Takeharu Nagai · Yuichi Togashi *Editors*

Minorities and Small Numbers from Molecules to Organisms in Biology

Toward a New Understanding of Biological Phenomena

Springer

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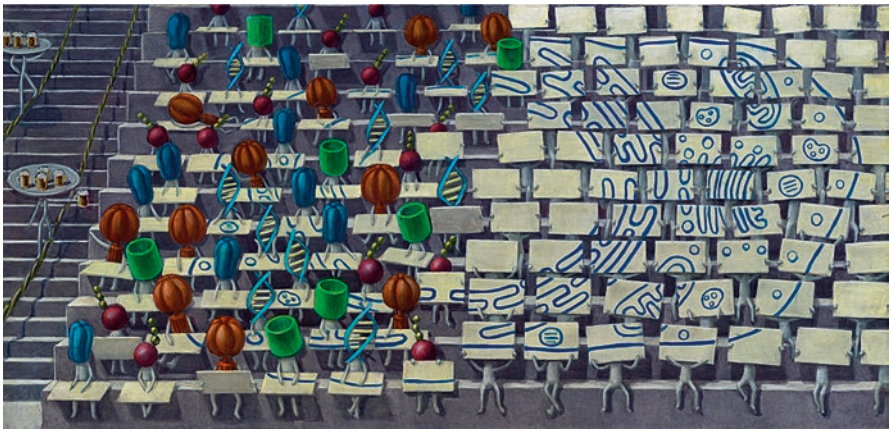

Illustration by Utako Kikutani. (Reprinted by courtesy of Project “Spying Minority in Biological Phenomena”)

Preface

There was a series called "Omoroi Biology (Interesting Biology)" in *Cell Technology*, a now-discontinued journal, in which I had an opportunity to thoroughly discuss the joy of science while looking back at more than 20 years of my life as a researcher in an article entitled "Behind Development of Fluorescent Probe Part 2–Four Seasons". I concluded this article as follows[1]:

> What is scientifically "interesting"? To me, it is the "ideas, inventions, and discoveries that change our perspectives". I believe the real thrill of science should be those. Since my words alone are not convincing, let me refer to the famous words by Dr. George M. Whitesides of Harvard University that I worship.
>
> "If the research that we do does not change the way people think, the project is a failure."
>
> Speaking of, I heard that the ultimate goal of his laboratory is "to fundamentally change the paradigms of science". Today, research is expected to be immediately beneficial and influence people's lives in Japan. However, I dare say I prefer continuing studies I find "interesting", although it does not immediately benefit people (…). Our laboratory has created "products" with our focus on "interest" and being the world's best and first. However, I think that we need to go further with the "degree of interest", which cannot be explained, at present, owing to page restrictions. However, I am convinced that unprecedented "interesting" facts lurk in "minority biology". I hope to elaborate on this topic on another occasion.

I wrote the above column in 2011 when the new field of study that I presented, "minority biology", just began. In this new field of science, "minority biology", we focus on the behaviour of minority in biological phenomena and understand biological phenomena from the perspective of how such behaviour of minority works on the overall biological system. The subtitle of the research topics was "pursuing biological phenomena weaved in a narrow gap between individual and the mass". We aimed to approach mechanisms and principles in which numerous elements in between individual and the mass work together and together impact the systems above and below. The frontispiece of this book is a graphic representation of this research to help you get a concrete image. The left half of this art shows several

[1] Takeharu Nagai, *Cell Technology*, Vol. 31, No. 12, 1390-1397 (2012).

types of biological molecules behaving individually, while the right half of this art shows these molecules working together to create cells and exerting their functions. If we remove individual biological molecules from cells and examine them with a microscope, we see them reacting and working in a "self-willed" manner. However, if we collect a large number of cells, lyse them, and observe a massive number of biological cells together, we could see much more apparently "accurate" reactions as a result of ensemble average. I assumed that understanding how we go from "self-willed" to apparently "accurate" would help understanding the relationship between behaviour of the minority and the overall biological system. "Minority biology" looks at traditional biological phenomena from such a "new perspective and beginning", in an attempt to develop research that leads to the creation of a new paradigm. In 6 years since then, many of my colleagues have developed amazing analytical methods, leading to the discovery of interesting phenomena and the birth of interesting ideas. This book was written to share our research results with as many high school/undergraduate students and young researchers as possible. I hope the book will have you amazed and excited with how "interesting" science is. I hope this book will show the real thrill of minority biology and science to as many people as possible.

Osaka, Japan Takeharu Nagai

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A Look at Functions That Emerge from Small Numbers

Takeharu Nagai

This chapter focuses on the cells that make up our bodies, and the effects that multitudes of biomolecules have within those cells, even when the biomolecules exist only in very small numbers. My goal is to trace how a minority event, the action of a small number of biomolecules within a cell, can exert major effects on the overall body. The recent American presidential election may be a good example. The unorthodox, ultra-radical Donald Trump, who was initially regarded as a fringe candidate, greatly betrayed expectations and won the presidency. If there had been an understanding of the principles of how minority factors work around us to accomplish transformations, there may not have been any "betrayal of expectations". The same can be said of the phenomena that occur in our bodies (and in this example, in terms of a disease). Here, a researcher named Yu talks to his mother about his work: how we can see the reactions among biomolecules in our cells, and how we can see the physiological phenomena that emerge as a result of these reactions.

(Yu, while in the Okayama City region having presented his work at an academic conference, visits his hometown of Wake)

Yu: Hey ma, I'm home!
Mother: Oh Yu! What're you doing here all of a sudden without even calling?
Yu: I was in the neighborhood for a research conference where I did a presentation, but it finished in the afternoon, so I thought I'd pop in.
Mother: Is that so. You gonna stay the night?
Yu: No, just stopping by for a bit.
Mother: I see. That's too bad. Since you came all this way, you should put your feet up.
Yu: I can't, ma. I've got a lot of work piling up, so I have to get back ASAP and take care of it. I just wanted to see you and you know, check up on you.

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Mother: Well, alright then. It is what it is, I suppose. I'm making tea now, I'll bring you some if you sit in the living room.

Mother: (While serving tea) So, what did you talk about at your conference?

Yu: I talked about the interactions between molecules in cells and how to observe their collective behavior.

Mother: Could you explain it to me in a way I can understand? I'd love to hear your talk. It's been so long.

Yu:(rubs hands together) Alright. I got this. Explaining it in a way you can understand is always good practice for my presentations, so it's good to do.

Mother: I always look forward to hearing what you have to say.

Yu: Aww, thanks, ma. I'm glad to hear that. And I'm glad I dropped by. First, I've got a question for you. Do you know about the general equilibrium theory of chemical reactions?

Mother: You've told me about it more times than I can count, so it's burned into my brain. It's the state in which a number of molecules equal to Avogadro's number (10^{23}), don't you call that a mole? - reacts in solution and reaches equilibrium.

Yu: Way to go, ma. That'll keep your mind sharp. If you understand that, the rest should make sense. I started my talk at the conference today with "Can we apply this theory to the chemical reactions that take place in cells?" Because the number of molecules in a cell isn't anywhere close to 10^{23}. For instance, do you know how many genes there are in a single cell?

Mother: That's obvious, isn't it? You've got one from your mother and one from your father, so that's two in total, isn't it? (See chapter "How Small Numbers of Long Genomic DNA Are Stored in Cells" by Maeshima).

Yu: Yup. The number is low enough that you can count it. But there have been experiments to see what happens when you react a huge number of genes and a huge number of proteins in a test tube. Scientists did that to examine the state in which an Avogadro's number of molecules reacts and reaches equilibrium. They reacted genes and proteins in conditions way out of touch with reality, and they used these data to infer what happens in cells; there have been lots of cases in life sciences where they've done that.

Mother: So you're saying that genes are an exception, and with other kinds of molecules, there are lots of 'em?

Yu: No, there are all kinds of other examples. Here's one: they found out that if you count the numbers of each type of protein expressed in *Escherichia coli*, there are so few expressed per cell for most types that you can count them all (See chapter "Thinking Small Numbers: When, Where, and How Many Molecules There Are in the Cell" by Taniguchi). In a small structure in nerves called a dendritic spine, there are molecules called AMPA receptor proteins that are closely involved in memory formation. There are so few of them per dendritic spine too that you can count them all (See chapter "Neuronal Synaptic Connections Organized by Small Numbers of Molecules" by Murakoshi). The number of clock proteins, which tick away a 24-h rhythm, is pretty low too (See chapter "The Flow of Time Inside the Cell: The Time of Days Given by Molecules Driving the Circadian Clocks" by Ode and Ueda).

Mother: Ho ho, so there are lots of examples, aren't there?

Yu: There are plenty more. The flu virus comes around every winter, but do you know how many particles it takes to trigger an infection in cells?

Mother: I don't have a clue.

Yu: Again, so few that you can count them all, apparently (See chapter "Invasions of Small Numbers: How Many Virus Particles Does It Take to Infect Someone with the Flu?" by Ohba).

Mother: Whoa. Is that right? I'd never have thought it!

Yu: A viral infection with so few particles that you can count them all out ends up affecting your whole body by triggering a nasty cold. Isn't that something? With numbers that small, probability and statistics as-is are no good. And the reactions won't reach equilibrium, either. Since conventional thinking won't get you anywhere, all you can do is work out a new way of thinking (See chapters "Rebellion by the Minority: Prophecies by Molecules on Paper and Computers" and "The Personality of Small Numbers: Do Molecules Have Personality?" by Togashi and Komatsuzaki).

Mother: Yeah, I reckon so. So how're they gonna do that?

Yu: You'd need to look at all kinds of biological phenomena at the molecular level and quantitatively confirm every single one of them (See chapter "Digital Bioanalysis" by Noji). To do that, you'd have to develop techniques for observing reactions that occur in cells.

Mother: Now how do they make these kinds of techniques??

Yu: There are all kinds of ways, but the one they're using right now is based on green fluorescent protein (GFP).

Mother: Oh, is it that glowing protein that Osamu Shimomura discovered in jellyfish and won a Nobel Prize for?

Yu: Yep, that's the one. At first, it was only green, but now scientists have developed proteins that emit a bunch of fluorescent colors from ultramarine to near-infrared. If you combine them well, you can see reactions in cells, such as how the concentration of calcium ion (Ca^{2+}) changes with cell activity.

Mother: How does it work?

Yu: This may be kind of difficult to understand, but you use a physical phenomenon called Förster resonance energy transfer (FRET). FRET occurs when two differently colored molecules like blue fluorescent protein (BFP) and GFP get within 10 nm of each other, and the excitation energy of BFP gets directly transferred to GFP by resonance. Anyway, when they're not close to each other, blue fluorescent light is emitted, and when they get close to each other, green fluorescent light is emitted.

Mother: Okay, but how does this FRET help you see Ca^{2+}?

Yu: For example, we know that Ca^{2+}-binding protein changes shape from long and stretched out to tangled and compact when binding with Ca^{2+}. You can combine that property nicely with FRET to design a Ca^{2+} sensor in which the color of the fluorescent light changes when Ca^{2+} binds.

Mother: Ho ho. Designing a sensor made only of proteins, and using the cell's own bio-molecules to inspire the design? That's outta this world.

Yu: With an artificial protein in which BFP and GFP are linked to both ends of the Ca^{2+}-binding protein, the Ca^{2+}-binding protein part is stretched out, and blue fluorescent light is emitted from BFP when there's no Ca^{2+}; but when there is Ca^{2+}, the Ca^{2+} binding protein changes to a compact shape, so BFP and GFP approach each other, FRET occurs, and green fluorescent light is emitted from GFP (Fig. 1). It's a Ca^{2+} version of litmus paper that turns red under acidic conditions and blue in basic ones.

Mother: Wow. But it's a modified protein, isn't it? How do you get it into the cell?

Yu: Proteins are like a string of beads, with the beads being different amino acids; you can write the order of the amino acids into genes. If you mix a solution of these genes with cells and apply an electric shock, small holes open up in the cells, and the genes go through them to get into the cells. Then, the apparatus in the cell read the genes to make the proteins.

Mother: Wow, it all sounds so easy. So what kind of phenomena did researchers see in cells with that fluorescent color-changing Ca^{2+} sensor?

Yu: They saw changes in Ca^{2+} concentration in response to cAMP (cyclic adenosine monophosphate) in an amoeba, which is a cellular slime mold. The reason they wanted to observe this is that we know this amoeba can tell apart small differences in the number of cAMP molecules and that it moves to places where there are more molecules. But humans can't tell apart small differences in numbers of cAMP molecules, no matter how elaborate a sensor you make; we can't do it with current techniques. But somehow amoeba cells can do it. They're otherworldly, aren't they? Scientists really wanted to figure out how amoebae do this thing.

Mother: What's all that got to do with Ca^{2+}?

Yu: It's been predicted for a long time that when an amoeba receives cAMP, it gets excited, and the Ca^{2+} concentration in its cell increases. But Ca^{2+} concentrations are low and can't be detected with conventional Ca^{2+} sensors. So, scientists made the world's most sensitive fluorescent Ca^{2+} sensor, which can detect ultra-small Ca^{2+} concentrations on the order of 10 nM (nanomolar; nano means 10^{-9}), and they introduced its gene into a cell. Then, when they observed it with a microscope, they were able to distinctly capture increases in Ca^{2+} concentration in response to an extremely minute volume of cAMP protein (less than 1 nM). But, unfortunately, we still don't know *how* amoebae can tell apart small differences in the number of cAMP molecules. We still need to do lots more experiments.

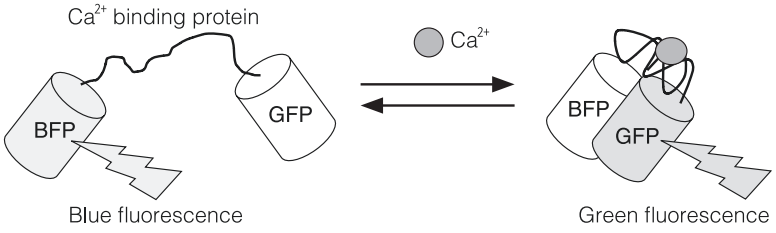

Fig. 1 Schematic of a fluorescent calcium sensor

Mother: That's too bad. I guess they just gotta continue probing! But have they managed to discover anything else?

Yu: What I just told you about involved a single cell that was magnified, but if you lower the magnification, you find something interesting. Normally, an amoeba lives as a single cell, but in a state of starvation, about 100,000 cells come together in a group. When they do, the "leader cell", which is probably in the center of the group, releases its cAMP; the cells that receive it get excited and then release their cAMP, and so on and so forth. In other words, these tens of thousands of cells form a bucket brigade. Then, as a result, other cells gather around the leader cell. Actually, no one had ever seen what happens with Ca^{2+} in that process.

Mother: So what did they see?

Yu: They saw that there are three patterns in those groups I mentioned. The first is a pattern where signals propagate outward from the center in concentric circles. The second is a pattern of propagation in a spiral. The third is a pattern where signals don't propagate, but instead glow for a moment and then end, like fireworks (Fig. 2). On top of that, they learned that this fireworks pattern seems to be related to the emergence of a leader cell.

Mother: Huh. What does that mean?

Yu: When they observed the fireworks closely, they realized that not every cell can launch fireworks; of the 100,000 cells, it seems that only a tiny minority of cells can do it.

Mother: Oh, so cells have personalities.

Yu: That's right. Just like people have personalities. Plus, around that cell (the leader candidate cell), the presence or absence of several cells (follower cells) that release their cAMP in response to the command to launch determines whether the signal is then conveyed to the surrounding cells (third wave cells). On top of that, once the signal is conveyed to the third wave cells, even if everything is quiet after that, cells will still come and gather.

Mother: So how many of these follower cells there need to be?

Yu: It's being studied as we speak, but it looks like the number is small enough that it can be counted out.

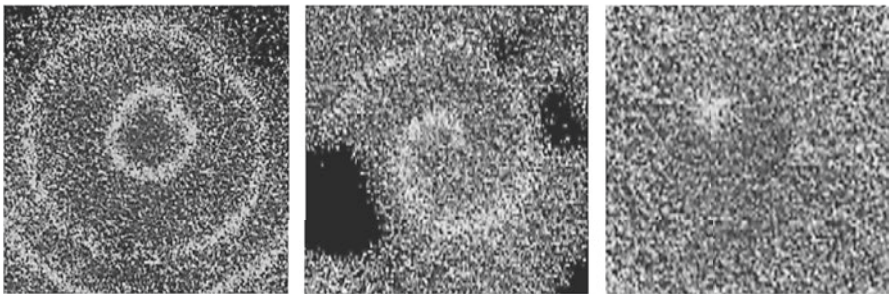

Fig. 2 The three patterns of calcium dynamics in grouping amoebae

Mother: Wow. So, you're basically saying that whether or not there's a number of supporters around a minor leader candidate cell determines whether it's confirmed as the leader cell?

Yu: Exactly. It's kind of the same in human society, isn't it? Oh yeah, there's an interesting TED talk (http://www.ted.com/talks/derek_sivers_how_to_start_a_movement) in which this guy named Derek Sivers talks about how to start a social movement. It's only 3 min, but the point is this: for kicking off discrete changes in group behavior, not only the first guy who dances shirtless and isn't afraid to be laughed at plays an important role, but also the first follower who comes after him. If you just lock that down, the third wave, the vast majority, instead of acting based on the popular will (mentality), get swept up by everyone around them.

Mother: (While watching the TED Talk on an iPad) Whoa ho, it really is like a grouping of amoebae, isn't it?

Yu: Sure is. If you want to start a revolution in human society, you'd think you have to act on the mentality of the people, right? But actually, you might be able to make a group act by a mechanism that's got nothing to do with that mentality. Donald Trump might have known that secret.

Mother: No way, he isn't that smart. Amoebae might be smarter than him.

Yu: With a single cell? That'd be something. If they really were smart, it'd be something of a paradigm shift. That's why I can't quit. Oops, I've stayed too long. I'm going to head out.

Mother: Thanks for the interesting talk. Be careful. Come around again soon.

Yu: See you, mom. You're getting on in years, so watch your health. Well, see you.

Mother: Come back soon, you hear?

Neuronal Synaptic Connections Organized by Small Numbers of Molecules

Hideji Murakoshi

In the late 1880s, Spanish neuroanatomist Santiago Ramón y Cajal (1852–1934) drew multiple sketches of cells. Having mastered Golgi's method (in which silver chromate formed by reacting silver nitrate and potassium dichromate is deposited in cells in "a part" of tissue), which was developed by Camillo Golgi, who was about 10 years his senior, Cajal aspired to demonstrate various neural cell morphologies in brain tissue [1, 2]. Have a look at one of them (Fig. 1, left). What do you think?

At that time, one theory held that the membranes of neural cells bordered on each other and were connected (the reticular theory; Fig. 1, middle); while another theory, the neuron theory, held that neural cells were discrete, unconnected units (Fig. 1, right). A great debate raged between supporters of these two theories. Golgi, who developed his eponymous staining method, endorsed the reticular theory, while his pupil Cajal supported the neuron theory. Which of them is correct?

Cajal Meets a Present-Day Neuroscientist (The Following Is Fiction)

(In the year 189X)

Cajal: Harrumph. Why won't Golgi understand what I'm telling him? His blasted reticular theory is wrong. All these sketches of cells I've done prove that I'm right! I'll give him a piece of my mind at the next conference. Today's another day to go to the university and sketch some cells. With my museum-worthy ultra-elaborate sketches and precise observations, I'll make this fool see that I'm right.

