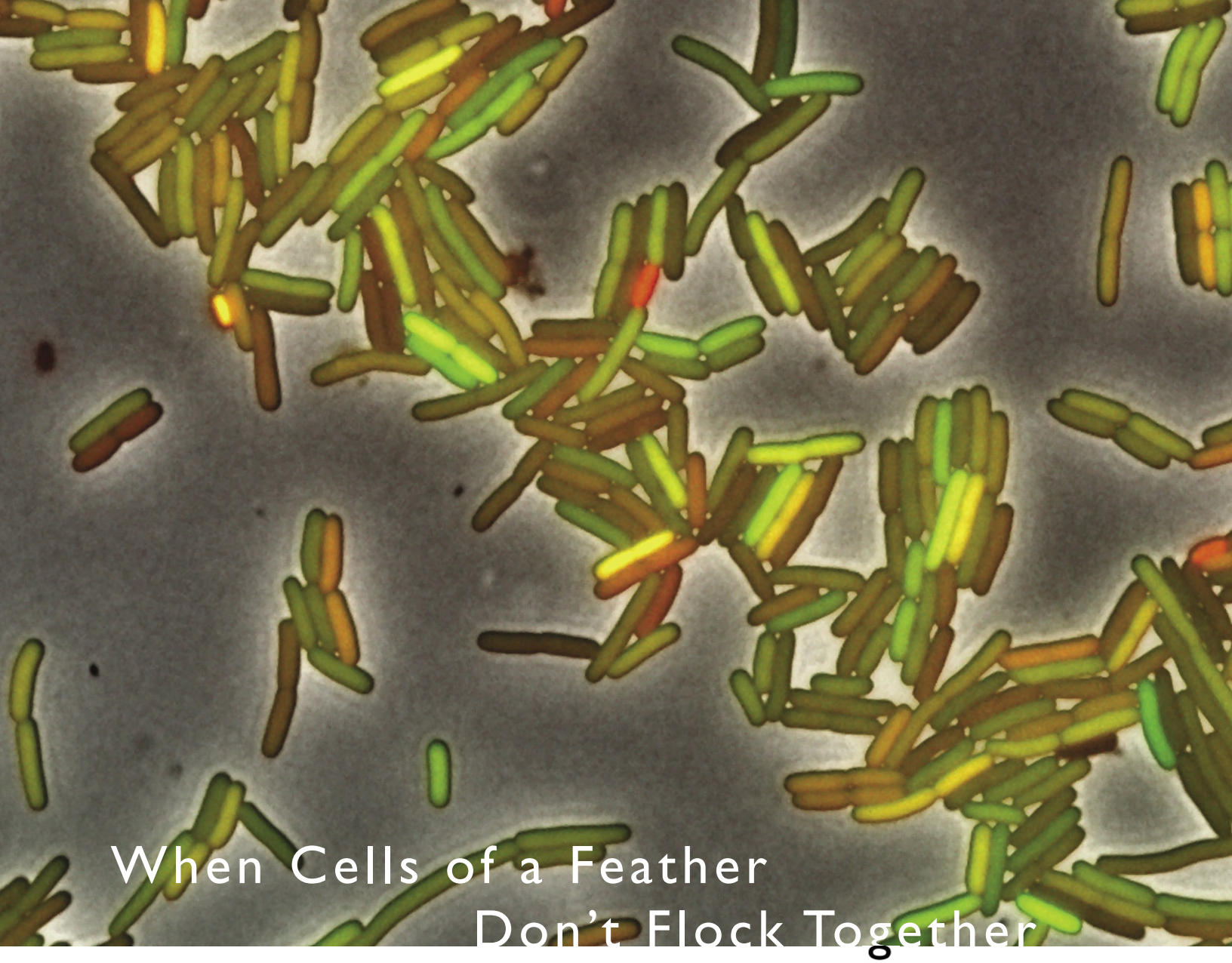


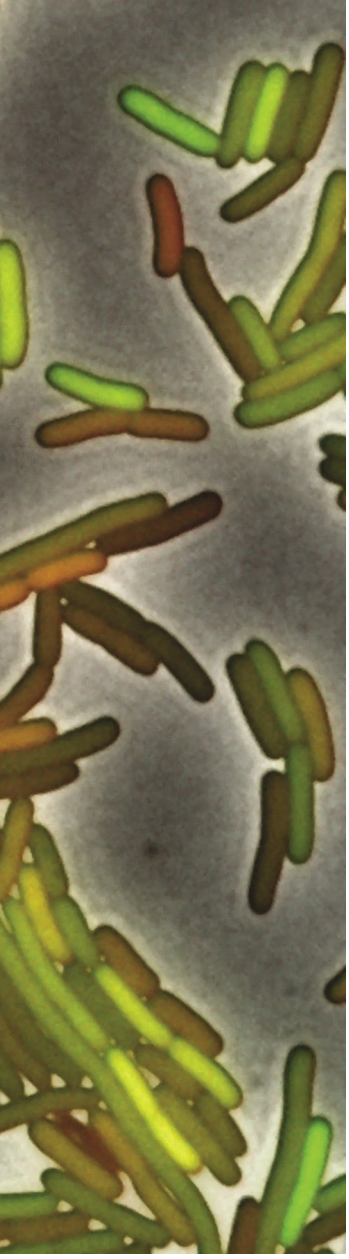
When Cells of a Feather Don't Flock Together

By Marcus Woo

In a scene worthy of a horror movie, pill-shaped bacteria cells glow green in the darkness. The ghostly cells squirm, grow, and divide, expanding their reach across the screen. Some shorten and turn into white spores, entering a dormant state from which they can come back to life decades—even centuries—later. Others temporarily glow red when they choose yet a different fate. But not all of the cells become spores, and only a few percent ever turn red at a given time. Michael Elowitz, assistant professor of biology and applied physics and Bren Scholar, and colleagues created these cells to explore the fundamental questions of how and when they decide to change. Applying the innovative approaches that won him a 2007 MacArthur Fellowship, the so-called genius grant, Elowitz wants to understand how a cell's genes work to make these decisions.

Even for simple organisms like *Bacillus subtilis*, this transformation process, called differentiation, remains a mystery. Cells choose different fates, even when they're genetically identical and grown in the same environment. As creatures become more complex, so do the choices. In mammals, for example, the power of embryonic stem cells lies in their ability to turn into anything, from blood to muscle to skin. Understanding the diversity of cell behavior could lead to new ways to attack cancer or develop new drugs, Elowitz says. If you want a drug to affect a cell a certain way, it helps to know how it behaves.

Elowitz's lab is peeking under the hood of the molecular interactions within the cell, where networks of genes, proteins, and other molecules work in concert to ensure the cell does what it's supposed to do. Called genetic circuits, these networks "form the foundation of all biology," says Gürol Süel, a former postdoc in Elowitz's lab and now an assistant professor at University of Texas Southwestern Medical Center. "Any biological



The colors of *B. subtilis* demonstrate variations in cell behavior from random fluctuations in the cells. Researchers engineered cells to glow green and red depending on which of two nearly identical genes were expressed. Fluctuations in gene expression, called intrinsic noise, cause the two genes to be expressed differently. Instead of a uniform yellow from equal parts green and red, the cells glow in shades of yellow, green, and orange.

process you can think of occurs as a result of interactions between biological molecules. There's no gene or protein that acts on its own—everything is interacting."

A Noisy Clock

Elowitz is part of a burgeoning field called systems biology, an outgrowth of molecular biology, which has mainly focused on the roles of individual molecules and their structures. Systems biologists, however, seek a broader understanding of how these parts underlie the mechanics of life. Traditionally, research papers would propose a series of interactions to explain a particular phenomenon, according to Elowitz. But this frustrated him, he says, because papers stopped short of quantifying the important components. Imagine someone giving you a cake recipe that told you to use flour, eggs, milk, and butter, but didn't tell you how much of each you needed. Given the inherent complexities of genetic circuits and biological systems in general, studying them in detail is a challenge. While a graduate student at Princeton, Elowitz decided to approach the problem from another direction—he set out to make his own simple circuit. After all, what better way to learn how to bake a cake than to try your own recipe?