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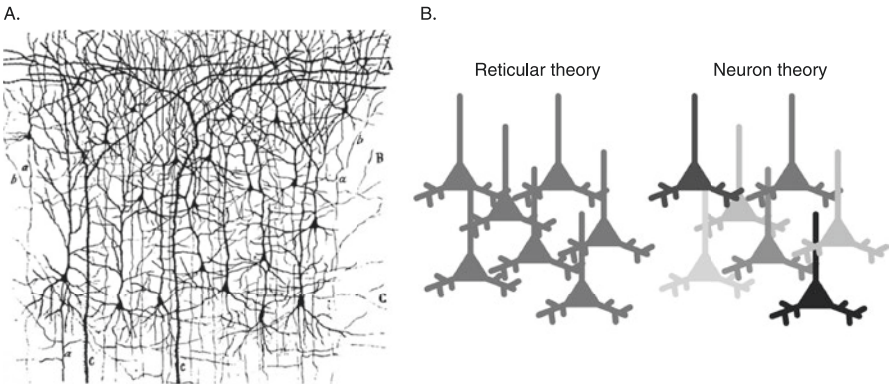

Fig. 1 (Left) Sketch of the cerebral cortex by Cajal. (From Ref. [3], Fig. 66). (Middle) The reticular theory. All cells are connected and have the same internal fluid. (Right) The neuronal theory. Individual cells are compartmentalized

The agitated Cajal flew out of his house. In his haste to get to university, he didn't notice that the ground was frozen, and he slipped, fell, and hit his head on a rock.

(In the year 201X. When Cajal regains consciousness, he finds himself in unfamiliar surroundings)
Cajal: … Hm? Where am I?
Student: Professor! Someone has broken into the lab!
Professor: What on earth is going on? That shouldn't be.

(The professor, a neuroscientist, enters the lab)

Professor: Who are you?!
Cajal: My name is Cajal.
Professor: Kahall? (I've heard that name somewhere before…) At any rate, I'm busy today. I'm supposed to talk to this student here about nerve cells. Could you get out of here?
Cajal: Nerve cells? (That's what I research, hey.)
Professor: Say what? Are you interested in nerve cells, too? In that case, you can stick around and listen too. I won't turn away anyone who's interested in neuroscience.
Cajal: Really? Then, I'll take you up on your offer. (I have no idea where I am. Must be a dream.)

Observation of Synapses with Electron Microscopy and Proof of the Neuron Theory

Professor: Well, today, I'll be talking about synapses. The word "synapse" is a coinage that originates from the Greek word for "conjunction"….

Cajal: It is. The word "synapse" was first used in a textbook in 1897 by Sherrington (a British physiologist), you know.

Professor: Yes, that's right. You sure do know your stuff. Back then, there were two theories about nerve cells: the reticular theory and the neuron theory. Based on several experiments, the neuron theory came to be supported by many people.

Cajal: According to my observations, neurodegeneration induced by transection doesn't spread this way and that in tissue. That's why I believe there must be partitions between cells.

Professor: …anyway, at that time, synapses couldn't be observed directly, so many people also advocated the reticular theory, which held that cells were connected by cytoplasm.

Student: It was discovered that there are gaps in the synapses that connect nerve cells in the 1950s, when they could be directly observed with an electron microscope, right?

Professor: That's right. Today, I just happen to have with me an electron microscopy image of a synapse. Would you like to have a look? (Fig. 2).

Cajal: Hey, wait just a minute! What the blazes is electron microscopy?

(The first electron microscope was developed by Max Knoll and Ernst Ruska at the Technical University of Berlin in 1931, which is why Cajal doesn't know what it is.)

Professor: What's your deal? You don't know what electron microscopy is? It's a method for observing samples that uses an electron beam with a wavelength (about 0.005 nanometers) that's much, much shorter than visible light (about 500

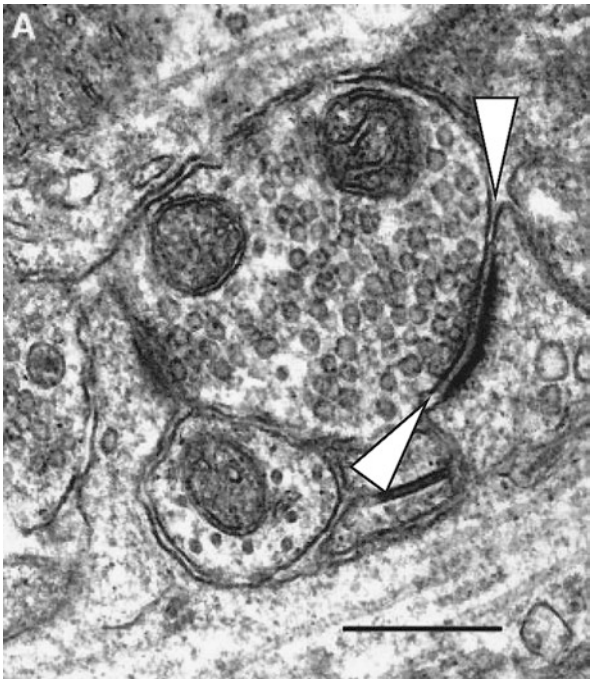

Fig. 2 Electron microscope image of an excitatory synapse. The white arrows indicate the synaptic cleft. (Partially revised from Ref. [4], Fig. 1A). The scale bar represents 400 nm. (Reprinted with permission from AAAS)

nanometers). The spatial resolution of a microscope is higher with a shorter wavelength. In other words, you can see much smaller things. The resolution of a light microscope is only about 300 nanometers, but the spatial resolution of an electron microscope is 1 nanometer. It lets you observe really small structures.

Cajal: Then that means this image shows the synapse in major detail! (I can see it much better than I can with the Zei and microscope I normally use!)

Professor: Exactly. Have a good look at the image. You see the gap in the synapse? That's the synaptic cleft. In other words, there's a partition between one synapse and the next; it clearly supports the neuron theory.

Cajal: So the neuron theory *was* right! It's definitive proof! Now that I have this, I'm going to cut that Golgi down to size!

Professor: Cut that Golgi down to size? What are you talking about? What a weird geezer.

Proteins in the Synapse

Professor: You can observe miniscule structures with an electron microscope, but looking at this image makes you really want to know what kind of molecules this structure is made of, doesn't it? One effective method for examining that sort of thing is fluorescence microscopy. By staining brain tissue with antibodies, you can find out what kinds of proteins are in synapses.

Student: I did that in practical training. First, you soak tissue in a solution of a primary antibody that recognizes the protein you're interested in. Next, you soak the tissue in a solution of a secondary antibody that's tagged with a fluorescent molecule and recognizes the primary antibody. Then you observe the tissue with a fluorescence microscope.

Cajal: So, instead of staining the cell itself, you use antibodies to stain a specific molecule inside the cell!

Professor: Exactly. Antibody staining is used for all sorts of things in biology. You could even say that the level of progress in antibody staining determines how much progress is made in a given field.

Cajal: So that's just how a long time ago, Nissl staining could only stain the somas of nerve cells, but the new Golgi method, which uses silver chromate, enabled staining of neurites for the first time and led to all kinds of findings.

Professor: The Golgi method was a long time ago, too. What a weirdo. Anyway, this method has revealed that all sorts of proteins are localized in synapses. For example, the major molecules in synapses include: adhesion molecules, which connect one synapse to the next; NMDA receptors and AMPA receptors, which channel ions; and scaffolding proteins such as PSD-95 for clustering various proteins. All of these different molecules play important roles (Fig. 3).

Cajal: What? So that's what the synapse is like, eh? I had no idea. Could you go into a little more detail? With my staining method, synapses just look like black spots. I'd been wondering what they were like on the inside.

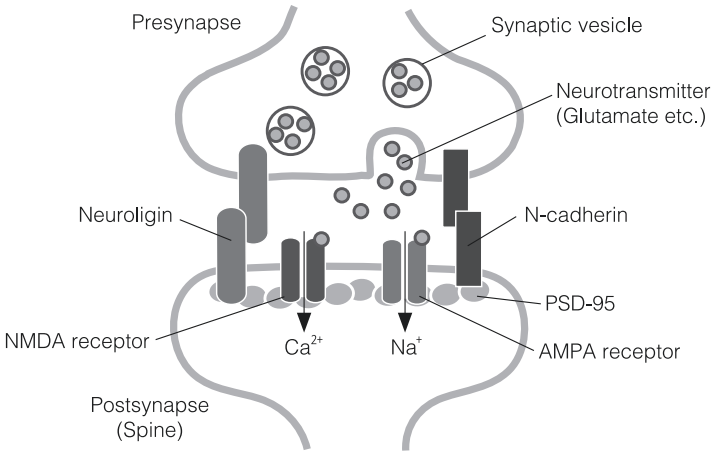

Fig. 3 Conceptual diagram of synaptic molecular mechanisms. (Drawn by Dr. Akihiro Shibata)

Professor: For example, there are protein molecules called N-cadherin and neuroligin. These molecules act as connectors between cells, i.e., the presynaptic and postsynaptic cells.

Cajal: Ho ho, I see. Now I understand how nerve cells connect to each other, but how do they send and receive signals? Since there's a gap in the synapse, they wouldn't be able to exchange information as is, hey?

Professor: Now you're on a roll, Mr. Kahall. Signals are exchanged using neurotransmitters. Neurotransmitters that are released by the presynaptic cell reach the postsynaptic cell; that's how cells get in touch with each other. Let's use the most well-known synapse, the hippocampal synapse, as an example. When there's a change in the difference in electric potential inside and outside the presynaptic membrane (depolarization due to a change in ion concentration inside and outside the membrane), glutamate is released. When glutamate binds to an AMPA receptor, sodium ions flow into the postsynaptic cell, i.e., into the dendritic spine, which triggers depolarization. Basically, glutamate is used to convey a change in electric potential in the presynaptic cell as a change in electric potential in the postsynaptic cell.

Cajal: I'd never imagined that it happens like that.

Student: Synapses are incredibly sophisticated, aren't they?

Professor: You'd think so. But when you really think about it, they're not.

Only a Small Number of Molecules Are Localized in the Synapse

Professor: The diameter of the synapse is extremely small. Let me put it this way: if the brain is a classroom 10 m^2 in area, then a single synapse would only be about 1 mm in size.

Cajal: I know that much.

Professor: That's just the setup. Now, let's calculate the number of proteins in a single postsynaptic cell, i.e., in the dendritic spine. We know that the density of proteins in the cell is about 4 mM. With a spinal volume of 0.2 femtoliters (fL), you can calculate 4 (mM) × 0.02 (fL) × 6 × 10^{23} = 48,000 proteins. Assuming that there are 5000 types of proteins in a nerve cell, there would be an average of only about 10 of each type of proteins. Besides such a small number of proteins, we also know that almost all proteins are diffuse in the cell. Basically, the state and reactivity of the spine temporally and spatially change every moment; it's believed that the spine never exists in the same state twice. That's substantively different from an elaborately crafted machine.

Cajal: I see. I had thought the function of the synapse was mechanical, but it seems that's not the case.

Professor: That's right. In fact, we now know based on fluorescence microscopy observation, electron microscopy observation, and all sorts of other results that there are only a few dozen NMDA receptors and AMPA receptors. Thus, the number of molecules that comprises these functions is not very high; when conducting research, I think it's important to keep in mind that the state of the dendritic spine is constantly wavering. The academic discipline that takes the perspective that constituent molecules are small in number is called minority biology.

Student: Why are there only dozens of NMDA receptors? Can there not be hundreds?

Professor: I guess that's like saying, "Hey, even if you only win a bronze medal, you still get to stand on the podium!"

Professor: But it's a good question. Why does the synapse function with relatively small numbers of molecules? At the moment, aside from the fact that it came to be that way in the course of evolution, we know nothing about it at all. I think that knowing whether unstable elements like synapses, with their states that change from moment to moment, are responsible for brain function leads to an essential understanding of the brain. For instance, synapse states being unstable may mean that it's easy to erase memories you want to forget and form new memories.

Professor: Today, we started with Cajal's sketches and took a general view of recent findings. If the real Cajal could see present-day research data80 years after his death, what would he say? He might say he's surprised at how little progress there's been.

Cajal: But I am Cajal…

Professor: …

Student: …

Professor: The sun has gone down, so I'm going to have to ask you to leave. I have a lecture early tomorrow morning. Well, bye.

Student: I'm leaving too.

Cajal: Hey, what am I supposed to do? Who am I? Where am I? A minority is one thing, but being all alone is something else!

References

1. McComas, A. J. (2011). *Galvani's spark: The story of the nerve impulse*. New York: Oxford University Press.
2. Bear, M. F., Connors, B. W., & Paradiso, M. A. (2006). *Neuroscience: Exploring the brain*. Baltimore: Lippincott Williams and Wilkins.
3. Defelipe, J., DeFelipe, J., & Jones, E. G. (1988). *Cajal on the cerebral cortex: An annotated translation of the complete writings (History of neuroscience)*. New York: Oxford University Press.
4. Kennedy, M. B. (2000). Signal-processing machines at the postsynaptic density. *Science, 290*, 750–754.

Invasions of Small Numbers: How Many Virus Particles Does It Take to Infect Someone with the Flu?

Yusuke Ohba

Infections by pathogenic viruses cause diseases in humans. One such example is the flu, which is caused by an influenza virus. Based on the number of cells in the human body, the number of invading virus particles is extremely small. When I think about the question of just how many virus particles it takes to infect someone with the flu, I always recall the rescue of Princess Leia in "Star Wars".

To deliver the plans for the Death Star, the Empire's massive super-weapon, to the rebel alliance, Luke Skywalker (hereafter Luke) and Obi-Wan Kenobi (Obi-Wan) head for the Alderaan System aboard the Millennium Falcon with the smuggler Han Solo (Solo), Chewbacca, R2-D2, and C-3PO. However, because Princess Leia Organa (Leia) refused to reveal the location of the rebel base, the Empire used the Death Star's super-laser to destroy the planet Alderaan as an example, and the debris of the planet floats through space as an asteroid field. The Falcon's hyperspace jump brings it right in the middle of this asteroid field, and the ship is pulled in by the Death Star's tractor beam. Though it is only the four of them (and two droids) invading the Death Star, Obi-Wan disables the tractor beam, and Luke, Solo, and Chewbacca rescue Leia from the detention block. Obi-Wan is slain in a duel by his former apprentice Darth Vader, but then…

Thus, a group of just four people and two droids went up against a massive number of enemies (primarily Storm troopers) and managed to rescue Leia. Here, we will listen in on a silly conversation between a father and his son about the story of a small group of brave heroes (a virus) taking on a massive enemy (the human body).

Ken: Ah-choo! I must've caught a bad cold…maybe it's because I was cold as I didn't sleep under a blanket yesterday?

Dad: You don't catch colds because you're cold. A cold is an infection, so you were infected by a pathogen. It's a bacterium or a virus that causes a cold.

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Ken: Whoa, really? I didn't catch a cold because I was cold!?
Dad: It's because your strength was down, so your body's immunity, its defense, was down; it couldn't fend off the infecting pathogen. So your body must have already been infected by some pathogens.
Ken: Whoa, no kidding. So you're saying that all kinds of pathogens attack me, but my body usually defends against them?
Dad: Ooh, good question. I don't know if they're all pathogens, but there are lots and lots of bacteria and viruses around us, so they're always getting into our bodies. Bodyguards in our bodies take them out.
Ken: Speaking of which, since the flu makes you break out in a fever like a cold does, is the flu related to pathogens too?
Dad: It is. The flu is caused by a virus called an influenza virus.
Ken: Just like the name says (heh)
Dad: That's true, viruses and bacteria usually get named for the diseases they cause.
Ken: There are lots of cases of flu in winter, right? People get flu shots at the start of winter. Why? Is it because it's cold and our bodies get cold?
Dad: Immunity definitely does get weaker when it's cold. But it's said that viruses live longer in the winter because the air is drier. So probably, what's happening is that lots of viruses attack the body.
Ken: So you're saying it's because people with the flu *cough* and *sneeze*, and the viruses in there live forever?
Dad: Exactly!
Ken: How many virus particles are there in those *coughs* and *sneezes*?
Dad: There are about 50,000 in a cough and 100,000 in a sneeze.
Ken: I don't know if that's a lot or a little…how many virus particles does it take to cause the flu or a cold in the first place?
Dad: As I said earlier, it depends on your immunity, how strong or weak your bodyguards are.
Ken: So, if you don't have immunity, it only takes one particle?
Dad: No one knows yet.
Ken: Hey, weren't you doing research on the flu? Then how do you not know?
Dad: Ugh, yeah, tell me about it. Okay, I'll find out.
Ken: How do you find out something that no one knows?
Dad: Thinking about that is my job.
Ken: Oh, right! You're a researcher, duh. So really, what are you going to do?
Dad: Actually, a great man I know, Professor Noji, developed a clever method for accurately counting influenza virus particles. I'll try asking him first!

In virus research, the volume of a virus is represented with a numerical index called a viral titer, which is the lowest concentration of a virus in a virus-containing solution that can infect cells. Therefore, the calculated viral titer changes greatly based on the type of target cell being infected or the method used to assess whether cells are infected (or, in some cases, the observer of the results). This makes it unlikely that the numbers of viral particles themselves are being represented "quantitatively". In response, Dr. Kazuhito Tabata, Professor Hiroyuki Noji, et al. of the

University of Tokyo developed an influenza virus particle digital counting technique that uses microchambers; this technique enables counting of the absolute number of virus particles (Chapter "Digital Bioanalysis"). This method has been used to demonstrate that a virus solution of 1 viral titer contains 2000 viral particles [1].

Ken: Dad, did you figure out how many virus particles there are in the flu?

Dad: We still have to work on that, but in my research with Professor Noji, we've learned a lot of interesting stuff!

Ken: Like what?

Dad: First of all, we found out that the number of virus particles we've been using in our experiments was impossibly large compared with the number it actually takes for someone to come down with the flu.

Ken: What do you mean?

Dad: Earlier, I said that there were 50,000 virus particles in a cough and 100,000 in a sneeze, right?

Ken: Yeah, uh, did you? I forgot…

Dad: Meh, that's fine, but yes. After counting the virus particles in the virus solution we use, when we recalculated for humans in the experiments we do, we found that we'd have to incubate the cells in the equivalent of 12 million coughs and 6 million sneezes.

Ken: How much is that? 12 million coughs times 500,000 particles? Um, uh, one, ten, hundred, thousand, ten thousand, hundred thousand, million, ten million, hundred million…that's impossible, right?

Dad: It is. Actually, just a few coughs will do it. So in our experiments, we had been incubating the cells in way more virus particles than necessary. It was a situation that's impossible in reality.

Ken: Then what the heck was the point!!

Dad: You're right. But that was common sense until now, so that was what we did.

Ken: By common sense, do you mean rules? It had to be that way?

Dad: Well, something like that. Think about it like this: it's a rule *for now*. By doing experiments according to the rules that had existed until now, we ended up with something that's impossible in reality, is what I'm saying. It's a classic case of losing sight of the truth because you're too caught up in common sense.

Ken: By the way, dad, I haven't done my homework yet, but doing homework is common sense, right? If I want to be a researcher who isn't caught up in common sense and makes a big discovery, does that mean I don't have to do my homework?

Dad: Don't be stupid. Big discoveries get made *because* of basic knowledge. And if you don't understand common sense, how can you judge whether you're caught up in common sense, hum? Besides, it's generally a good idea to live your life according to common sense. The only times it's better not to get too caught up in common sense are for important things like research! After this, you do your homework like you're supposed to!!

Ken: Okay, okay, okay (sob) So, what happened with your research?

Dad: Right, so we ran experiments with fewer and fewer virus particles. When we did, we found out that whether you get infected with the flu doesn't depend on viral titer, as everyone had always said; what matters is the number of virus particles.

Ken: Isn't that obvious?

Dad: It definitely is if you look at the results first, but that's not what common sense said until now.

Ken: It really is good not to get caught up in common sense!

Dad: I guess so. So even if you make the number of viral particles lower and lower, the cells still get infected with the flu. We ultimately found out that it only takes about 10 particles.

Ken: So with 10 particles, you get the flu?

Dad: Like I said earlier, we still have to do more experiments before we can pin down the number to 10. But it's definitely somewhere between a few and 20. We also discovered something even more interesting than that: the mechanism of infection is completely different depending on whether there are fewer than 20 viral particles per cell or 20 or more (Fig. 1).

Ken: What do you mean? How is it different??

Dad: Well…when there are fewer than 20 particles, the virus infects cells in such as a way as to remain really quietly hidden and lurks there. But when they propagate in the cell, they can infect surrounding cells in numbers of more than 20, and the infection increases.

Ken: Can a virus not multiply on its own without a cell?

Dad: Right. I didn't mention that, did I? There are all kinds of pathogens, but most of the ones that cause disease in humans are bacteria and viruses. The biggest difference between them is that bacteria can multiply on their own but viruses can't unless they infect a cell.

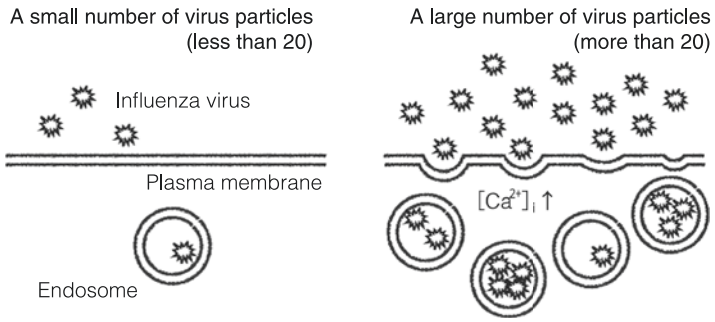

Fig. 1 Influenza viruses are internalized into host cells via endosomes by utilizing cellular phenomena called endocytosis. When the number of virus particles is small (less than 20), viruses are internalized into cells very slowly and can infect cells subtly. However, when the number of particles is large (more than 20), intracellular calcium concentration ($[Ca^{2+}]_i$) is increased by exposure to advance viruses, which resulted in upregulation of endocytosis (in other words, the gate of the cell at the plasma membrane is forced open). Viral particles efficiently enter into cells through this gate

Ken: So viruses need cells.

Dad: Right, so viruses aren't classified as life. They can't do anything on their own. Like you!

Ken: Shut up, fool!

Dad: Hahaha. Anyway, getting back on track, we had seen in our experiments what happens when there are 20 or more particles, but viruses also have a method that we did not know about for invading cells when they are fewer than 20 particles.

Ken: Then hasn't all your research until now been meaningless?

Dad: That's not true. We had already found out in our research that calcium in the cell is important in influenza virus invasion, and it turns out that calcium in the cell also seems to be the key to the difference between infection with few particles and infection with many particles.

Ken: That's good!

Dad: And there's also immunity, so being infected isn't necessarily the same thing as getting sick. Even if there are 20 or more viral particles, if you have effective medicine, it'll team up with your own immunity and be able to take out the virus. Tamiflu (note: Tamiflu is a brand name; the name of the substance is oseltamivir) is such a drug, and it actually works.

Ken: But the other day, there was something on TV about Tamiflu *resistance*. I didn't know the word *resistance*, but apparently, existing medicines don't work.

Dad: That's my TV-addicted boy (heh). Yes, because Tamiflu suppresses influenza as it comes out of the cell. It works for cases where there are 20 or more particles.

Ken: Then you should use a medicine that works when there are fewer than 20 particles!

Dad: Exactly! So I'm going to research that from now on!

Ken: Are you going to win a Nobel Prize?

Dad: Of course, I'm aiming for that in my research. I have to be careful not to get too caught up in common sense. Say, don't regular people with common sense do their homework!?

Ken: Okay, okay (grumble)

To plunge into the heart of the enemy when you're overwhelmingly outnumbered, you need some sort of breakthrough. What was the breakthrough in the rescue of Princess Leia in Star Wars? Luke and Solo put on the armor of Storm troopers they had killed to pose as the enemy, then handcuffed Chewbacca and pretended to escort him as a prisoner to sneak deep into the Death Star. At this point, there was no big commotion in the Death Star. This is how it is when a cell is invaded by fewer than 20 viral particles. If the heroes had been caught at this point, Luke wouldn't have fired proton torpedoes into the Death Star's exhaust port at the Battle of Yavin, and the Death Star wouldn't have been destroyed.

When Luke and Solo reached the detention level, they were no longer able to get by on deception and were forced into a blaster battle. Word of the commotion went around the Death Star; the imperial troops were ordered to capture the intruders and

were put on high alert. This is like the shift into ≥20-particle infection mode, where the human host initiates an immune response in an attempt to expel the virus.

Ultimately, Luke and Solo rescued Leia from solitary confinement, got out of a number of dangerous situations (aside from Obi-Wan being slain in his duel with Vader and becoming one with the Force), and finally managed to escape the Death Star. The rebel alliance, now armed with the knowledge of the Death Star's weak point, attacked its exhaust port and destroyed the Death Star. Viruses that can proliferate in humans who have been treated with vaccines or Tamiflu learn about this inhibitory effect and possess the capacity as "new" viruses to outstrip the effect. These are the "resistant strains" that everyone fusses about. To avoid giving rise to such resistant strains, the key is to elucidate the mode of infection when there are fewer than 20 viral particles and to find measures to combat it (Fig. 1).

Reference

1. Enoki, et al. (2012). Label-free single-particle imaging of the influenza virus by objective-type total internal reflection dark-field microscopy. *PLoS One, 7*, e49208.

Rebellion by the Minority: Prophecies by Molecules on Paper and Computers

Yuichi Togashi

Here is a cup of coffee. If you divide it in half, both halves will be the same coffee. If you divide it in half again, it will still be the same coffee.

Of course, we know that coffee is made of a mixture of molecules such as water and caffeine. However, the number of molecules in a cup of coffee is immense; therefore, there is no difference in properties between a cup and a drop of coffee. Counting all of the individual molecules would be inconvenient, so they are grouped together in vast units of approximately 6×10^{23} (Avogadro's number); this number of molecules is referred to as 1 mole. Two moles of hydrogen molecules and 1 mole of oxygen molecules react to produce 2 moles of water ($2H_2 + O_2 \rightarrow 2H_2O$), while 0.02 moles of hydrogen molecules and 0.01 moles of oxygen molecules would react to produce 0.02 moles of water. Thus, if the overall volume is changed but the proportions are constant, the same behavior will be exhibited.

Or will it? What happens when you divide a cup of coffee? Do individual molecules never show themselves in our daily lives?

A Simple Question

AProfMiki: (…now that I've written this, I wonder how the students will react to it. Let's see.)

PasserbyBill: Is this college-level content? Everyone knows that. Is this old bat senile or what?

Miki: (Oh geez. I guess he's being so rude out of the gate because we're on the internet. Is he one of my students?) Bill, it seems that you read it. Does everyone know that, huh?

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Bill: (Oh, she read my post) We learn that in high school chemistry.

Miki: Are you a student? Yes, that's what the textbooks say, but haven't you ever thought there's more to it than what it's in the textbooks? Did you learn about how big the cells in living things are?

Bill: I don't know what biology has to do with chemistry, but I think I've heard that human cells are 20 μm or something like that.

Miki: Correct. The exact size depends on the cell, but it's normally about that size. So, how many molecules are there in a cell?

Bill: Uh, let me do some calculation…10 μm cubed is 10 to the minus 12th liters, and a liter of water is about 55 moles, so that's about 3×10 to the 25th molecules, so multiply those and you get 10 to the 13th…so there are tens of trillions. That's a lot.

Miki: Ooh, you're fast. Well done. That's about the total number. So, there are lots of water molecules, but how about DNA?

Bill: 46 strands, duh.

Miki: Right. For humans, there are 46 double-strands of genomic DNA (chromosomal DNA), but they're not all the same. How about proteins?

Bill: Proteins…you get them from eating beef and pork and stuff, so I guess there's a pretty good number?

Miki: Well, yes. If you take them all together, there are a lot of them. In human cells, there are a few billion proteins; bacteria are smaller, and *Escherichia coli*, for example, has a few million proteins [1]. But there are lots of types of proteins; for some types, there are only a few molecules. With *E. coli*, there's not even one molecule in the cell for some types of proteins [2]. In that case, you can't divide them in half.

Bill: But if there's only one of a molecule in the cell, it wouldn't really mean much, would it?

Miki: What about DNA? You only have one Y chromosome; nothing would change if it got destroyed?

Bill: Ah…but that's an extreme example. Come on.

Miki: Certainly, the reason why a difference in one molecule affects a lot of molecules is that DNA is a "template"; it's involved in lots of chemical reactions without ever changing itself. Have you ever heard of any other molecules that can be involved in lots of reactions without ever changing?

Bill: I learned that in chemistry. Things that don't change in reactions are catalysts. In living things, enzymes, yeah?

Miki: Correct. You could even say that cells are driven by enzymatic reactions. There are lots of them. If there's only a little of something at first, a small change could lead to a big difference.

Bill: Come on, what kind of difference? Isn't there some formula or something in a textbook?

Do Minorities Change Big Groups?

Miki: Unfortunately, there are all kinds of life forms and enzymes, so there's no convenient equation that you can apply to all of them. But you can think about this with a simple example, and there's even a theory that has been made.

Bill: "Theory" sounds complicated. Is it actually simple?

Miki: Like I said earlier, one molecule can have a big effect in an enzymatic reaction, so let's think about a model that incorporates that.

Miki: Now, you have two kinds of molecules, A and B. Let's think about autocatalytic reactions between them, $A + B \rightarrow 2A$ and $A + B \rightarrow 2B$; and let's say that the reaction rate constants, k, for both reactions are equal. Let's also say you have the non-catalytic reactions $A \rightarrow B$ and $B \rightarrow A$, and the reaction rate constants, u, for both reactions are also equal. Now, let's call the total number of molecules N and the volume of solution V (etc.) [3–5].

Bill: This doesn't sound simple at all.

Miki: Oh, sorry, I slipped into academic conference presentation mode. So…let's try thinking about it in terms of a game with players split into two teams (Fig. 1). Two players go one-on-one, and the loser joins the winner's team. Assuming both players are equally skilled and they both thus have a 50–50 chance of winning, if both teams start with the same number of people, what do you think will happen?

Bill: If everyone has a 50–50 shot, I guess the numbers wouldn't really change.

Miki: What about if you have two people? One on Team A and one on Team B.

Bill: How could you call those teams? If they play once, they'd end with 2 people on one team and 0 on the other.

Miki: Exactly. Sometimes, everyone on the same team will lose, and that'll be the end of it; the fewer people there are, the more likely it is. If the game ended like that, it'd be boring, so let's add a rule where you occasionally have a traitor who will jump to the other team. That's the model I was describing earlier.

Bill: What kind of stupid game is this? It makes sense for someone from the losing team to jump to the winning team, but if someone who was winning broke away alone, they'd get beaten up, wouldn't they?

Miki: It's possible the team could randomly go on a winning streak and start a revolution, isn't it? Actually, if the probability of betrayal is low, one team would win big for long, and things would occasionally get flipped. A 50–50 outcome would actually be quite rare. Of course, if everyone was a traitor, you'd just get a lot of coming and going between sides, and a 50–50 outcome would be pretty likely (heh).

Bill: This isn't a game at all, for crying out loud.

Miki: If the probability of betrayal is the same, then a 50–50 outcome is likely if you have lots of players; but if you have a small number of players, the outcome is likely to be skewed. Even if you only change the number of players without changing the rules, their behavior will change. If you have one traitor, the outcome could flip. This is an example of a "small number effect".

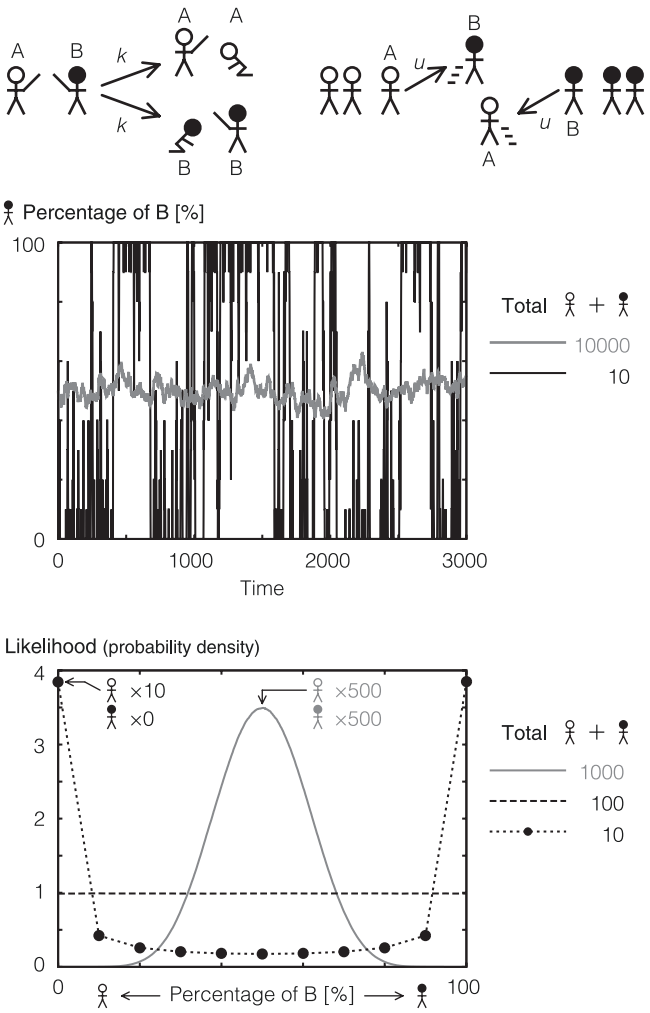

Fig. 1 (Top) Conceptual diagram of a model. (Middle) Changes in the percentage of B. With a large number of molecules (10,000), the percentages of A and B are roughly 50%. However, if the number of molecules is small (10), the percentages change drastically; the reactions cease for some time, when only one type of molecule remains. (Bottom) The "likelihood" (probability density distribution) of the percentages of B. When there are large numbers of molecules, the percentage is close to 50%; when the number of molecules is small, it becomes likely that only one type of molecule will be present (0% or 100%; because numbers of molecules are integers, the only values that can actually occur are those indicated with a ●, which occur at intervals). When there is an intermediate number of molecules (such as 100), all the percentage values are almost equally likely to occur (Based on the model in [5], with the parameters changed. Total concentration $N/V = 1$, $k = 1$, $u = 0.01$)

Bill: Well, I get it, but what does it have to do with living things?

Miki: I hear that a lot when I do research with these kinds of abstract models. It doesn't matter what A and B are as long as they follow the same rules, so similar things should happen everywhere. But I guess you still don't believe me. Let's try a more enzyme-like model, then.

Miki: Now, we have two types of molecules, A and B. Let's say we have their respective inactive forms, A and B; and their active forms, A^* and B^*. Both molecules undergo spontaneous activation, $A \rightarrow A^*$ and $B \rightarrow B^*$ (with respective reaction rate constants, r). The active forms react with each other, $A^* + B^* \rightarrow A + B^*$ and $A^* + B^* \rightarrow A^* + B$ (with respective reaction rate constants, s), which inactivates one of the molecules (etc.) [6].

Bill: The point being…?

Miki: Um. This time, you don't have people moving between two teams. Occasionally, some of them will suddenly activate…get pissed off, and then fight with someone on the other team who's also pissed off, and the loser goes quiet (Fig. 2).

Bill: Man, that got violent all of a sudden, gran…lady.

Miki: Meh. Enzymes switch between active forms, which participate in reactions, and inactive forms, which don't react; some enzymes are even saboteurs that would land us in trouble if they were always active. In any case, if both teams start with the same number of people, what do you think would happen?

Bill: I guess you're asking for the percentage of pissed-off people…active molecules. I should be able to write an equation for it.

Bill: If the concentration of A is [A], then the activation reaction rate is r[A]. For it to go back to its inactive form, you need one molecule each of A^* and B^*, so if you multiply those concentrations, I guess you get $s[A^*][B^*]$. Change stops when the going and the coming are the same, so you can connect them with = and solve the equation.

Miki: Exactly. You write the chemical reaction rates with the concentrations of the components, and the concentrations should stop changing when the activation and inactivation balance out. The total of the active and inactive molecules doesn't change, so you can solve the equation easily.

Bill: Yeah. But I had to do that a million times when I was studying for entrance exams, and now I hate it.

Miki: (Huh? If you are in college, you should have learned about differential equations. Connecting reaction rates with changes in concentration per unit of time,

$$\frac{\mathrm{d}\left[\mathrm{A}^*\right]}{\mathrm{d}t} = r\left[\mathrm{A}\right] - s\left[\mathrm{A}^*\right]\left[\mathrm{B}^*\right] = r\left(\left[\mathrm{A}_{\mathrm{total}}\right] - \left[\mathrm{A}^*\right]\right) - s\left[\mathrm{A}^*\right]\left[\mathrm{B}^*\right]$$

$$\frac{\mathrm{d}\left[\mathrm{B}^*\right]}{\mathrm{d}t} = r\left[\mathrm{B}\right] - s\left[\mathrm{A}^*\right]\left[\mathrm{B}^*\right] = r\left(\left[\mathrm{B}_{\mathrm{total}}\right] - \left[\mathrm{B}^*\right]\right) - s\left[\mathrm{A}^*\right]\left[\mathrm{B}^*\right]$$

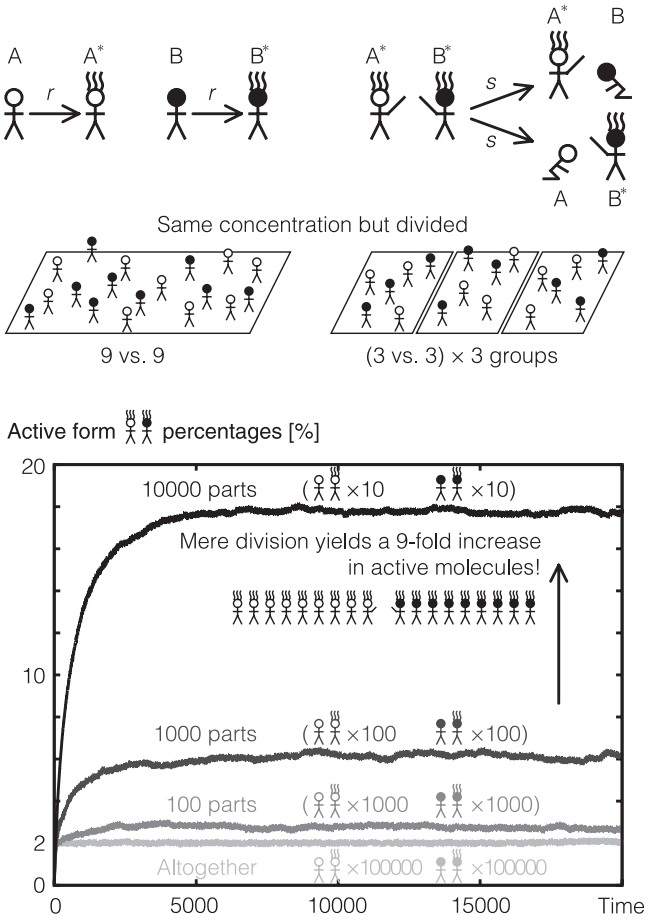

Fig. 2 (Top) Conceptual diagram of a model. Consideration of all molecules together in a large container versus division into equal parts. (Bottom) Changes in percentages of active forms (A^* and B^*) within all containers. First, all molecules were in an inactive form (A and B). When large numbers of molecules are grouped together, the percentage of active molecules is nearly 2%, as predicted by a reaction rate equation; however, when groups of molecules are divided into small parts, the percentage of active molecules increases (Concentration $[A_{total}] = [B_{total}] = 1$, $r = 0.0004$, $s = 0.98$)

(However, $[A_{total}] = [A] + [A^*]$ and $[B_{total}] = [B] + [B^*]$; they do not change). These are called reaction rate equations. If you solve them, not only do things balance out, but you can also follow changes.)