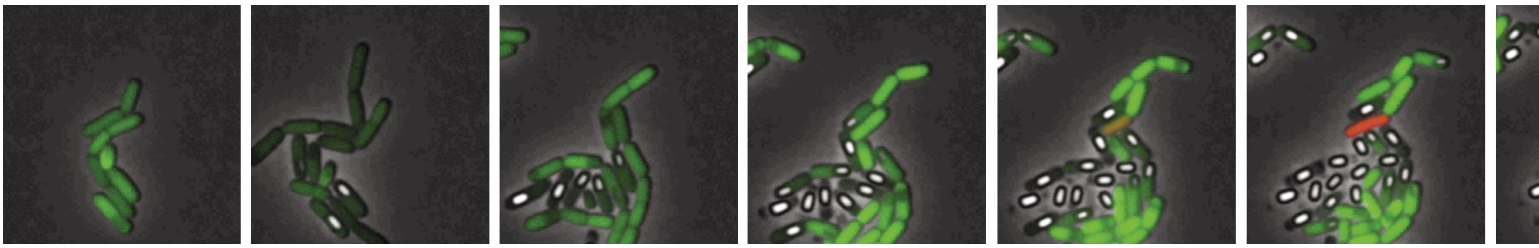
In 2000, Elowitz and his thesis advisor, Stanislas Leibler, designed and built a simple biological clock based on proteins called repressors, which turn off specific genes. As with all proteins, repressors are made when their genes are turned on, so the researchers engineered a set of three genes that turned each other on and off, like a three-way rock-paper-scissors game. In such a loop, rock turns off scissors. But scissors turns off paper, so if scissors is off, paper remains on. Paper, on the other hand, turns off rock, so if paper is on, rock turns off. This then causes scissors to turn back on, and the entire sequence cycles through as each gene switches on and off. One of the genes was engineered to light up when on, so that, when the researchers inserted their homemade circuit into a bacterium called *Escherichia coli*, the cells pulsed. Elowitz and Leibler called their circuit the repressilator, a combination of the words "repressor" and "oscillator."

The repressilator was an exciting proof-of-principle experiment, showing that the genetic circuits underlying cellular behavior can ultimately be understood and manipulated. Researchers had tinkered with genetic circuits before, but this was the first made entirely from scratch that also had a dynamic function. "We were very happy at first—wow, we were able to create this oscillator," Elowitz recalls. But they soon noticed something odd.

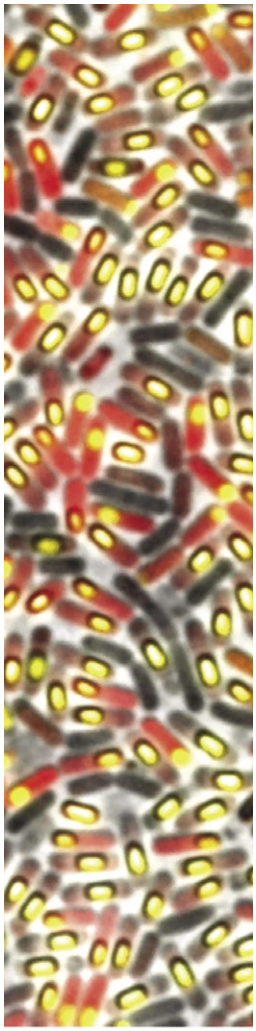
To study the repressilator in detail, they had made black and white movies of the *E. coli* cells. Each film began with a single cell, dark and faintly visible, at the center. The cell began to divide, and its offspring pulsed, casting an eerie glow on their neighbors. But none of the cells pulsed in sync with the other—even though all of the cells had identical genes. "If the oscillator were behaving exactly the same in all the cells, they would all remain in perfect synchrony," Elowitz says. "But they clearly don't—they get out of synchrony really fast."

Perhaps this shouldn't have come as a surprise, since biology—and the life of a cell—is known to be complicated and messy. Gene expression—the process by which a gene turns on to make a protein—is itself a muddled affair. Consisting of many biochemical reactions, gene expression is, in essence, a mechanical dance. Although choreographed well, it may be ungainly and missteps are inevitable. Molecules called transcription factors bind to specific DNA sequences and help genes get made into proteins. But they may not bind properly, or they may fall off the strand before their job is done. Some of the molecules involved may number fewer than 100—or even 10—making these missteps even more pronounced. As a result, a genetic circuit doesn't work the exact same way every time. There's a certain amount of inherent noise. Like the random variations that cause static in your AM radio signal, this noise was likely the culprit that knocked the represillators out of sync.