Miki: So, here, if you increase the number of people…that is, without changing the concentration, and you increase the volume (with players from both teams mixed well inside that volume), what will happen?

Bill: The reaction rate equations only have concentration in them, so nothing would change.

Miki: Correct. That means that whether you react them all together in a big container or divide them into a bunch of small containers, the combined result should be the same.

Bill: You're talking about the small number effect again. This time…huh?

Miki: It's hard to predict intuitively. It's easier to understand if you do a computer simulation.

Bill: Is that for solving the reaction rate equations you were just talking about?

Miki: Actually, when the number of molecules is small, instead of solving this equation, you have to follow each individual reaction in order. Which reaction occurs and when are determined using random numbers, as only the probability is known (to put it in an easier way, by rolling dice; this is called stochastic simulation).

Bill: I don't really get this randomness. It sounds like a hassle.

Miki: If it's only this, you can do it on a smartphone.

(This is going to get a bit technical. Assuming the contents are mixed well, the reaction rate will be determined by how many there are of each type of molecule, so it won't change until the next reaction occurs and the numbers of molecules change. The probability of a reaction occurring in the next second is the same as the probability that a reaction will occur in the next second after 100 s during which nothing occurs (conditional probability). In this case, the interval until the next reaction occurs follows an exponential distribution, so an exponentially distributed random number should be used to determine after how many seconds the next reaction will actually occur; you can skip the interval in which no reaction occurs.)

Miki: To make it easier to understand, let's say you have a situation where there are 100,000 people on team A and 100,000 people on team B, a situation where you divide them into 100 equal parts to get 1000 versus 1000; and a situation where you divide them into 10,000 equal parts to get 10 versus 10. We'll set it up so that the reaction rate constant is the same in all of those situations and when you consider it in terms of a reaction rate equation, the percentage of active molecules is 2%.

Miki: So when you do that simulation, it comes out like this (Fig. 2). Even though all we did was to divide the players into smaller groups, the percentage of active molecules increases for some reason. Even though the percentage of active molecules is only 2% when you have 100,000 versus 100,000, when you split them into 10,000 groups, the percentage is 9 times higher.

Bill: Why?

Miki: To use the metaphor I gave earlier, even if everyone on a team is pissed off, if they don't have any opponents, they stay pissed off while they wait. If you have a lot of people all together, there will be opponents somewhere; but if there are only 10 people, 2% means there are only 0.2 opponents. Oftentimes, there isn't anyone.

Bill: What a bunch of morons. Oh, molecules don't have brains or anything, I guess. But does that kind of thing happen in living things?

Miki: Haven't you ever seen a drawing of a cell in a biology textbook? There are lots of small things like pouches in a cell. If cells are divided into such small parts, that alone might be enough to cause changes in how reactions occur.

(By the way, we just considered a situation in which the contents in a pouch are mixed well, but there's also the issue of how molecules are distributed when they're not mixed well. A different small number effect may occur as a result of not only the overall minority of molecules but also low density (being sparse).

For example, let's say that enzyme X produces a separate molecule Y, but Y falls apart immediately and can't go far. If different Xs are far away from each other, Y will settle only around X. In this state, if reactions occur between two Ys or between X and Y, the reaction will be faster than it would if Ys were uniformly distributed [7, 8].)

Small Numbers of Molecules, Cells, Individuals…

Bill: By the way, are we only talking about stuff inside cells?

Miki: Good question. In my explanation, I used people instead of cells, but when you consider that molecules come together to form cells, which come together to form individuals, which come together to form societies and ecosystems, there can be similar issues among all of them.

Bill: For example?

Miki: There's an example where small number effects were discussed using a chemical reaction model to represent ant behavior [4]. Though you can't fully explain the relationships among various cells in an individual by saying they're the same as chemical reactions, it is possible for minority cells to stir something up. It's being researched as we speak.

Bill: Is calculation-based research like the stuff you explained earlier still going on?

Miki: Looks easy, doesn't it? It's been going on for more than 10 years, but there's still a long way to go before scientists come up with a theory that can be applied to situations in which all sorts of reactions are involved like in real cells, or before they connect a theory like that to what happens on the level of individuals or societies [9, 10]. I don't run experiments, but I often talk to people who do.

Bill: Then even if I were to jump in now, I guess there's still a lot of interesting stuff left to study. I've still got time before I take my college entrance exams, so I'll think about it.

Miki: Oh, so you *are* a high school student. There's still interesting stuff left, in between math and physics and chemistry and biology and geoscience and informatics and politics and economics…so study hard, and don't skip over anything just because you don't need it for your entrance exams.

Bill: Will do. Thank you much.

Miki: (Whew. I thought he was just an impertinent dope, but he knows a lot, and he can do really quick calculations. He might be an elite-level student. I hope he

comes to my lab while he's still got so much energy. Let me take a look at his profile…)

What the heck is this? "AI Student at High School. There's no one inside" "The latest artificial intelligence engine developed by our company is tweeting"?

Aaaah, I've been had. But he could start a revolution.

References

1. Milo, R., & Phillips, R. (2016). *Cell biology by the numbers*. New York: Garland Science.
2. Taniguchi, Y., Choi, P. J., et al. (2010). Quantifying *E. coli* proteome and transcriptome with single-molecule sensitivity in single cells. *Science, 329*(5991), 533–538.
3. Ohkubo, J., Shnerb, N., & Kessler, D. A. (2008). Transition phenomena induced by internal noise and quasi-absorbing state. *Journal of the Physical Society of Japan, 77*(4), 044002.
4. Biancalani, T., Dyson, L., & McKane, A. J. (2014). Noise-induced bistable states and their mean switching time in foraging colonies. *Physical Review Letters, 112*(3), 038101.
5. Saito, N., & Kaneko, K. (2015). Theoretical analysis of discreteness-induced transition in autocatalytic reaction dynamics. *Physical Review E, 91*(2), 022707.
6. Togashi, Y. (2014). Theoretical studies of reaction networks involving small numbers of molecules [in Japanese]. *Seitai no Kagaku, 65*(5), 450–451.
7. Shnerb, N. M., Louzoun, Y., et al. (2000). The importance of being discrete: Life always wins on the surface. *Proceedings of the National Academy of Sciences of the United States of America, 97*(19), 10322–10324.
8. Togashi, Y., & Kaneko, K. (2004). Molecular discreteness in reaction-diffusion systems yields steady states not seen in the continuum limit. *Physical Review E, 70*(2), 020901.
9. Togashi, Y., & Kaneko, K. (2001). Transitions induced by the discreteness of molecules in a small autocatalytic system. *Physical Review Letters, 86*(11), 2459–2462.
10. Nakagawa, M., & Togashi, Y. (2016). An analytical framework for studying small-number effects in catalytic reaction networks: A probability generating function approach to chemical master equations. *Frontiers in Physiology, 7*, 89.

The Personality of Small Numbers: Do Molecules Have Personality?

Tamiki Komatsuzaki

In high school physics, you learn about various subjects, from the movement of objects in terms of speed, acceleration, and the law of conservation of energy to thermodynamics, which explains congregations of lots of particles (~10^{23} particles). On the one hand, in the case of the former, a situation (state) at a given moment is unambiguously determined by the object mass, position, speed, and so on. In thermodynamics, on the other hand, you learn that this state is determined by temperature, pressure, volume, and the number of particles, all of which are shockingly small variables. When lots of particles assemble, the behavior of individual particles (molecules and atoms) is said to be similar to that of an individual in a crowd, meaning that the molecules do not have a unique behavior. But is that true? This chapter features a conversation between Yuu, a 17-year-old high school girl in Sapporo who is busy with her school's basketball team; and her father, who researches life sciences and chaos in a university. Through their conversation, we'll take a look at the latest research suggesting that molecules may have individual personalities after all.

What Is Personality?

Yuu: Dad, you said before that you study chaos, and now you're studying life sciences. What the heck are you researching?

Dad: Ah, yes. I'll tell you about what I've been researching lately: the possibility that molecules have personality.

Yuu: Sounds kinda interesting. Personality is easy to picture with a person or an animal, but molecules having personalities? That doesn't sound like it has anything to do with chaos.

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Dad: I'll get to its relationship with chaos a bit later. First, we have to define "personality". When do you use the word "unique"?

Yuu: With people, if someone is unique, I guess it means that there's something about them that's different from normal people.

Dad: Can you think of another word that's easy to understand that represents a normal person?

Yuu: You'd say they're average. Like, in basketball, next to someone with average height, someone who's super crazy tall has advantages that other players don't have (like being able to dunk). I guess you could call that unique.

Dad: Right you are. Also, if you want to find the average height of the players, it would be difficult to judge who is "uniquely" tall if you only have a sample of 2–3 players; you'd need to tackle the question in terms of a difference from the average with a large sample of tens, hundreds, or thousands of players.

Yuu: Basketball is a sport in which your team scores by shooting and you defend against the other team's shots under a constant set of rules, but even if players have certain unique factors, I guess they're not always necessarily relevant to the game.

Dad: Nice perspective. For instance, the length of a player's hair has no effect on the outcome of the game, so a player with hair a meter long and a player with a shaved head might be unique, but they have nothing to do with winning or losing a game.

Yuu: The conversation has been normal so far, and I get it, but what does that have to do with the personalities of molecules?

Dad: Molecules also have aspects that correspond to the outcome of a game. I want you to think back to chemical reactions you learned about in chemistry class. Let's use enzymatic reactions as an example. In an enzymatic reaction, an enzyme molecule E meets a substrate molecule S and helps to convert it into another molecule, P. The molecule E itself doesn't become another molecule. Instead, its function is to help and is called "catalyzing (the reaction)". Here, the "outcome of the game" for the enzyme molecule is which enzyme molecule E can help substrate molecule S be converted into molecule P the fastest.

Yuu: But even though they're all enzyme molecules, if the numbers or kinds of atoms or the chemical bonds are different, their abilities to help would be different too, wouldn't they?

Dad: Right. But what I mean here by the personality of a molecule is whether it can "win games often" despite having the exactly same composition and chemical bonds as another molecule; in other words, there may be some molecules with the same composition and chemical bonds that convert the substrate molecule to another molecule quickly, and some that don't.

Yuu: I had thought they were all the same.

Dad: Then, I'll try to explain how researchers assert that molecules may have personalities.

Do Molecules Have Personalities?

Dad: Over the past dozen years, advances in genetic engineering have made it possible to detect how molecules change shape by rigging a (specific wavelength of) light to be released whenever the molecule changes its shape.

Yuu: What kind of experiments do they do about molecules possibly having personalities?

Dad: A group led by Professor Xie at Harvard University was able to observe enzymatic reactions, I just talked about, in solution at the level of a single molecule [1]. To be specific and as shown in Fig. 1, the enzyme molecule called β-galactosidase is immobilized on a bead so that it doesn't "run away" from the region being irradiated with light and observed. Also, β-galactosidase reacts with a substrate called resorufin-β-D-galactopyranoside, which moves around a lot in solution, to convert it into a light-emitting molecule called resorufin. The names of the molecules aren't important, so we'll call the enzyme molecule E, the substrate molecule S, and the resorufin generated from the reaction P*. The generated molecule P* diffuses and immediately leaves the small region that is being observed; from the point of view of the observer (who is focused on the region of observation), the light vanishes immediately after it appears and does not appear again until another molecule S interacts with the enzyme molecule E and is converted into molecule P*. The interval without light corresponds precisely to the waiting time of the reaction (of enzyme molecule E).

Yuu: So, it's just like cormorant fishing. The cormorant lights up at the "moment" it catches a fish, and it's dark until it catches the next fish. The cormorant fishing master ties a rope to the cormorant so it doesn't escape; he plays the role of the bead.

Dad: Exactly. That's a good, clear analogy. I'll use it in my class sometime. To add to it, the bead itself is attached to a glass coverslip. I guess it would correspond to the boat that the fishing master is in. So, Professor Xie's group investigated the

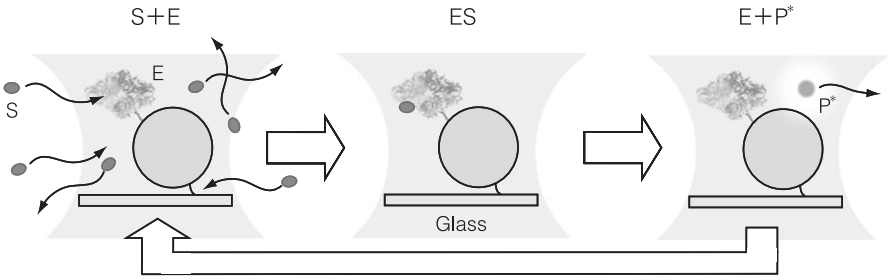

Fig. 1 A single enzyme E is immobilized on a coverslip in the confocal detection volume and immersed in a solution with a constant concentration of substrate S. (S + E) The diffusion stage in which a freely moving nonfluorescent substrate S searches the catalytic sites of the enzyme E. (ES) The enzyme-substrate complex is formed. (E + P*) The bound substrate is converted into the fluorescent product P*, then quickly diffuses out from the confocal detection volume

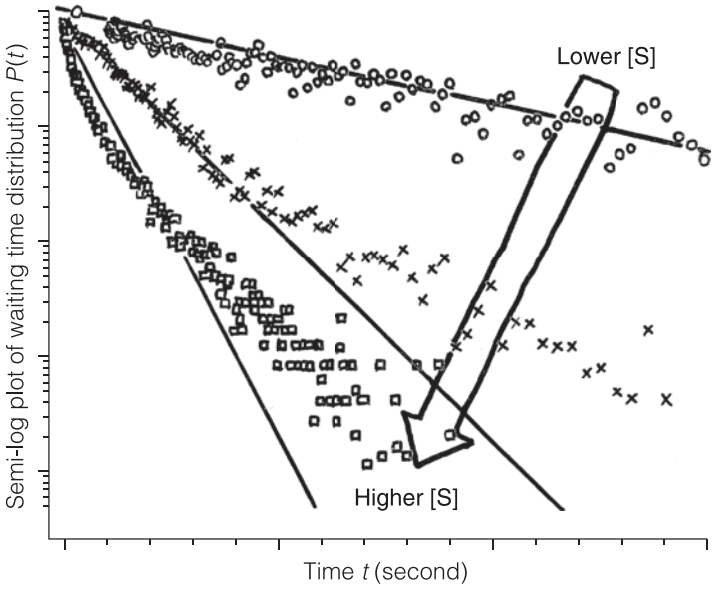

Fig. 2 When substrate concentration increases, the waiting time distribution turns to non-single exponential distribution from a single exponential distribution

distribution of waiting times of one enzyme molecule E, and they examined the correlations between waiting times by changing the concentration of substrate molecule S. As shown in Fig. 2, Professor Xie's group created a semi-log plot with waiting times, t, on the x-axis, and the distribution of reaction waiting times (histogram), $P(t)$, on the y-axis. Assuming that the waiting time distribution is an exponential distribution, $P(t) \approx Ae^{-\gamma t}$ (where A and γ are constants), which conforms to an exponential function, the semi-log plot should be a straight line with a negative slope $-\gamma$. In other words, Fig. 2 shows that as the concentration of substrate molecule S increases, waiting times change from an exponential distribution to a non-exponential distribution. Professor Xie's group also discovered that significant positive correlations emerge between one waiting time and the next.

Yuu: Huh. How does that tell that molecule E has personalities?

Dad: Hey now, hold your horses. To put it plainly, the reaction waiting time is determined by the (diffusion) time taken for substrate molecule S to encounter enzyme molecule E and by the (reaction) time required from the encounter to the reaction; so, the distribution is predicted to reflect that. Assuming you'll learn the mathematical background in college, having an exponential distribution means there's one (effective) timescale that "represents change", while having a non-exponential distribution means there are two or more such timescales.

Yuu: But, if there's an exponential distribution when the concentration of substrate molecule S is low, doesn't that contradict having multiple timescales, like for diffusion and reaction times?

Dad: A low concentration of substrate molecule S means that there isn't much of substrate molecule S around enzyme molecule E, so it takes much longer for substrate molecule S to encounter the enzyme molecule E. In other words, the diffusion for this encounter happens at the slowest overall speed, so there's effectively only one timescale. On the flip side, when the concentration of substrate molecule S is high, diffusion doesn't necessarily determine the distribution of waiting times, so it ends up being a non-exponential distribution.

Yuu: What does it mean that there's a positive correlation between one waiting time and the next?

Dad: It means that if the waiting time for an enzymatic reaction at a given "point" in time is short (or long), the waiting time at the next "point" in time will also be short (or long). Significant positive correlations only happen when the substrate concentration is high. Professor Xie's group figured that, assuming that the time scale of reaction S→P* depends on the shape of enzyme molecule E at that "point", there could be multiple timescales, which means the conversion time could be explained with a non-exponential distribution.

Yuu: In the cormorant fishing analogy, it would mean that the cormorant posture would determine how fast it catches a fish, right?

Dad: Your explanations are better than mine and really easy to understand! When the substrate concentration is high, the next substrate molecule S often arrives before the enzyme molecule E can change its shape, so from the point of view of enzyme molecule E, another substrate molecule S suddenly comes in when it's trying to change its shape, and it ends up reacting with the new substrate molecule S with almost the same shape as before. The reaction time can change depending on shape, so the interpretation was that the distribution of reaction waiting times is non-exponential with positive correlations between one reaction waiting time and the next.

Yuu: I see. So "the personalities of molecules" refers to molecules with different shapes, and it means the shape determines how fast it converts substrate molecule S. In other words, shape is the key to "winning at the molecule game".

Dad: That's right. Also, the reaction time has to be faster than the timescale for the molecule to change its shape. If it's not, there won't be any correlation between one reaction waiting time and the next.

Molecular Personalities Inside Cells

Yuu: But if the number of enzyme molecules is about equal to Avogadro's number, then with the issue of which molecule would promote the fastest reaction, i.e., which molecule would "win the game", they'd all be averaged out, so it seems that there's no point in discussing the personalities of molecules.

Dad: Exactly. That's terrifically insightful. In everything we've talked about so far, we've ultimately been considering a single enzyme molecule. In high school chemistry class, you learn the concept of concentration, i.e., the number of

molecules in a unit of volume (e.g., 1 liter), right? The concept of concentration presumes the congregation of a whopping 10^{23} molecules, so differences in the shapes and movement of individual molecules, i.e., the personalities of the molecules, are considered to be averaged out.

Yuu: I totally get it now. To turn it around, if you have a group of way less than 10^{23} molecules, individual personalities might show themselves.

Dad: Yes, exactly! Researchers are working hard every day to shed light on that issue as we speak. In 2010, a man named Taniguchi published a paper with Professor Xie and colleagues about their work on actually counting out the numbers of individual proteins in *Escherichia coli* [2]. They found that the average number of each type of protein per *E. coli* cell only ranges from 0.1 to 1,000, which is far smaller than Avogadro's number; in other words, the fact that the average number of a given type of protein molecule per group of *E. coli* is less than 1 means that some *E. coli* cells don't have that type of protein in it at all.

Yuu: So that means if you look at proteins inside a cell, you might see the personalities of different molecules!

Dad: That's right. But there are still technical difficulties in observing changes in the shapes of individual molecules in a cell; science hasn't got to the point where we can do that. My colleagues and I recently developed a technique called data science that uses observed data to abstract the number of molecules, the different shapes they take, and how fast they move back and forth based on the patterns of light flickers emitted by the molecules [3].

Yuu: I feel like I finally understand your work a little. I'll ask you about data science another time. You said your work has to do with chaos, but it hasn't come up in this conversation yet.