Biologists have long suspected that cells and their internal biochemistry might be inherently noisy. But in a 2002 paper, Elowitz, then at Rockefeller University, and colleagues were able not only to identify and quantify noise in cells, but to separate it into two components they called intrinsic and extrinsic noise. Extrinsic noise is due to fluctuations throughout an individual cell—differing concentrations of the transcription factor, for instance. Intrinsic noise comes from the process of gene expression itself. To isolate these two kinds of noise, the researchers put two nearly identical genes into the same cell. The researchers engineered each gene to make a different color-coded



This series of time-lapse frames shows the dynamics of cell behavior over the course of nearly two-and-a-half days. The cells that are expressing ComS glow green, and the white shapes are spores. After the cells have divided some, one cell begins to glow red, indicating competence. During competence, the cell can't divide, although it still grows. Only when the cell leaves its competent state is it able to divide again.



The fluorescent proteins in this image track sporulation in *B. subtilis*. Cells about to sporulate turn red, while the part that's about to become the spore turns yellow.

fluorescent protein, so that they could track the gene's expression. This powerful technique, now commonplace, was only made possible when biologists cloned fluorescent protein genes from jellyfish off the west coast of North America more than a decade ago. Although many scientists have used fluorescent proteins and movies to study cells, the Elowitz Lab is among the first to use them in a highly quantitative fashion. Much like how oscilloscopes allow electrical engineers to measure an electrical circuit, these movies allow biologists to measure a genetic circuit, Elowitz says.

If the noise were mainly extrinsic, it would affect the genes in the same way, and the researchers would see equal amounts of each color in any particular cell. For example, if the two colors were red and green, then every *E. coli* cell would appear a uniform yellow. Alternatively, if the noise were mainly intrinsic, then each gene would behave differently. You would now expect intermediate shades such as orange-yellow and goldenrod as well as red and green cells. The cells behaved as the team had hoped. The researchers engineered a strain dominated by intrinsic noise, and the cells glowed a whole spectrum from red to green. Likewise, for colonies with minimal noise, the cells were all yellow. The researchers also found that cells were more susceptible to intrinsic noise when genes were expressed at low levels. "Noise is not just some mysterious fluctuation that causes cells to be different from each other," Elowitz says. "We can understand why cells are different from each other, and we can break [noise] into these different components."

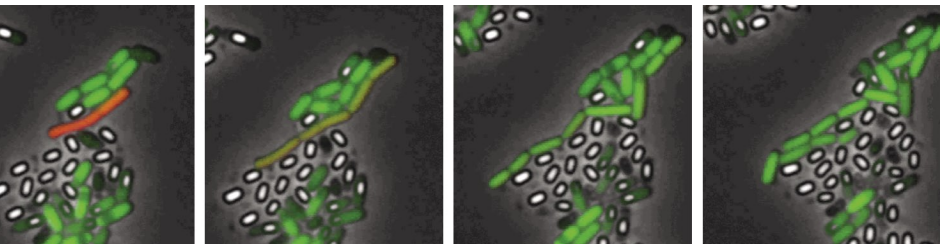
These direct measurements of noise demonstrated its importance, but what wasn't clear was how noise influenced a genetic circuit and, subsequently, cellular behavior. The question was twofold: how does a cell suppress fluctuation when it wants to do something accurately, and how can a cell use the noise to its advantage? The answer to the latter, at least, lay in another bacterium: *B. subtilis*.

It's Just Like Flushing a Toilet

Commonly found in soil, *B. subtilis* is sometimes used to make a type of fermented soybean eaten in Korea and Japan. But Elowitz and his colleagues were interested in what *B. subtilis* did when there were no soybeans to ferment. When faced with stress—such as a lack of nutrients—*B. subtilis* sometimes assumes a state called competence. In its competent state, *B. subtilis* can take in stray strands of DNA that happen to be floating around. Many scientists say competence is bacterial sex, a means for it to exchange genetic material. Others contend that it allows *B. subtilis* to eat the DNA as food.

But what interested Elowitz was that competence, like many other types of cellular differentiation, is not a predetermined fate. Instead, each cell has a certain probability of becoming competent or not. Even when they have the same genes and are put in the same environment, only a few percent of the cells ever become competent at a given time. This is good for the bacteria, because *B. subtilis* can't divide when competent. If all became competent at once, then none could divide, spelling doom for the colony. The researchers also discovered that the competent state is temporary. After less than a day, a competent cell returns to normal. But how do these cells, which are genetically identical, know which ones should become competent, and how do they know when to turn back? "It's very mysterious," Elowitz says. "All the cells are supposed to be the same, but they all do something different, even when you control the system as carefully as possible."

The key player is a transcription factor called ComK. ComK activates the genes needed for competence as well as the gene that makes ComK itself, which is written as *comK*, forming a positive feedback loop. A second player is a protein called ComS, made when one cell sends another a chemical signal; the reception of that signal activates



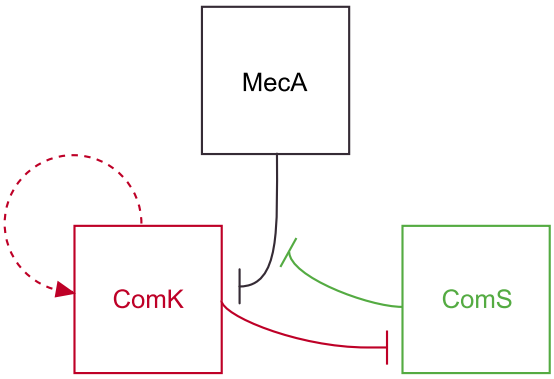


Diagram of the basic competence circuit. The key player is ComK, the protein that triggers competence. ComK, which also promotes itself, as indicated by the dotted arrow, blocks the expression of ComS, as indicated by the T-shaped arrow. The third player is called the MecA complex, an enzyme that destroys both ComK and ComS. When enough ComS is produced, MecA goes after ComS instead of ComK, allowing enough expression of ComK to start competence. In this way, ComS prevents MecA from blocking ComK.

the gene—known as *comS*—that makes ComS. Finally, a third party called the MecA complex joins the dance. MecA causes a protease, an enzyme that destroys proteins, to target both ComK and ComS.

Similar to the repressilator, the three proteins work together in a network of negative and positive feedback loops. By destroying ComK, MecA blocks competence. But when enough ComS is expressed, MecA becomes occupied zapping ComS instead of ComK. The production of ComS, then, indirectly supports competence by acting as a decoy for MecA. It also turns out that, through a series of intermediate interactions, ComK blocks the expression of ComS, which prevents any more decoys from distracting MecA. MecA then can attack ComK, eventually shutting down competence.

Although past research had unearthed this batch of interactions, no one had been able to explain the subtle interplay between each step. Here was another case of a cake recipe consisting only of a list of ingredients. In 2006, however, then-postdoc Süel, along with Elowitz, graduate student Louisa Liberman, and Jordi Garcia-Ojalvo from the Technical University of Catalonia in Spain, were able to mathematically model the competence dance. The model didn't get bogged down in every single biochemical reaction, but captured the essence of the genetic circuit. It showed that the cell's decision to differentiate relied on random noise—the same kind of fluctuations that prevented the repressilator from pulsing in sync among all the cells. In other words, to determine which cell would become competent, *B. subtilis* drew straws.

"What's great about this," Elowitz says, "is that we went from the repressilator, where noise was an annoyance—we found it wasn't working precisely because of the noise—to a natural differentiation circuit where the cell has actually taken advantage of this seeming annoyance to produce a system that can control the probability of whether something happens."

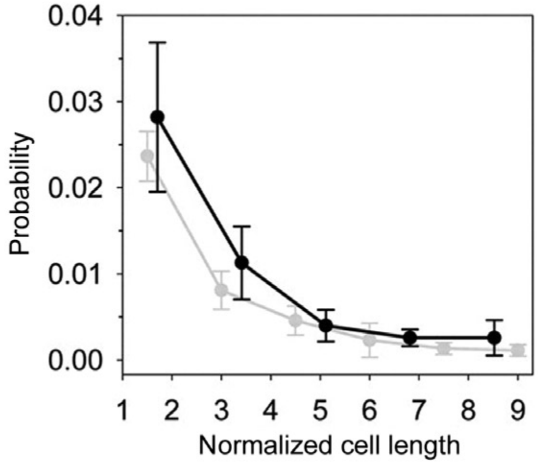
To develop their model, the researchers used fluorescent proteins that glowed different colors when ComK and ComS were expressed. The brightness of each color corresponded to how much ComK and ComS was being made, with the glow of ComK heralding competence. They then took snapshots of the bacteria colonies every 20 minutes or so, allowing them to track each cell.