Dad: I told you that if you have a number of molecules equal to Avogadro's number, their personalities may be averaged out, and the situation (state) of the congregation of molecules can (probably) be expressed with concentration. To be precise, a massive number of molecules equal to Avogadro's number is not important in itself. What matters are the complex collisions among the molecules, the way the molecules change shape because of those collisions, and other complex interactions among the molecules; thus, the point is that *the behavior of individual molecules is completely impossible to predict*.

Yuu: It doesn't really make sense to me.

Dad: That's understandable. After all, in the motion of objects you learn about in high school physics, you learn that if you determine mass, position, velocity, and force (which emerges as a result of interactions), you can solve and accurately predict the position and velocity of an object after a certain amount of time, t. "Solvable" here refers to the solution for an equation of motion; for instance, position $x(t)$ with respect to time t can be expressed as a function of t. For example, the motion of a pendulum is "solvable", so even if you have a congregation of Avogadro's number of pendulums, you can completely predict their behavior at any given time. But in the real world, cases like that are very rare; unsolvable motion equations are far more common. The concept that establishes this is "chaos". However, chaos can be strong or weak. When it's strong, the detailed

behavior of individual molecules is utterly impossible to predict; and expressions in terms of probabilistic descriptions, i.e., the number and density of molecules per unit of volume, are justified. However, chaos isn't always strong. So, there's a stochastic view in which probabilistic descriptions are justified, and there's a deterministic view in which chaos isn't so strong, but the truth must be somewhere in the middle.

Yuu: If there are differences in the level of chaos, does that mean that individual personalities are apparent in some groups of molecules and not apparent in others, even if those groups have the same number of molecules?

Dad: That's a great question! I think that's correct. But no one has figured it out for sure yet.

Yuu: One more thing, dad: why did you get interested in the personalities of molecules?

Dad: In human society, I think there's more potential for development in the overall system when you have an appropriate number of unique individuals than when you have a disjointed mass of nothing but uniform, average people.

Yuu: You could say the same about the make-up of a basketball team. The team is better overall when you have all kinds of unique players.

Dad: I think the question of the roles that unique small numbers of things play and how they form systems with potential for development is interesting. I like your unique traits more than anyone. As you experience college and adult life and learn the rules of the world, I want you to develop your unique personality.

References

1. English, B. P., Min, W., et al. (2006). Ever-fluctuating single enzyme molecules: Michaelis-Menten equation revised. *Nature Chemical Biology, 2*, 87.
2. Taniguchi, Y., Choi, P. J., et al. (2010). Quantifying *E. coli* proteome and transcriptome with single-molecule sensitivity in single cells. *Science, 329*, 533.
3. Li, C.-B., & Komatsuzaki, T. (2013). Aggregated Markov model using time series of single molecule dwell times with minimum excessive information. *Physical Review Letters, 111*, 058301.

Distinguishing and Searching for Minority Cells: Small in Number, But Large in Effect

Katsuyuki Shiroguchi

The human body is believed to be made up of approximately tens of trillions of cells. Many cells are tens of micrometers in size; thus, given that the human body is more than a meter in size, it makes sense that there would be so many cells. Now then, among these many cells, very small numbers of cells (in some cases, even one cell) can sometimes exert massive effects. For instance, there is the idea that there are a few immune cells of a certain type with special properties that act to effectively protect the body from foreign substances. There is a body of research that focuses on such cells, which are important despite being a minority. Here, a baseball-loving junior high school student Rick and his father have a conversation about the nature of cells that are important despite being minorities, and how to distinguish minority cells.

(One afternoon during summer break, Rick's father was on the internet)

Dad: Hey, Rick! There are apparently cells in the body that can destroy cancer cells. Did you know that?

Rick: Nope. Wait, then why do people get cancer?

Dad: Haha, such a pointed question out of nowhere. It's because there apparently aren't many cells that can destroy cancer cells.

Rick: Huh? People get cancer because there aren't many cells that can destroy it? That's kind of confusing. But it's good to know that we have these cells in the first place. Can't they put them to good use to treat cancer?

Dad: I wonder. Why don't we look into it a little more together?

Rick: Huh? Now? You said you'd play catch with me. But it sounds interesting, and it might be perfect for my summer research project, so why not?

Dad: Alright, let's do it!

Rick: T cells that kill cancer cells??? What are T cells?

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Dad: Heh heh. I know what they are. I learned about them in biology in college. T cells are a type of immune cell. You know what immunity is, right?
Rick: That thing where if you get a disease once, you won't get it again?
Dad: Well, yeah, that's immunity too. T cells have an important function in the immune system.
Rick: What kind of function?
Dad: Huh? Hang on, hum…
Rick: Come on, dad, didn't you study biology?
Dad: Well, that was a long time ago (heh). Hey, here it is. It says T cells are basically individual cells that each have different receptors. The receptors are apparently the key.
Rick: What's a receptor? (Fig. 1)
Dad: It's apparently a protein on the cell membrane whose shape and properties are determined by a gene sequence. Do you know what a gene sequence is?
Rick: I've heard of genes. Are you talking about a bunch of A, G, C, T, and whatever all lined up?
Dad: Right, right. The order of those A's, G's, C's, and T's determines the properties of the receptor. What I'm saying is that there are lots of T cells in the body, and their properties are determined by the sequences of the genes that make up their receptors. The key point here is that only cells with special receptor sequences can attack cancer cells.
Rick: No kidding. That was a pretty good explanation, dad (heh). So, there are T cells that can attack cancer cells, but there aren't many of them? Can't they use those cells for treatment?
Dad: Oh, right. Actually, some people are thinking of searching for these T cells that can attack cancer and using them in treatment.
Rick: I thought so! It would be awesome if they could!
Dad: It would. But it doesn't seem so easy.
Rick: Why not?

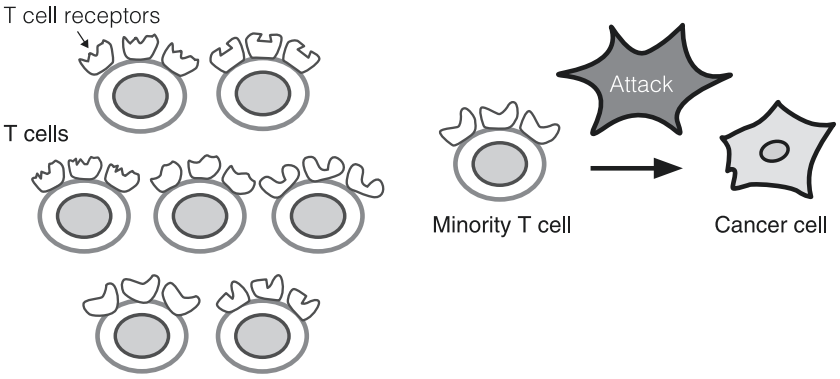

Fig. 1 There is a minority of T cells that attack cancer cells

Dad: Because there are lots of T cells, and it's apparently difficult to distinguish which ones can attack cancer cells. It seems that there is only a few of them.

Rick: It's really something that a minority of cells could mean the difference between life and death. I wonder how you can distinguish them.

Dad: With T cells, apparently, one way of doing it is to examine the receptor gene sequences that determine the properties of the cell. You determine the sequence of genes with a method called "gene sequencing". By the way, we owe our ability to sequence genes to the great work of a man named Frederick Sanger. He won the Nobel Prize in Chemistry in 1980. Gene sequencing is also referred to as the Sanger sequencing method.

Rick: Huh. So you mean you should use the Sanger method to determine the receptor gene sequence of a T cell to figure out whether it can attack cancer?

Dad: Right. But with a receptor gene sequence, even in the important part alone, there are apparently about 500 A's, G's, C's, and T's lined up in all; if even one of them is changed, it apparently changes the properties of the receptor.

Rick: Then you have to determine the sequence without even one mistake? That sounds really hard. But it's important to be able to distinguish cancer-killing T cells properly, isn't it?

Dad: It is, but aren't you forgetting an important point?

Rick: Huh? What is it?

Dad: That there is only a small number of T cells with receptors that can attack cancer. To find them, you have to sequence the receptor sequences for a whole lot of T cells.

Rick: Oh, yeah. You have to find a small number of cells among a whole lot of cells, don't you? That sounds even harder. But can't you do it with the Sanger method?

Dad: No, because it apparently takes time. Instead, there's a recently developed next-generation sequencer that apparently lets you examine lots of T cell receptor sequences in just a few days. To give you an example, the sequencing of the entire human DNA sequence with the Sanger method would have taken more than 10 years. But with the next-generation sequencer, you can do it in about a week.

Rick: That's amazing. It seems that research will jump ahead light-years in one shot.

Dad: It does. I feel like this technological revolution could change the world. Actually, thanks to this next-generation sequencer, we're starting to be able to distinguish these minority T cells.

Rick: Huh. Technological revolutions are incredible. So, the age of finding cancer-attacking minority T cells left and right is just around the corner.

Dad: No, I think there's a big difference between "we've done it" and "we're starting to do it". Strides in research are happening every day, but it's going to be a while before we're all the way there, and I'd imagine researchers are working their hardest towards that goal.

Rick: I wonder when they'll be able to do it.

Dad: It depends on how hard researchers work from here on out.

Rick: Huh. It seems like worthwhile research. Research that's useful to people is cool.
Dad: Why don't you try your hand at it?
Rick: Naah. For now, I'll think about it while we play catch. Are we going to play or what?
Dad: Let's do it!
(Rick and his dad are taking a break)
Dad: You've gotten better. Do you like baseball?
Rick: Yeah. Especially pitching.
Dad: By the way, we were just talking about distinguishing important T cells, but it's important to distinguish cancer cells too, you know.
Rick: I agree, but it seems that it'd be hard to find the first cancer cell. After all, humans are made up of lots and lots of cells, right?
Dad: In cancer research, one of the things they're focusing on is cancer cells that have to do with metastasis. If a single cancer cell goes around in the blood and winds up somewhere else, cancer cells will increase in that other place, too. That's what you call recurrence.
Rick: …Can't they just find the cancer cell as it's going around in the blood and take care of it?
Dad: That's the goal researchers are working towards. First, there's the issue of how to distinguish the cancer cell.
Rick: Huh. How are cancer cells different from regular cells?
Dad: I told you a little about this earlier, but apparently, there are differences in the DNA sequence, which is represented by the order of A's, G's, C's, and T's.
Rick: In that case, they should figure that out. Just a minute ago, you said that they can examine human DNA in about a week, right? How many A's, G's, C's, and T's are connected altogether in human DNA?
Dad: 3 billion.
Rick: Say whaaaaat!? One, ten, a hundred, a thousand, ten thousand, a hundred thousand, a million, ten million, a hundred million, a billion. That's 10 digits. Wow, that's a lot. But it's amazing they can figure all that out.
Dad: But for a while, they've been able to use ten thousand or a hundred thousand cells with the same DNA sequence to examine all 3 billion bits of the DNA sequence. But if you're trying to distinguish cancer cells, you have to examine 3 billion A's, G's, C's, and T's from one or two cells.
Rick: Huh. That's even harder.
Dad: But they recently figured out a way to do it [1, 2]. It's amazing that they can distinguish a single cell, isn't it? Even when there are cells with different sequences mixed in, they can examine the differences in each cell like, "this cell has this sequence, and that cell has that sequence". That's how they can find cancer cells.
Rick: Wow. It's awesome that they can figure that one with a single cell.
Dad: It's the same with cells that might metastasize. They don't always know from the start that it's a cancer cell, so they have to examine as many cells as possible. Just like the T cells I was telling you about earlier.

Rick: Isn't it hard to examine the whole 3 billion-base sequences of so many cells? Can the next-generation sequencer do that too?

Dad: As you might expect, it's apparently impossible. So when they want to examine a thousand cells or more, they focus on a hundred-base or thousand-base part of the sequence that's liable to change when a cell becomes cancerous.

Rick: I see. But it still sounds hard to examine a thousand or ten thousand cells one by one. Wouldn't the cells get mixed up, and then you'd lose track of which cell the sequence was from?

Dad: You're perceptive. To avoid that, a hundred-base part of the sequence, for example, from the same cell has an artificially prepared identical DNA sequence attached to it [3, 4]. They examine both this attached DNA sequence and the DNA sequence from the cell simultaneously. This attached DNA sequence is called a barcode. At supermarkets and places like that, you often see barcodes stuck on the merchandise, right? They're used to count the number of the same item and to differentiate between similar items. In the same way, DNA barcodes are used to differentiate between cells when sequencing genes (Fig. 2).

Rick: Huh. So they developed a method for examining lots of cells at once, too. Wouldn't it be amazing if they could use all these techniques to find the first cell to turn into cancer, not metastatic cancer cells?

Dad: It would. But it's apparently still hard to find the first cancer cell in the body.

Rick: But they keep being able to do more and more new things, so they might be able to do pull it off someday.

Dad: You're right. It's important to never give up and to work hard with the belief that you can do it. Just like with practicing baseball!

Rick: Ah ha ha. Maybe so. Now that you mention it, I guess a small number of cells having a big effect is like a pitcher. The pitcher's performance has a huge impact on the game.

Dad: You're right about that.

Rick: But if the pitcher is struggling, it's important for the team to band together and to help him out. They can cheer him on and try harder on defense and stuff like that. Maybe there are cells like that.

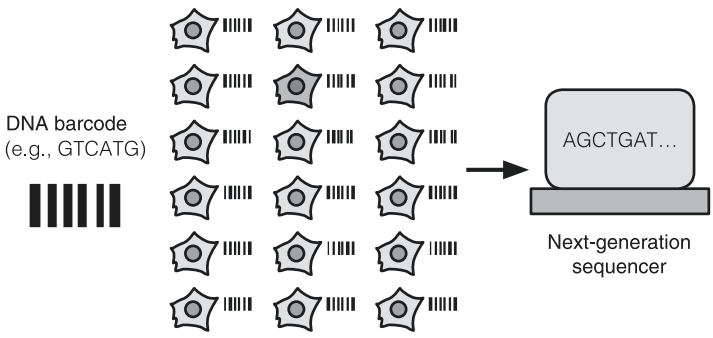

Fig. 2 Cells are labeled with barcodes to distinguish minority cells

Dad: That's an interesting notion. There might be different types of cells around the important minority cells that protect and help the important cells. Maybe there's research being done on those kinds of cells too.

Rick: Minority cell research seems important, but next time, I want to pitch, so catch for me!

Dad: Yes sir. You sure do love baseball. It's good you found something you love. You need to thank your manager and your coaches.

Rick: I do. They taught me the importance of respect and teamwork too!

References

1. Zong, C., Lu, S., Chapman, A. R., & Xie, X. S. (2012). Genome-wide detection of single-nucleotide and copy-number variations of a single human cell. *Science, 338*, 1622–1626.
2. Ni, X., et al. (2013). Reproducible copy number variation patterns among single circulating tumor cells of lung cancer patients. *Proceedings of the National Academy of Sciences of the United States of America, 110*, 21083–21088.
3. Shiroguchi, K., Jia, T. Z., Sims, P. A., & Xie, X. S. (2012). Digital RNA sequencing minimizes sequence-dependent bias and amplification noise with optimized single molecule barcodes. *Proceedings of the National Academy of Sciences of the United States of America, 109*, 1347–1352.
4. Ogawa, T., Kryukov, K., Imanishi, T., & Shiroguchi, K. (2017). The efficacy and further functional advantages of random-base molecular barcodes for absolute and digital quantification of nucleic acid molecules. *Scientific Reports, 7*, 13576.

Digital Bioanalysis

Hiroyuki Noji

Molecules are grains. There's no such thing as 1.5 molecules. Until now, there has been no need to be concerned with this graininess (discreteness) in bioanalysis. However, as micro/nano processing technology has recently become easily available, we can now get a sense of this graininess, which is now used in a new set of measurement methods termed "digital analysis". This chapter introduces digital analysis. Just how do you feel this graininess? Is there anything to be gained by this graininess? We will get a glimpse into digital analysis through a dialogue between Hiro, a university professor researching digital bioanalysis, and his son Tomo. Tomo is bored whenever his father gets that self-satisfied look and wants to explain something about his work; he likes video games much more than science. On top of that, he's right in the middle of his rebellious phase. Will he listen to everything his father has to say?

What Is Digitalization?

Hiro: Tomo, let's talk about digital bioanalysis. You know, what I research.
Tomo: Huh? Where did this come from? I didn't ask for it, and I'm playing a video game, so leave me alone.
Hiro: Professor T edited this book; he says I should introduce research in a dialogue format, so be my partner. Come on.
Tomo: Man, buzz off. And what the heck do you mean by "digital" anyway? If it's just processing data measured with a computer, you don't need to go out of your way to say "digital".
Hiro: Do you know the original meaning of "digital"? It means discretizing data to process it. "Discretizing" means temporarily converting data to values separated

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by fixed intervals. A classic example of discretization is rounding off numbers with decimals into integers. Your video game console temporarily discretizes your operation of the controller to process the information. All the information is represented in binary format as zeros and ones. This is called binarization…

Tomo: Ugh, here we go again. I know all that already (but only vaguely). This is boring. I'm going to play my game.

Hiro: Come on, listen. Please?

Tomo: Alright, back off. I'll give you just 5 minutes.

Hiro: Alrighty then. So, digital bioanalysis means digitalizing (binarizing) signals from the molecule you want to measure as zeros or ones and measuring the number of "one" signals.

Tomo: ?? What do you mean by "signals from the molecule you want to measure"?

Hiro: It can mean a lot of things. For instance, if there's some fluorescence clinging to the molecule itself, the light is the signal.

Tomo: I guess you're talking about GFP (green fluorescent protein).

Hiro: Atta boy. It's the protein that Osamu Shimomura won a Nobel Prize for discovering. That's not what I want to talk about, though. Today, I want to talk about enzymes. Do you know what an enzyme is?

Tomo: Yeah, I do. Like digestive enzymes, right?

Hiro: Right, they're proteins that catalyze certain chemical reactions. An enzyme makes a certain molecule (called a substrate) go through reactions until it becomes a different molecule. You learned at school about amylase, which breaks down the starch in rice into glucose. If you take the molecule that the enzyme acts on and, say, make it emit fluorescent light, you can know where the enzyme is.

Tomo: Huh. But you might want to measure something other than an enzyme, wouldn't you?

Hiro: Right. But, as I'll get into later, you can use enzymes to detect molecules other than enzymes. Listen until I'm finished, okay?

Tomo: Remember, I said 5 minutes. So what does that have to do with binarization?

Hiro: If you set up a beaker that has an enzyme in it and another beaker with no enzyme, then in the beaker with the enzyme, reactions will occur, and the fluorescence will get stronger and stronger; but in the beaker with no enzyme, there won't be any light. You binarize the outcomes based on the intensity of the fluorescence like this: if there's light, that's a 1; if there's no light, that's a 0.

Tomo: I already knew that. That's a normal experiment. What's new about it?

Hiro: This is done with a single enzyme molecule. When there is 0 molecule, there's no signal, so the value is 0; when there's 1 molecule, there's a signal, so the value is 1.

Tomo: I'm not sure, but an enzyme molecule is super-duper small, isn't it? Can you detect it?

Hiro: You're sharp. And you're right. Normally, it's difficult. This is where my technique comes in.

Tomo: (Here we go again. He's going to brag about his research)

Smaller Is Better

Hiro: An enzyme is an efficient catalyst,[1] but it can only catalyze reactions at an average of 10 times per second. In other words, when you can only use a single enzyme, it can only yield 10 reaction products in 1 second. For instance, let's say you have 1 enzyme in 1 cubic centimeter of solution (= 1 mL or 1 cc). Even if you were to wait for 1 minute, concentration of the reaction product would be only 1 aM (a = atto, 10^{-18}), or 10 to the negative 18th power moles per liter. There's no apparatus that could detect that.

Tomo: I don't understand 10 to the negative 18th power, but anyway, it's a super-duper low concentration, right? Then, you should increase the concentration.