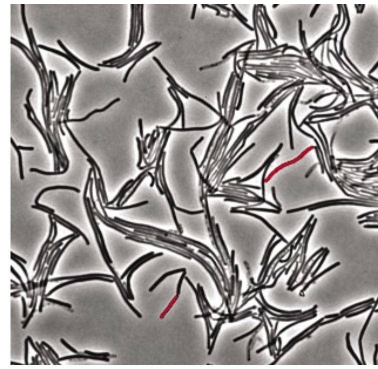
Their analysis showed that the competence circuit was an "excitable system," in which random fluctuations trigger some process after crossing a particular threshold. A simple example of an excitable system is jiggling the handle of a toilet. "Once in a while, you're going to jiggle a little too hard, and it'll just cross the threshold, and it'll flush," Elowitz explains. Another characteristic of an excitable system is that the strength of the trigger doesn't affect the system. Pushing the toilet handle harder doesn't make the flush more powerful or last longer. In the competence circuit, the expression of ComK is inherently noisy—like jiggling a toilet handle—and once in a while, enough will be expressed to trigger competence. But more ComK doesn't cause the cell to become competent more often or for a longer time.

The competence circuit is a self-controlling one. Once the system turns on, it sets up a chain of events that eventually turns itself back off. "The system can spontaneously flick on, but when it's on, it starts to activate something that will build up and build up," Elowitz explains. "When it goes to a high enough level, it'll shut the whole thing down." The competent state happens while MecA is busy gobbling up ComS, and additional ComS isn't being made because ComK is repressing it. Over time, ComS dwindles—in this case, what's "building up" is the depletion of ComS. Once MecA runs out of ComS to destroy, it goes after ComK. Since ComK is the trigger for competence, repressing ComK returns the cell to its normal state.

This work is among the first to show how cells can use noise to their advantage. Evolution has created an excitable system that allows just the right fraction of *B. subtilis* cells to become competent. But even though the model is consistent with what *B. subtilis* does, how do you know for sure that noise is indeed the driving force behind differentiation? Elowitz points out that the decision making could be hiding in an unknown interaction. The researchers continued tweaking and tuning the genetic circuit, and in an elegant experiment showed that without noise, competence was not possible, Elowitz says. "This was kind of the proof that noise was necessary."

Left: As the size of the cells increase, the probability of their becoming competent decreases, as predicted by the model. The experimental results (black line) are consistent with computer simulations (gray line).
Far right: An image of the extra-long *B. subtilis.* The red cells are competent.



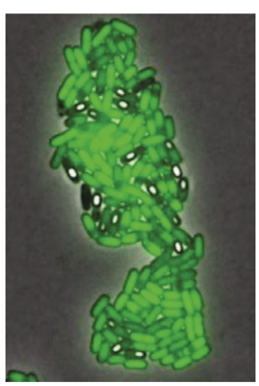


From Süel et al., *Science*, vol. 315, p. 1718, March 23, 2007.

Tuning Out the Noise

Noise is all about numbers. If you want to know America's favorite ice cream flavors, but only ask 10 people, you probably won't have much confidence that your results are representative of the general population. But if you ask 100,000 people, the results will surely be more accurate. With more numbers, the uncertainty—i.e., the noise—goes down. Noise is prevalent in the cell because cells are tiny, and molecules like ComK number in only the tens or hundreds. To boost the numbers, Süel, graduate student Rajan Kulkarni (PhD '06) , Jonathan Dworkin of Columbia University, Garcia-Ojalvo, and Elowitz reduced noise by engineering versions of *B. subtilis* that were up to nine times their original lengths, and thus contained nine times as many molecules like ComK.

To make the extra-long bacteria, the researchers introduced a mutation that prevented the cells from fully dividing. Now, during division, each cell would double in size, but not split off. Since the molecular concentrations remained the same throughout, with bigger cells came more molecules and therefore less noise. The researchers' model predicted that with reduced noise, the cells would not become competent. Fewer fluctuations meant a smaller chance that enough ComK would be expressed to trigger differentiation. To return to the toilet analogy, you probably won't flush the toilet if you barely jiggle the handle. In fact, the probability of a cell becoming competent went from about 3 percent to less than a half percent as the cell lengthened.



A group of green-glowing *B. subtilis*, signifying that ComS is being expressed. A few cells have become white spores.

The team also discovered that the minimum, or basal, expression level of the *comK* gene controls the frequency of competence, and the basal expression level of the *comS* gene independently controls the duration of competence. In the context of the model, this makes sense, since the amount of the ComS protein acts like a timer in the circuit; the cell is competent until MecA finishes off ComS and turns to ComK. Likewise, ComK controls the decision to enter competence in the first place.