Hiro: And how can you do that?

Tomo: You should just speed up the enzymatic reaction, right?

Hiro: That sounds good, but it's hard to make an enzyme work faster. For example, even if you raise the temperature by 10 degrees Celsius (18 degrees Fahrenheit), this would normally only double the speed. So how can you increase the concentration more dramatically?

Tomo: Just increase the temperature, duh.

Hiro: The enzyme would fall apart like a soft-boiled egg.

Tomo: Then you should reduce the volume.

Hiro: Ding ding ding ding ding!

Tomo: That's pretty simple.

Hiro: Indeed it is. But you have to make the volume dramatically small.

Tomo: How small?

Hiro: About the volume of a bacterium. 1 mL is 1 cubic cm, but a bacterium is 1 μm (1/1000 mm), so 1 cubic μm is 1 femtoliter (fL). Femto is 10 to the negative 15th power. With a volume that small, 1 enzyme left alone for 1 minute will yield 1 μM (μ = micro, 10^{-6}) of reaction product (Fig. 1). You might not be familiar with that unit itself, but if you hear that it's 10^{12} times[2] more concentrated than what I just mentioned, you can understand the effect intuitively. If the reaction product has fluorescence, you can see it easily with a microscope.

Tomo: Okay, so how do you make a tiny beaker?

Hiro: There are lots of different ways, but I only have 5 minutes, so I'll tell you how I do it. I make small drops of water in oil and stick them to a glass substrate.

Tomo: Small drops of water in oil, huh? Like salad dressing?

Hiro: Oh ho ho, that's my boy! You're really catching on. Yes, if you mix water and oil and shake it, you can see lots of small drops of water. Really tiny ones.

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[1] General term for a substance that dramatically accelerates a specific chemical reaction. Includes everything from manganese dioxide, which accelerates the breakdown of hydrogen peroxide, to enzymes. It does not increase or decrease in volume from before to after the reaction, and it itself does not change.

[2] The length of a side of the reaction vessel has shrunk from 1 cm to 1 μm, i.e., 10^4 times; thus, the volume has been reduced by the third power of that.

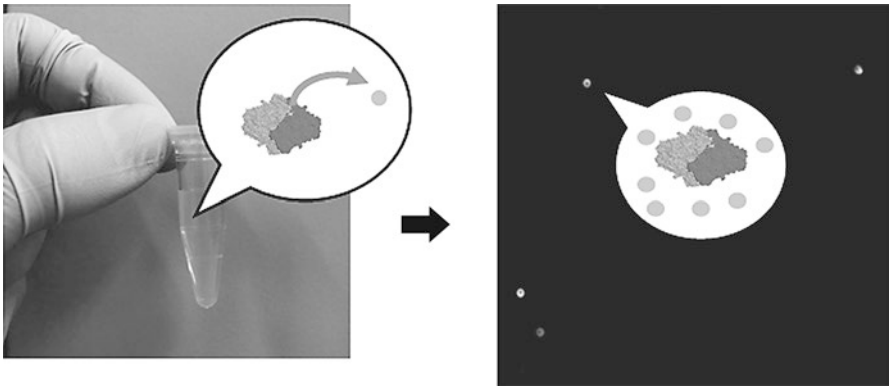

Fig. 1 Concept of single molecule detection of enzymes by downsizing the reaction volume. Conventional reaction tubes are too large for the single molecule detection (left). When the reaction mixture is partitioned into an extremely small reactor like 1 fl that is a cube of 1 μm and smaller than the conventional test tube by the factor of 10 to the power of the minus 12th, the detection should be feasible (right). Even if the enzyme solution is largely diluted, one can find enzyme molecules as fluorescent reactors

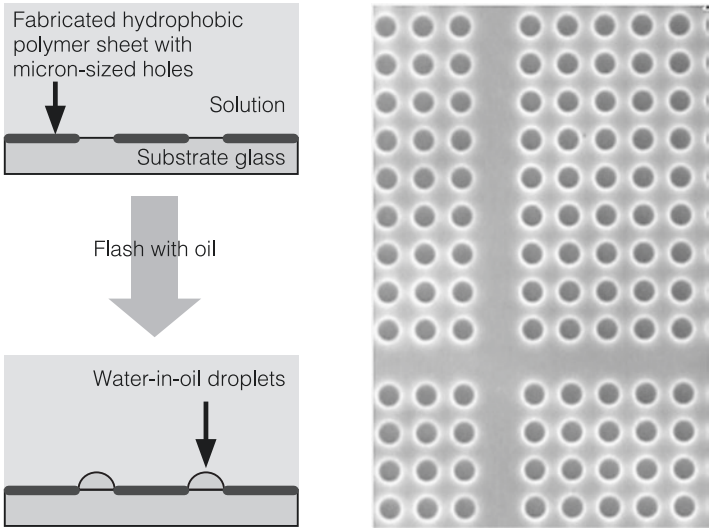

Fig. 2 Schematic image of micro droplet preparation (left) and the optical microscopic image viewed from top (right). The diameter of the droplet is 5 μm

Tomo: (It's fine that he's praising me, but it doesn't look like he's going to wrap this up anytime soon) How do you stick them on to the glass substrate?

Hiro: You get a glass substrate and, on the surface, you apply a very thin coat of a substance that gets along with oil (Fig. 2). Then, you make very small holes in that substance to partially expose the surface of the glass. The surface of the glass

gets along with water, so that's enough for water droplets to stick to it. If the hole is a micron in size, the water droplets that stick to the glass will be the same volume as a bacterium.

Tomo: So how exactly do you use it?

Hiro: First, you put a solution of a mixture of enzyme and reactant on the glass surface. Then, you remove the excess solution with oil. When you do, micron-sized water droplets are left on the surface.

Tomo: How do you trap an enzyme?

Hiro: You just dilute the concentration of the enzyme solution. When the concentration of enzyme is low, like 0.1 enzyme per reactor, then out of 10 reactors, only one will have a single enzyme in it. Then, you can probabilistically trap exactly one enzyme molecule.

Tomo: Seriously? That's BS. You can only get one at a time?

Hiro: (Wow, he hit me right where it hurts. Uh oh…) Um, it's actually still pretty difficult. But it's better not to encapsulate molecules one-by-one in a single reactor. The number of reactors with enzymes trapped in them changes according to the concentration, so you can count them up and calculate the precise concentration.

Tomo: Huh. So you take not having a technique and turn it to your advantage.

Hiro: Don't say that. It's simple, but it's better. This is what lets you do digital bioanalysis.

Tomo: So you're saying…

Hiro: So I'm saying, you set up a bunch of micron-sized reactors, and you probabilistically trap a single enzyme molecule in them. Once the enzyme is trapped, it'll start a reaction on its own, and reaction product will build up. The volume of the reactor is extremely small, so the concentration of reaction product increases rapidly; if the reaction product is fluorescent, you can easily see light coming from it with a microscope. You can digitalize the signals like this: if the reaction product doesn't light up, it's 0; if it does light up, it's 1. If you add up all the ones, you'll know the number of enzyme molecules and their concentration in the initial solution.

Tomo: That took a while. But I get it.

What Is That Good for?

Tomo: But that's a small technique. How can you be happy about detecting just one enzyme?

Hiro: Detecting even one enzyme is plenty of reason to be happy! Haven't you heard the phrase, "The usefulness of the useless"[3]? To find something useful, you have to find something that's useless at a glance. And in a case like this, it's really useful.

[3] From "The Zhuangzi"

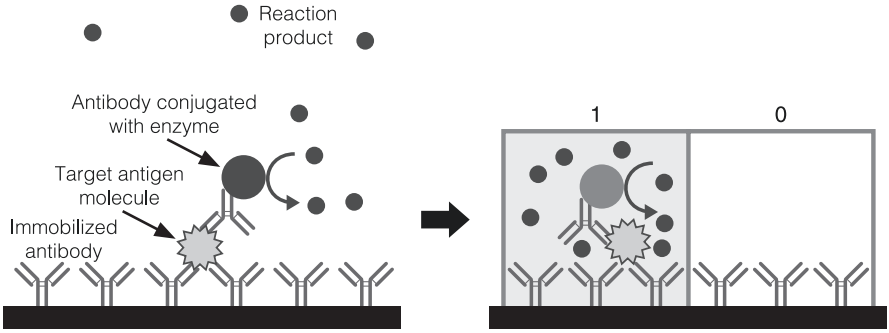

Fig. 3 Normal ELISA (left) and digital ELISA (right). The reaction itself is the same in both systems. First, target antigen molecules are captured with antibody immobilized on substrate surface. Then, antigen molecules are reacted with enzyme-conjugated antibody. After washing unbound enzyme-conjugated antibody, reaction substrate is introduced to initiate the reaction that produces fluorescent products. In digital ELISA, the reaction is to be initiated after portioning the reaction mixture into extremely small reactors. Reactors shows digitized fluorescent signal in all-or-none fashion

Tomo: (Oh crap, I pushed his button) Yeah, yeah, I got it.

Hiro: Listen up. First, enzymes are used in all sorts of places. In most cases of hemodiagnosis in hospitals, pathogens and viruses are tagged with molecules called antibodies and detected by enzymatic reactions.

Tomo: Antibodies? Tag? Speak English.

Hiro: Okeydokey, I'll tell you about enzyme-linked immunosorbent assay (ELISA). It's the most commonly used method. You have to prepare two types of antibodies and one type of enzyme. At this point, an enzyme that makes more fluorescent product is chosen, and conjugated with one of the two types of antibody. The other one is stuck to the surface of some substrate or microparticle. This isn't quite what happens, but for now, to make it easy, let's say it's anchored to the substrate.

Tomo: It's still pretty complicated. Can't you draw a diagram or something?

Hiro: Uh, have a look at Fig. 3. For instance, the target virus is trapped by an antibody stuck to the substrate, and then it's bound by the enzyme-tagged antibody. Whatever isn't bound to the target is washed away. Then, the volume of the target will be proportional to the volume of the enzyme. At this point, the volume of target will also be proportional to the fluorescence from the reaction product yielded by the enzyme, so you can use that as a basis to predict the molecular mass of the target.

Tomo: Aaaah, okay, gotcha. That's a pain, but it's not hard to understand. So digitalizing this means…

Hiro: Basically, the bottom surfaces of the reactors…

Tomo: Shut up, let me say it. You use that precious reactor array you love to brag about, so the bottom surfaces of the reactors have trapping antibodies stuck to them, right? So when the target virus concentration is low, then there's a good

chance that only one virus is trapped in the reactor. Then, the enzyme-tagged antibody sticks to the virus from above. All the molecules that aren't stuck to the virus are washed away. Last, you put in a molecule called the enzyme substrate, and you seal the top with oil. When you do that, fluorescent light builds up in the reactor. That's pretty much it, right? Then you digitalize it as 0 or 1 based on the intensity of the fluorescence, and you count up all the ones.

Hiro: Bingo! Well done! That's my boy!

Tomo: (Knock it off) And you can find the causes of diseases in single units.

Hiro: That's right. There have been cases in which virus- and disease-marker molecules have been detected by a single particle or a single molecule easily, but actually, in most cases, it's been a massive pain and hasn't been suitable for practical use. But the underlying principle of this method is really simple, so it is suitable for practical use.

Tomo: Ooh. Then have you put it into practical use?

Hiro: Well…that's what I'm working on really hard right now.

Tomo: If it really is such a great technique, then roll it out already.

Hiro: Okay.

Tomo: I'm busy with my game, but you need to get busy rolling out your technique and making money.

Hiro: Okay…

How Small Numbers of Long Genomic DNA Are Stored in Cells

Kazuhiro Maeshima

Our cells contain a nucleus with an incredibly small volume of ~ 1 picoliter (10^{-12}, or one trillionth), inside which 2 m of genomic DNA is folded. There are two sets of this genomic DNA in the cell. How is genomic DNA stored in the cell? Also, how is information from specific genes searched for and retrieved? This issue is the most basic example of "minority biology". In this chapter, we will explore this topic through a conversation between a biologist, K, and his daughter, high-school student S.

S: Biology class is boring. There's so much stuff we have to memorize. Today, DNA came up in class.

K: Biology is really interesting, I'll have you know (heh). What did you learn about DNA?

S: That it stands for deoxyribonucleic acid, and that it acts as the blueprint of life. It has four bases, adenine (A), cytosine (C), guanine (G), and thymine (T), lined up like a ladder (Fig. 1, top). The sides of the ladder are linked by phosphates with negative electric charges. That's what makes it a nucleic *acid*. The bases make pairs, A to T and C to G, joined by hydrogen bonds, and the ladder is twisted into a double helix (Fig. 1, middle). And a row of three bases corresponds to an amino acid. For instance, if you have a DNA base sequence of TTT-TCT-TAT-TGT-CTT-CCT-CAT-CGT, you get an amino acid sequence of phenylalanine–serine–tyrosine–cysteine–leucine–proline–histidine–arginine. Then, the amino acids are linked up to form a protein.

K: So human genomic DNA has a sequence of 3 billion bases; how much information do you think can be stored in it? Let's do a little calculation. There are 3 billion (3×10^9) of the four bases A, C, G, and T. This is a simple permutation, so that's $4^{(3\times10\text{^}9)}$ pieces of information. $4^{(3\times10\text{^}9)}$ is $2^{(6\times10\text{^}9)}$, so if you convert the information into a unit, it corresponds to 6×10^9 bits. Since there are 8 bits in 1

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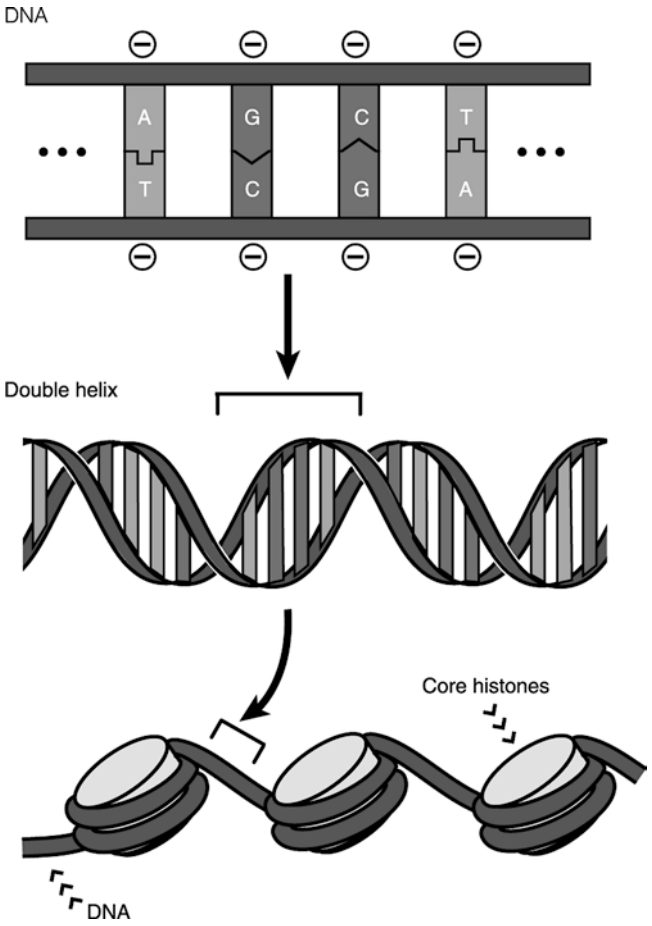

Fig. 1 (Top) Bases make pairs between two DNA phosphate chains, forming a ladder. The phosphates chains, the sides of the ladder, are negatively charged. (Middle) The ladder is twisted, forming a double helix of 2 nm in diameter. (Bottom) The negatively charged DNA is wrapped around positively charged spools called core histones

byte, you end up with about 750 megabytes (0.75 gigabytes). That's about the same as one CD, which can store about 700 megabytes.

S: Isn't that actually really small? My iPhone has 64 gigs of storage.

K: Indeed, it's only a tiny fraction of your iPhone storage capacity. But the nucleus of a cell is only about 10 μm in diameter. If you divide the amount of information by the volume of the nucleus…you get about 7.5×10^{14} bytes/mm^3. That's an insanely high density. If you do the same calculation for a CD, you get about 5.1×10^4 bytes/mm^3. For a Blu-ray, it's about 1.8×10^6 bytes/mm^3; and for a USB flash drive, it's still only 6.3×10^8 bytes/mm^3. So, the DNA information in a cell is a million times denser than in a flash drive.

S: Wow, DNA is a serious memory device!

K: It is. So, the question of how all 2 meters of human genomic DNA is stored in cells is super important. What did you learn in school about how DNA is folded?

S: A super-long DNA molecule with negative charges is wrapped around protein spools called histones with positive charges to make what's called a nucleosome (Fig. 1, bottom). The spools are called core histones; they include two sets of the histones H2A, H2B, H3, and H4. The nucleosome is folded regularly into a twisty spiral to make what's called a chromatin fiber (Fig. 2, left). Then, the

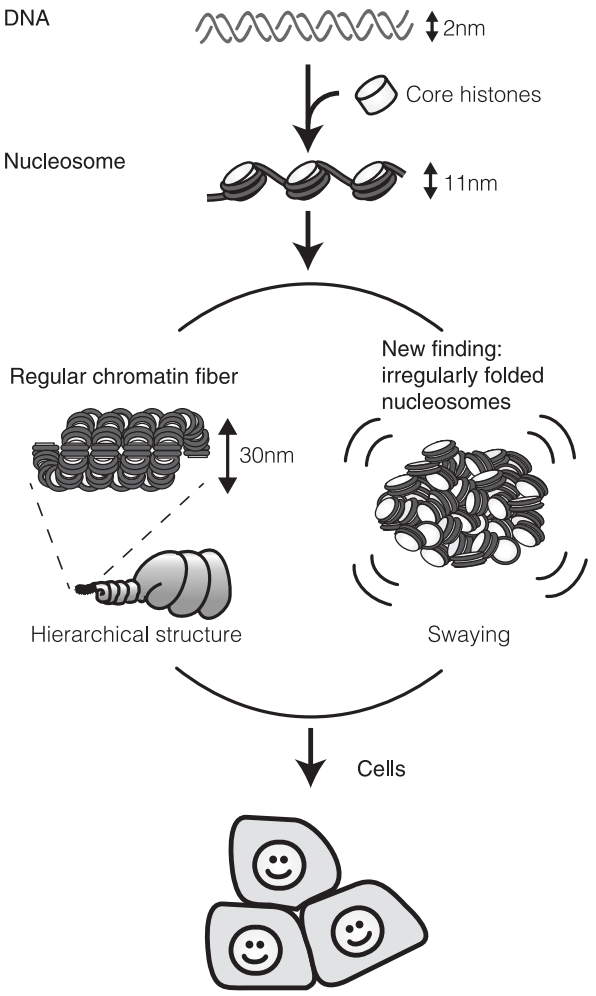

Fig. 2 DNA (first tier) is wrapped around spools called histones to form a nucleosome (second tier) of approximately 11 nm in diameter. This nucleosome was long believed to be folded regularly to form a 30-nm chromatin fiber (third tier, top left). Furthermore, it was proposed that this chromatin fiber is wrapped into a spiral of 100 nm, 200–250 nm, or 500–750 nm in length to form a hierarchical structure (building block structure) of regular spirals (third tier, bottom left). However, nucleosomes have recently been found to be stored irregularly (in a somewhat sloppy state) inside cells (third tier, right)

chromatin fiber is spun into a twisty spiral even more to make what's called a hierarchical structure. I guess that's basically it.

K: Okay, what do you think about this structure?

S: It's really, really pretty. Cells seem, like, so organized. But to be honest, it looks hard to unravel. When you want info from a gene, what the heck does the cell do? The hierarchical structure is pretty and all, but if the gene you want is at the back, it might be tough for the cell…you'd have to unravel the whooole thing.

K: Indeed you would. This model of chromatin fiber was proposed by a great scientist named Aaron Klug in 1976 [1], and it later became a standard in biology textbooks. But recently, we and others have discovered that nucleosomes aren't so regular; in fact, they get folded in a surprisingly sloppy manner (Fig. 2, right) [2].

S: How did you discover that?

K: By using a method called cryo-electron microscopy, in which cells are flash-frozen, thin-sliced, and can be observed with an electron microscope in a near-living state. We were not able to see the chromatin fiber and the hierarchical structure [3].

S: But if you thin-slice the cells, you might miss the hierarchical structure.

K: The electron beam used in electron microscopy can't pass through the cell if you don't thin-slice it, so our observation was also confirmed with a method called X-ray scattering. This method works with thicker objects and can be used to examine the regularity of all sorts of structures. If you expose a structure of assembled proteins to X-rays, you get scattering patterns that conform to the regularity of the structure. At a facility in Hyogo Prefecture, Japan called SPring-8, we exposed cell nuclei and chromosomes to synchrotron radiation, which is powerful X-rays, and examined the scattering patterns, but we didn't find any proof of the presence of chromatin fibers or a hierarchical structure. What we did find was a big structure (bundle) of irregularly folded nucleosomes (Fig. 2, right) [4].

S: Wow. I had no idea nucleosomes were folded in such a sloppy way in the cell. Then, why did Dr. Klug propose a model of regular chromatin fiber?

K: Actually…regular chromatin fiber is made in a test tube under special conditions (low salt concentration) [1]; that photo has been in textbooks for a long time. If you add a little more salt, the nucleosome gets folded irregularly into a large structure. Nucleosomes form big structures like chromosomes, so they've always been folded irregularly.

S: Ooh, I see. But…but if the folding is too sloppy, wouldn't the long DNA get tangled up in the cell? That'd be bad for the cell, wouldn't it?

K: It's fine. Cells have a bunch of proteins called type II topoisomerases that unravel tangled DNA.

S: Wow. Type II topoisomerases are important proteins, huh?

K: They are. They're especially important in cells that divide frequently, so there are lots of them in cancer cells. So, drugs that inhibit the function of type II topoisomerases are being used as anti-cancer agents.

S: If the nucleosomes are folded sloppily, it seems that it would be easy to make bundles of nucleosomes and unravel them. Are there any other advantages?

K: Let's see. If you make regular hierarchical structures, then when you try to search for information and use it, you find out that a lot of parts are hidden. On

the other hand, if the nucleosomes are stored just a little sloppily, individual nucleosomes can move better, and I guess it's more convenient for searching for information. Like, it's flexible and dynamic.

S: Cool.

K: And recently, we've been able to actually observe nucleosomes swaying around in living cells.

S: How?

K: There are more than 30 million nucleosomes in a single cell. It's impossible to see all of them at once, so they're tagged at intervals with proteins that emit fluorescence (fluorescent proteins). With this technique, we can see about 100,000 of the 30 million nucleosomes. Then, when you use a special fluorescence microscope, you can observe the movements of individual nucleosomes in living cells (Fig. 3, left). With this microscope, you can illuminate just the parts that are hidden in the cells (oblique illumination), like shining a spotlight on a really dark stage. The white spots in the photo show individual nucleosomes. And if you look at how each individual nucleosome moves, you can see that it moves 50–60 nm in a very short interval of 30 milliseconds. This represents about 5 nucleosomes. You can tell that the nucleosomes are actually swaying (Fig. 3, right).

S: Wow, they really are swaying. But how do they move?

K: We don't know the details yet. But it's probably Brownian motion. It's a swaying motion caused by lots of water molecules colliding with the nucleosome.

S: You mean it doesn't use ATP or energy? I guess it's an energy saver.

K: Basically, yeah. I think that's an amazing thing about cells. Also, a computer simulation tells us something even more interesting: large proteins involved with gene switch can move around freely among the nucleosomes (faint color balls in right panel of Fig. 3) [5]. It's as if every person on a crowded train moves a little

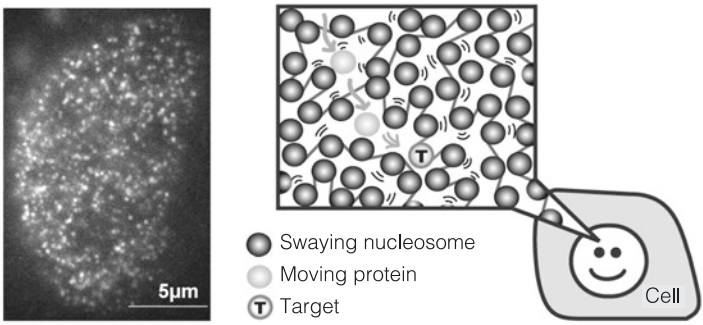

Fig. 3 (Left) Histone H4 in nucleosomes in the cell is tagged with a fluorescent protein called PA-GFP. The cell is then observed with oblique illumination using a special fluorescence microscope [5]. Each white fluorescent spot is an individual nucleosome. (Right) Nucleosomes (thick color balls) are stored irregularly in the cell. The swaying (short, quick movements) of the nucleosomes grants them a greater degree of freedom, and proteins (faint color balls) can move more freely. Consequently, the proteins can reach the target gene more quickly, thereby promoting search for information

bit, the passengers in the back can get off. And even if you reduce the number of nucleosomes and free up space, it's easier for the proteins to move if they sway.

S: You mean this makes it easier for the proteins swaying around to reach their target genes (Fig. 3, right)? And they save energy too. Plus, it means they can find information more easily!

K: Exactly. When hierarchical structures are made, a lot of genes and their information end up being hidden. That's a problem when you're trying to find information. But if the nucleosomes are folded irregularly with a certain level of sloppiness, they'll have a higher degree of freedom. It makes information easier to find.

S: So we recently found that out because we can now actually observe the swaying of individual nucleosomes in living cells, right? And if the nucleosomes sway, it helps the proteins inside them move around better.

K: Also, because of this swaying, various parts of the DNA occasionally end up outside the nucleosome. In other words, they're not hidden anymore. Swaying really helps make information easier to find. We know that the cell nucleus has a part called euchromatin, which has lots of genes and where information is often decoded; and a part called heterochromatin, which doesn't have many genes. Heterochromatin is near the nuclear membrane, but we know that the movement of the nucleosomes in it is suppressed [6]. Where there's not much information, nucleosome movement is suppressed, and it may be blocked (inactivated). Apparently, the movement of nucleosomes regulates access to information.

S: So that's the mechanism for searching and retrieving necessary information inside cells. And it saves energy to boot. That's awesome!

K: It is. It might end up leading to the development of brand-new memory devices and information search systems. It could change the world. There's still a lot of interesting things about DNA in cells.

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References

1. Finch, J. T., & Klug, A. (1976). *Proceedings of the National Academy of Sciences of the United States of America, 73*, 1897–1901.
2. Maeshima, K., Imai, R., Tamura, S., & Nozaki, T. (2014). *Chromosoma, 123*, 225–237.
3. Eltsov, M., Maclellan, K. M., Maeshima, K., Frangakis, A. S., & Dubochet, J. (2008). *Proceedings of the National Academy of Sciences of the United States of America, 105*, 19732–19737.
4. Nishino, Y., Eltsov, M., Joti, Y., Ito, K., Takata, H., Takahashi, Y., Hihara, S., Frangakis, A. S., Imamoto, N., Ishikawa, T., & Maeshima, K. (2012). *The EMBO Journal, 31*, 1644–1653.
5. Hihara, S., Pack, C. G., Kaizu, K., Tani, T., Hanafusa, T., Nozaki, T., Takemoto, S., Yoshimi, T., Yokota, H., Imamoto, N., Sako, Y., Kinjo, M., Takahashi, K., Nagai, T., & Maeshima, K. (2012). *Cell Reports, 2*, 1645–1656.
6. Nozaki, T., Imai, R., Tanbo, M., Nagashima, R., Tamura, S., Tani, T., Joti, Y., Tomita, M., Hibino, K., Wendt, K. S., Okada, Y., Nagai, T., & Maeshima, K. (2017). *Molecular Cell, 67*, 282–293.

Formation by Small Numbers: Minority Biological Scenarios in Correlations Among the Structure, Dynamics, and Function of Nuclear Chromosomes

Akinori Awazu

"Molecular minority" shows itself in the microscale world, such as in cells and organelles. In this world, the effects of shapes and containers (margins) of molecules play various roles in biological activities. As one such example, this chapter introduces nuclear chromosome dynamics recently elucidated by studies that have used mathematical models. Our guide for this introduction is a conversation between Purine and Pyrim.

Scenarios of the Structural Formation of Nuclear Chromosomes

Purine: In any case, there really are lots of different organisms on Earth, huh? And they all have totally different shapes and ways of life.

Pyrim: Where the heck did that come from? Well, anyway, you're right. But one thing all organisms have in common is that they're made up of cells. And one thing most cells have in common is that they have DNA, which encompasses genes, serving as blueprints for RNA that contains the information for making proteins.

Purine: DNA is a string-shaped molecule (macromolecule) made up of the four nucleic acids adenine, guanine, thymine, and cytosine, right?

Pyrim: Right. And the order these four nucleic acids are connected in is called the DNA base sequence. Different organisms have different base sequences, which results in differences in the types and amounts of proteins that organisms can make, giving rise to all kinds of differences among organisms. There are unicellular organisms such as *Escherichia coli* and yeasts, and there are multicellular organisms such as humans. The cells of a multicellular organism can't survive if

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they're each on their own; but by working together, with different types of cells having different specialized roles, they can form an individual organism (one that's active, intelligent, and able to adapt to various environments) capable of processing information more complex than that of unicellular organisms.

Purine: By the way, when a cell divides, an exact copy of it is made, so the cell has the same DNA after it divides, right? So, each individual multicellular organism starts out as a single cell called a fertilized egg that then divides, and then almost every single cell of a multicellular organism has the same DNA, right? Then why are there so many different shapes and types of cells that make up the human body if they all have the same DNA?

Pyrim: Because even if cells all have the same DNA and the same genes, the genes that every cell actually uses are different, which means cells make and use different types and amounts of proteins. Every cell uses the optimal combination of genes it needs to serve its own role.

Purine: Whoa! But how do they use only an optimal set of genes?

Pyrim: It's similar to the way humans live their lives. They put the things they need frequently in places where they can use them easily, and they put things they don't use on shelves and such places; it helps them to be efficient at the things they need to do. Cells are considered to do the same thing; they hide the genes they don't need so that they can use only the genes they do need.

Purine: But how?

Pyrim: Let's stop here to take a look at the properties of DNA in a multicellular organism. In the case of humans, when a cell approximates a spherical shape, its diameter is tens of micrometers; inside the cell is a nucleus with a diameter of about 10 μm, and inside the nucleus is DNA. A human cell usually has 46 DNA strands, each one only about 2-nm thick. Although that's extremely thin, the length of all the strands adds up to about 2 m. That's really long compared with the "container" it's in, the nucleus.

Purine: Wouldn't it get tangled?

Pyrim: You'd think so, but in the nucleus, histones and other types of proteins bind to the DNA, which gets folded in such a way that it won't get tangled and stored as chromosomes (Fig. 1). Here, the DNA and the local structure composed of proteins are called a "chromatin structure"; these chromatin structures are known to take on many different shapes. Also, some domains have genes that aren't really needed; all kinds of proteins bind to these domains and turn them into a tightly packed form of chromatin called "heterochromatin". This makes it hard for the proteins that read genes (transcription factors and RNA polymerase) to reach those regions; even if the proteins manage to get there, it's hard for them to unzip the double helix and read the DNA. Conversely, DNA regions with necessary genes have a chromatin structure called "euchromatin", a loose string formation that makes it easy for gene-reading proteins to access the DNA. Chromosomes are formed by alternating connected sections of heterochromatin and euchromatin.

Purine: I get it. Nuclear DNA is made up of structures called heterochromatin and euchromatin so that the necessary genes can be used properly.

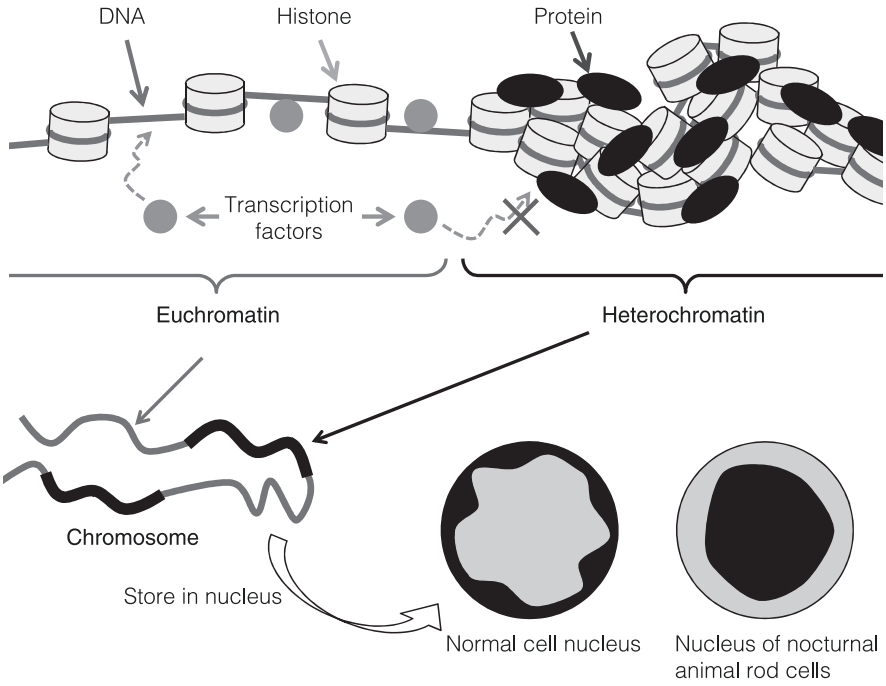

Fig. 1 Illustrations of euchromatin and heterochromatin structures, and cell species dependent intranucleus chromatin distributions

Pyrim: Of course, there's more to it than that, but these chromatin structures are an important aspect of the mechanism of gene regulation. Also, in most cells, heterochromatin tends to be present mostly near the nuclear membrane, i.e., on the outer part of the nucleus, meaning that euchromatin is mostly located in the inner part of the nucleus (Fig. 1). This is due to a protein called lamin B receptor (Lbr) that anchors the heterochromatin to another protein called lamin, which binds to and shapes the nuclear membrane.

Purine: How did those proteins come to be used, and why does the inside of the nucleus possess this structure?

Pyrim: This is only conjecture, but it's thought that when multiple strings of euchromatin congregate near each other, genes that need to be read are congregated near each other, which allows gene-reading proteins to access lots of genes more efficiently.

Purine: Yeah, it's the same way humans work on things; they can work more efficiently when they shove what they don't need off to the side and gather what they do need close together.

Pyrim: By the way, there are exceptions: there are also cells that don't have proteins such as lamin or Lbr. A typical example is the retinal cells of nocturnal mice. The same thing can be seen in the cells of several other animals. The chromatin structure of these cells are the opposite of that of most cells, with the euchromatin

domain near the nuclear membrane, while the heterochromatin domain is distributed in the center of the nucleus (Fig. 1) [1, 2].

Purine: Based on what you said earlier, it surely seems likely to make reading of the genes inefficient…

Pyrim: Going by what I explained earlier, you would certainly think that gene reading would be inefficient, but there may be bigger priorities for retinal cells and other cells than increasing the efficiency of protein production. The retinal cells of nocturnal mice need to be efficient at absorbing weak light from the outside environment. Calculation of the direction in which light moves inside the retinal cells indicates that light can be absorb more efficiently if the heterochromatin domain is in the center of the nucleus [1].

Purine: Oh, how clever! By the way, I get that heterochromatin is distributed near the nuclear membrane when proteins such as lamin and Lbr are present, but why does heterochromatin congregate near the center of the nucleus when those proteins aren't present? In the absence of those proteins, wouldn't there be a uniform distribution of heterochromatin and euchromatin in the nucleus because of Brownian motion?

Pyrim: It certainly does seem that it should be the case. So, how does heterochromatin congregate in the center of the nucleus? There isn't a clear answer yet, but a scenario has been proposed based on theoretical discussion using mathematical models. The key to this scenario is that the shape of the nucleus constantly changes.

Purine: The shape of the nucleus changes? But drawings of cells in textbooks and other materials show the nucleus as a hard sphere…

Pyrim: Of course, it depends on the type of cells. In mouse embryonic stem cells and fibroblasts, scientists have observed nuclei constantly changing shape due to the effects of the cytoskeleton, which is made of microtubules and other filaments and binds to the nucleus; and the proteins [3]. Even bigger changes are observed in mouse retinal cells that don't have lamin, which shapes the nuclear membrane [4].

Purine: Wow, really? But what has the nucleus changing shape to do with heterochromatin congregating in the center of the nucleus?

Pyrim: Unlike the euchromatin, the heterochromatin has a condensed structure in which various proteins are densely bound to the DNA, so when it moves in the nucleus, it meets greater resistance from surrounding fluid than the euchromatin does when it moves; as a result, it's thought that the heterochromatin can't move easily. When you consider that and the changes in the shape of the nucleus, it suggests that the heterochromatin domain likely condenses towards the center of the nucleus.

Purine: Uhhh, but why?

Pyrim: When the nucleus changes shape, from the point of view of the local chromosomes, the position of the nuclear membrane changes. When the nuclear membrane locally moves away from the center of the nucleus, the chromatin moves via Brownian motion to fill the void that has been created. When that happens, the heterochromatin experiences a stronger resistance than the euchroma-

tin and therefore, on average, moves more slowly; as a result, the void created by the movement of the nuclear membrane is filled mostly by euchromatin. Conversely, when the nuclear membrane locally moves closer to the center of the nucleus, the chromosomes are pushed by the nuclear membrane towards the center of the nucleus with their structure mostly intact. Thus, changes in the shape of the nucleus, i.e., repeated local movement of the nuclear membrane, causes the euchromatin to move to the edge of the nucleus, which results in the heterochromatin accumulating in the center of the nucleus (Fig. 2).

Purine: I see. But in that case, it seems that the relationships among the size of the nucleus and the lengths of the chromosomes and chromatin would be important in the formation of that type of chromatin distribution.

Pyrim: Exactly. Each extremely long chromosome fits tightly into the nucleus, which is a small container. Let's consider a different situation: for example, if instead of string-like chromosomes, the nucleus was to be occupied by an equal volume of particulate molecules that moved more easily or less easily than chromosomes; or if each chromosome was to be extremely short relative to the size of the nucleus, in which case they would resemble particulate matter from the point of view of the nucleus. In this case, as the nuclear membrane moves locally, as I explained earlier, many fast-moving particles (or chromosomes with large amounts of euchromatin) do indeed end up near the nuclear membrane. However,

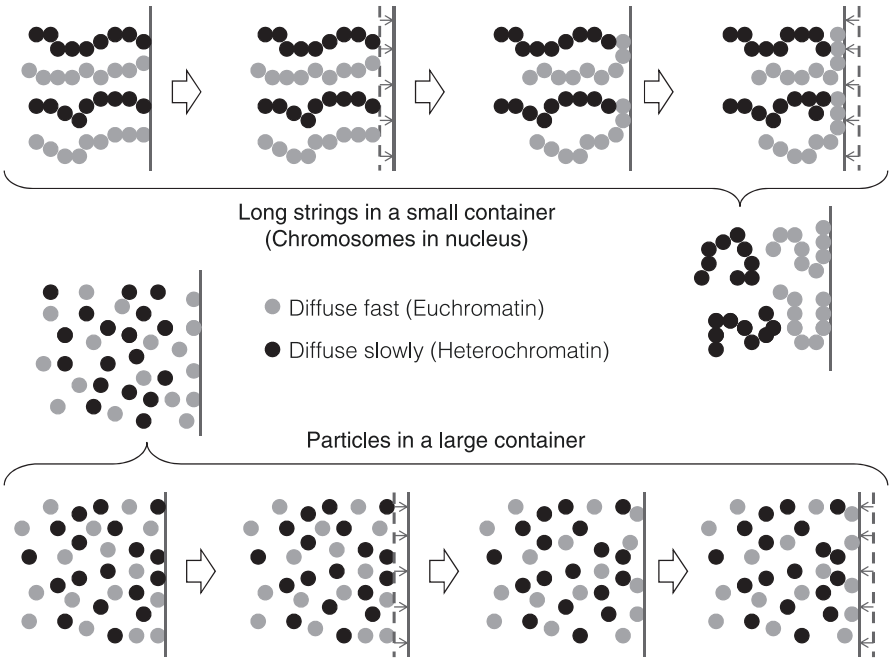

Fig. 2 Diffusive motions of euchromatin and heterochromatin with the motion of container wall (nuclear envelope), and changes in behavior depending on the shape of contents

particles that are some distance away from the nuclear membrane are unaffected by its movement, meaning that many particles with different speeds of movement remain uniformly distributed and that slow-moving particles (or chromosomes with large amounts of heterochromatin) don't accumulate near the center of the nucleus (Fig. 2).