By tweaking the two basal expression levels, the researchers tuned *B. subtilis* to become competent more often or for a longer time. Increasing the basal expression level for ComK by about 20 times its normal amount caused all cells to become competent. By increasing the basal expression level for ComS, the team was able to make most of the cells remain competent for about 40 hours, compared with a normal duration of about 20 hours.

This finding is more than a bioengineering novelty, however. The fact that competence frequency and duration can be tuned independently may be critical for a bacterial species' evolutionary survival. Scientists still don't fully understand the reasons behind competence, but if the reasons were to enhance genetic diversity, then competence would allow *B. subtilis* to evolve a response against a stressful environment. Say that the time the organism spends in competence is already evolutionarily fine-tuned. If a more stressful environment favors more *B. subtilis* to become competent, then the organism can evolve in that direction without affecting the optimized duration. According to Süel, it's like designing a race car: "Maybe you want to improve the braking performance but don't want to sacrifice the steering."

The team then wanted to see what would happen if they added another feedback loop to the

competence circuit. They inserted a protein called Rok that represses the expression of ComK. They anticipated that the addition would spur the cells to leave competence faster, and indeed that's what happened. Instead of 20 hours, most of the cells were competent for only about 14 hours. But then the modified circuit offered a surprise: there was less variability among the cells than in the normal circuit. Remember that since every system has a certain amount of noise, every measurement features an inherent variability. Even though most of the normal cells were competent for about 20 hours, a few would remain in that state for 10 or 30 hours. In the new circuit, more cells stayed competent for similar amounts of time.

"What's important is the creativity of the experiment. Can we make simple, beautiful, and elegant experiments that push this field into new directions, or allow us to see things in a simpler, clearer way?"

The researchers were able to explain this enhanced precision with their model. Normally, when a competent cell returns to its normal state, there are few ComS molecules, since its depletion is what allows MecA to gobble up ComK and send the cell back to normal. But with the addition of Rok to help block ComK, the cell returns to its normal state earlier, while there are still many ComS molecules left. Since ComS governs competence duration, and the reengineered circuit has left a higher number of ComS molecules, there is less noise in the distribution of duration times. "This is weird for biological circuits," Elowitz says. "Normally, when you mess around with these genetic circuits, you're going to screw them up in some way."

Toward Something Crazier?

The ability to understand a genetic circuit at such a deep level is an accomplishment in and of itself, but this work has broader implications. Many other single-celled organisms, not to mention cells in multicellular organisms, differentiate on a probabilistic basis. Additionally, being an excitable system is not unique to the competence circuit. Biological systems at scales other than the gene level are also excitable—the firing of neurons in the brain, for example.

"You have a tree, a rat, or a human—they're all governed by proteins, DNA, and RNA," Süel says. "The rules for how these molecules work together and give physiological behavior at the cellular level seem to be conserved among all organisms. Maybe the details are different: the organism has more genes or different interactions. But the approach, the thinking, and the tools we're using are not limited to this one particular bacteria."

In the past, biologists were primarily interested in looking at whether specific genes are expressed or not, the researchers say. Now, scientists want to learn about the dynamics of gene expression—how fast does the gene express itself, and at what level? How does it change over time?

According to Elowitz, researchers now have enough biochemical data about genetic interactions to develop mathematical models that can make quantitative predictions. Elowitz, trained in physics, winnows complexity into simplicity. A complicated network of interactions, the competence circuit, has been distilled into two equations describing the dynamics of ComK and ComS. "Sometimes a physics point of view lets you ask questions you otherwise wouldn't ask," says Elowitz.

Perhaps it was this kind of interdisciplinary thinking that earned him a MacArthur Fellowship, which, as its website says, is given to "talented individuals who have shown extraordinary originality and dedication in their creative pursuits and a marked capacity for self-direction." The distinction includes $500,000, given over five years, for the individual to do with as he or she pleases.

The recognition, he says, was a nice pat on the back. "It's encouraging," he says. "It makes you stop and think about what's important, and what's important is the creativity of the experiment. Can we make simple, beautiful, and elegant experiments that push this field into new directions, or allow us to see things in a simpler, clearer way? It makes you ask yourself, are we doing enough? Are we doing the right things? Are we doing things that are too easy?" He says he hasn't decided what to do with the money yet, but the fellowship nevertheless propels him forward. "People think what we did so far is good," he says. "We have to do something crazier now." □

From left to right: Gürol Süel, Michael Elowitz, and Jordi Garcia-Ojalvo.

PICTURE CREDITS: 28-33 — Elowitz lab; 31 — Doug Cummings