Purine: Right. It definitely seems as though that's what would happen.

Pyrim: Conversely, chromosomes in the nucleus are long compared to the scale of the nucleus, and they lead from near the nuclear membrane to an area adjacent to the center of the nucleus. Therefore, differences in the movement of euchromatin and heterochromatin near the nuclear membrane result in a change in their relative positions; this effect propagates to the area adjacent to the center of the nucleus. As a result, the euchromatin domain throughout the nucleus tends to move to the edge of the nucleus, while heterochromatin accumulates near the center of the nucleus. In fact, this state has actually been observed in a mathematical model of chromosomes that represents the heterochromatin and euchromatin domains as macromolecular domains with different characteristics like I described earlier [5].

Purine: I see. So, the structure inside the nucleus is the result of changes in the shape of the nucleus and the fact that chromosomes are long compared to the nucleus.

Pyrim: Also, as I explained earlier, accumulation of the heterochromatin near the center of the nucleus is considered unsuitable for efficient reading of necessary genes. However, it's actually theoretically presumed that heterochromatin accumulates near the center of the nucleus when chromosomes are left alone. It is suggested that in most cells, the molecular evolution of lamin and other proteins has resulted in accumulation of heterochromatin on the edge of the nucleus, which has led to efficient transcription of genes.

The World Where Molecular Minority Shows Itself

Pyrim: By the way, in microsystems, the effects of molecular minority are prominent. I'll explain the characteristics of a microsystem. To the "container", the "content" seems extremely large. The "character" of the content comes from its length, size, and how easily it moves; while the "character" of the container comes from things like its movement. The characters of the content and the container determine the overall properties of the microsystem. You could say that the structure of nuclear chromosomes is also formed by these characteristics of microsystems.

Purine: Huh? Where the heck did that come from? Minority?

Pyrim: For example, a regular bottle of water, like the ones people carry around with them, is an aggregation of about 10^{23} water molecules. The overall appearance of the water and its physical properties (its viscosity and how easily

it warms up) doesn't change even if the shape or the size of the bottle changes slightly or if it's shaken slowly.

Purine: Right, right.

Pyrim: This is because in the liquid of the bottle, the number of molecules in contact with the "margin" of the bottle, i.e., the walls and the bottom, is far smaller than the number of molecules not in contact with the margin, so the overall properties of the liquid are determined by the interaction between the molecules that make up the liquid, and the effects of the shape of the bottle are negligible (although if you shake the bottle violently, foam will form, and the properties of the liquid may change). By the way, let's think about the nucleus of a cell as a container and the chromosomes in it as its contents. Human cells have 46 chromosomes. To the container (the nucleus), the contents (the chromosomes) seem extremely large, so the interaction between the container and the content is about the same as the interaction of the contents among themselves. As a result, the shape of the container and differences in the shapes, lengths, and mobility of the individual contents, i.e., the character of the contents, seem to have a major effect on the overall properties of the system.

Purine: Now that you mention it and with what you were saying earlier about the arrangement of heterochromatin, the character, like differences in mobility and changes in the shape of the nucleus, is key, isn't it? I guess that type of character dominates in such a small world.

Pyrim: That's right. The character dominates and determines the overall fate of the system. This is the characteristic of the microworld, in which molecular minority shows itself.

Recognition of Homologous Nuclear Chromosomes

Purine: In situations in which molecular minority dominates, it seems that differences in the shapes of the molecules would have a big effect on the system's behavior, yeah? What kind of stuff do people think happens in nuclear chromosomes?

Pyrim: I think all sorts of things happen, but I'd like to introduce a scenario in which the individual shapes of chromosomes enable search and recognition of homologous chromosomes in the synapsis that happens between homologous chromosomes during meiosis.

Purine: Synapsis?

Pyrim: In typical cells in multicellular organisms, paternal and maternal sequences in the nucleus have (almost) the same chromosomes (plus sex chromosomes). These chromosomes are called homologous chromosomes. For example, there are 22 pairs of homologous chromosomes in the nucleus in humans. Also, cells that form germ cells bind neighboring pairs of homologous chromosomes in the nucleus during meiosis; this pairing is called synapsis. After this synapsis, segments of the paternal chromosome and the maternal chromosome are exchanged

through a process called homologous recombination. Although paternal chromosomes and maternal chromosomes are homologous, their sequences are not completely identical, so this exchange of sequences increases genetic diversity.

Purine: Hmm. Many things are going on with couples, parents and children, huh?

Pyrim: Actually, this process of synapsis and homologous recombination happens even in yeast, which is a unicellular eukaryote. In yeast, when two cells mate and their nuclei fuse, pairs of homologous chromosomes are formed in the nucleus, and synapsis and homologous recombination occur (Fig. 3).

Purine: By the way, you casually mentioned "neighboring pairs of homologous chromosomes", but there are multiple types of chromosomes with different sequences in the nucleus, right? Those chromosomes definitely have complex structures, but they're just aggregations of molecules, right? So how do they recognize homology between themselves and other chromosomes and search for chromosomes homologous to themselves in the first place?

Pyrim: Actually, it still isn't well understood. However, in several types of organisms, it's known that when synapsis occurs, the nucleus itself undergoes a staggering change in shape or intense translation and rotation for a long period of time [6]. The most well-known example is called "horsetail movement", which happens during synapsis in fission yeast; the nucleus is stretched into an elongated ellipsoid and swings from one end of the cell to the other like a horse tail (Fig. 3). When this movement occurs, it's thought that the chromosomes are also stretched into long strings.

Purine: Does that help the chromosomes recognize homology?

Pyrim: Here's the type of mechanism that has been proposed. First of all, let's note that homologous chromosomes strongly resemble each other in terms of local shape. As for the structure of chromosomes, nucleosomes are first distributed in a DNA sequence-dependent manner [7]; chromosomes are then shaped by various small molecules and proteins binding to nucleosomes and DNA regions that have not been arranged into nucleosomes. So, the resulting chromosome structure is greatly affected by DNA sequences; of course, different sequences result in different structures, while homologous chromosomes with similar sequences have similar shapes. These string-like chromosomes with their locally complex

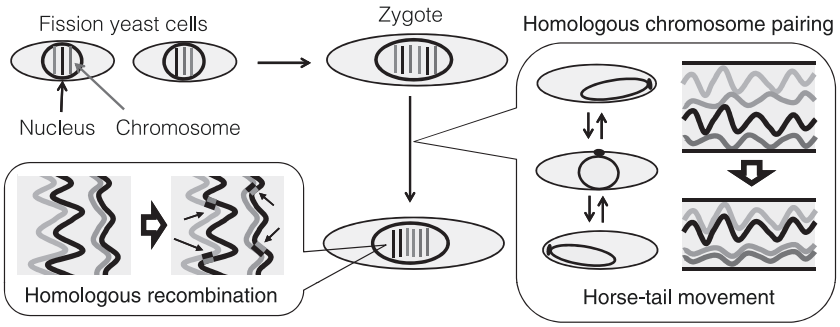

Fig. 3 Illustration of homologous recombination of fission yeast meiotic prophase

structures are confined in the elongated nucleus. At this point, the nuclear membrane is moving constantly, which puts constant pressure on the chromosomes, so a compact state is the most stable state. Now then, how do chromosomes reach their most compact state?

Purine: If they're all extended into strings, I guess maybe they could overlap a little and become compact if chromosomes with the same shape were next to each other (Fig. 3).

Pyrim: That's right. On the contrary, if the shapes of the overlapping chromosomes are different, they can never become compact. When identically shaped string-like objects, i.e., homologous chromosomes, are next to each other in the nucleus, they pack into the most compact shape possible, which is considered to be their most stable state. This has been confirmed in a mathematical model simulation in which string-shaped molecules with different configurations were enclosed in a narrow cylindrical space [8].

Purine: I see. So in regard to the question of how chromosomes can search for and recognize homologous chromosomes, intermolecular differences in shapes that stand out by the minority of molecules play an important role.

Searching for the Wellspring

Purine: By the way, we've been talking about the behavior of nuclear chromosomes in terms that are used in human society and communication, like "efficiency", "searching", and "recognition", but these are just macromolecules we've been talking about.

Pyrim: You're right. But it's tempting to anthropomorphize the behavior of these groups of "just macromolecules" because it's the wellspring of biological activity. Now then, how does this wellspring bubble up with physicochemical processes? The concepts and dimensions that emerge from a perspective called minority biology could very well be a wellspring for future ideas to answer that question.

Purine: Minority, huh? The importance of small amounts, huh? But I'm happier when I have a lot of delicious pudding.

Pyrim: But when you mix in small portions of lots of different spices, the flavor gets deeper, more complex, and more delicious!

References

1. Solovei, I., et al. (2009). Nuclear architecture of rod photoreceptor cells adapts to vision in mammalian evolution. *Cell, 137*, 356.
2. Solovei, I., et al. (2013). LBR and Lamin A/C sequentially tether peripheral heterochromatin and inversely regulate differentiation. *Cell, 152*, 584.

3. Talwar, S., et al. (2013). Correlated spatio-temporal fluctuations in chromatin compaction states characterize stem cells. *Biophysical Journal, 104*, 553.
4. Nickerson, P. E. B., et al. (2013). Live imaging and analysis of postnatal mouse retinal development. *BMC Developmental Biology, 13*, 24.
5. Awazu, A. (2015). Nuclear dynamical deformation induced hetero- and euchromatin positioning. *Physical Review E, 92*, 032709.
6. Koszul, R., et al. (2009). Dynamic chromosome movements during meiosis: A way to eliminate unwanted connections? *Trends in Cell Biology, 19*, 716.
7. Awazu, A. (2017). Prediction of nucleosome positioning by the incorporation of frequencies and distributions of three different nucleotide segment lengths into a general pseudo k-tuple nucleotide composition. *Bioinformatics, 33*, 42.
8. Takamiya, K., et al. (2016). Excluded volume effect enhances the homology pairing of model chromosomes. Nonlinear theory and its applications. *IEICE, 7*, 66.

Dividing Small Numbers: The Discreteness and Distribution of Molecules in the Cell Membrane

Hiroaki Suzuki

Cells vary in size; bacteria and other prokaryotic cells are approximately 1 micron, whereas plant, animal, and other eukaryotic cells are approximately 10 microns and more. These small containers are packed to the gills with DNA and all types of proteins. Although some types of molecules are present in abundance, the genome (all the genes that that cell possesses) is maintained as one set or two sets. How did cells obtain these universal characteristics and properties? Present-day cells have become complex entities as a result of evolution, but what were the first cells on earth like? Hints for solving this mystery have emerged from studies in which simple cell-like objects (artificial cells and cell models) are produced in a laboratory. Let's now listen in on a conversation among *Escherichia coli* (E), which is a prokaryotic cell; a human cell (H), which is eukaryotic; and an artificial cell (A) produced in a test tube (Fig. 1).

The Physical Properties of Cells

E: Hi, everyone. I'm *E. coli*. Most people think of me as a germ or a pathogen, so people usually hate me. But I'm normally harmless, and I'm pretty useful to you!

H: Hello. I'm a human cell. We take on quite different forms and functions depending on where we are in the human body, but we all have the same information in our genomes.

E: Yo, human cell, you're way bigger than me. I'm jealous because you've got all kinds of organelles and stuff. I'm nothing but a bunch of DNA and proteins stuffed in a cell membrane pouch.

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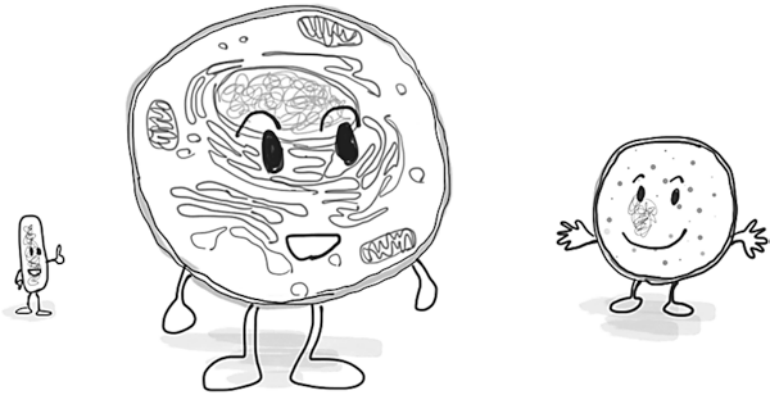

Fig. 1 (From left to right) *E. coli*, a eukaryotic cell (human cell), and an artificial cell

H: Now, now, you still have quite a few things, like a cell wall and flagella. And many of the basic mechanisms of cells have been determined thanks to you and your kind, haven't they?

E: Speaking of the mechanisms of cells, there's a book written by humans called "Molecular Biology of the Cell" [1] that's got a lot of detailed stuff about all kinds of substances, genes, and mechanisms.

H: Yes, I was surprised to see that they examined us in so much detail.

E: But I've got a question about something a lot simpler. Like, have you ever thought about a really long time ago, right after our ancestors were born? I've got about 4000 genes, but cells didn't acquire so many genes overnight, right?

H: You're right. In the very beginning, cells would have been simpler aggregates of molecules.

E: Gotcha. Organic matter was formed on Earth, and it linked up to make macromolecules, which developed a mechanism to make copies of themselves. People think that it was important at that time that macromolecules were enclosed in a small container made of membrane [2].

H: Because if there had been no container, the copied macromolecules would have been scattered in liquid. If they're in a narrow space, the increased concentration of molecules stays high, which would let macromolecule synthesis and other chemical reactions progress efficiently.

E: Yeah, but you and I get copied whole and multiply with macromolecule synthesis and copy reactions in our cell membranes, right? So I wonder how they did it in the beginning.

H: How indeed. In my case, cell division is elaborately controlled. The copied chromosomes are pulled along microtubules and arranged neatly on both sides of the cell, and then the cell divides on a division plane. I'm usually not conscious of it, but I think it's amazing, if I do say so myself.

E: Bacteria like us don't have that kind of amazing control mechanism, but we can make proper whole copies of ourselves. But I'm not conscious of it either….

Artificial Cells (Cell Models)

A: Hey, it sounds like your discussion has stalled out. I'm an artificial cell made of a combination of molecules as parts. There's been research recently about what you were just talking about.

E: Whoa, you're pretty simple, huh? It looks like you've got DNA and proteins in a nice, round, smooth membrane, but in low concentrations. And you're almost transparent. Are you really a cell?

A: That's a harsh question, but I'm a cell model. For instance, before you actually make a car or an airplane, you make a model to check the aerodynamic characteristics, right? In the same way, there's research in which they use models that partially recreate the characteristics and functions of cells. Let's not get into complex matters such as the definition of a cell here, okay?

H: What kind of research?

A: A British research group cultivated bacteria such as *Bacillus subtilis* and *E. coli* that lack cell walls. They found that although the cells ended up with all kinds of shapes that had lost a set amount of thickness and length, they were still somehow able to proliferate. Basically, even if cells don't have set shapes or advanced division systems, they can still grow, divide, and produce descendants.

E: Wow, so they lose their cell walls and get all squishy? That sucks…

H: Wow, how surprising! I understand that as long as they have a functioning metabolic system to synthesize necessary molecules, they can still grow to a large size, but I wonder how they divide.

A: They speculated the mechanism of uncontrolled proliferation by citing artificial cell researches [3]. *"In outline, shape perturbations leading to fission can be generated simply by increasing the vesicle* (a lipid bilayer bag) *surface area to volume ratio. (…) Shape distortions and fission can also be promoted by intravesicular nanoparticles or macromolecules, suggesting a possible role for cell constituents, particularly the nucleoid, in promoting fission."*

E: Uhh, I don't really get it. Go into more detail.

A: To do that, though this might sound far afield, I'll start by explaining osmotic pressure.

How Osmotic Pressure Is Generated

H: Osmotic pressure is that thing in science textbooks. It's the phenomenon where, if you partition a container with a semipermeable membrane and put in a solvent (for example, water) on one side and a solution (for example, sugar water) on the

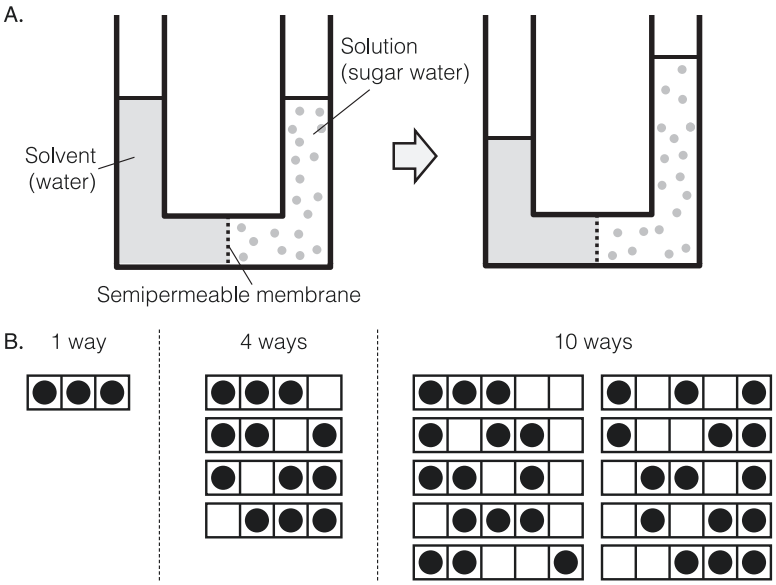

Fig. 2 (**a**) When a solvent and a solution are put in on either side of a semipermeable membrane, osmotic pressure is generated, causing the water level of the solution to rise. To be consistent with the second half of the discussion, the area containing solvent only is shown in light gray. (**b**) When there is more space, the number of possible arrangements of molecules increases sharply

opposite side, the surface of the water will rise on the solution side (Fig. 2a). The semipermeable membrane is made of something like cellulose that only the solvent can pass through. When the surface of the liquid on the solution side rises, the hydrostatic pressure increases accordingly; this is called osmotic pressure.

E: I experience osmotic pressure all the time. My body is packed to the gills with ions and macromolecules, but outside, the water usually has a low concentration of that stuff, so my body is always trying to expand. It's thanks to the cell wall that I don't burst.

H: Plant cells also become firm when soaked in fresh water. But I wonder what that has to do with what we're talking about now.

A: That's the phenomenon, but could you explain why it happens?

H: Physics textbooks explain that how the pressure of gases is generated. Gas molecules flying around in a container constantly collide with the walls and exchange momentum; the sum of that momentum is pressure.

E: But with osmotic pressure, there're water molecules on both sides of the semipermeable membrane, and molecules collide with the membrane like you were just explaining, right? On one side, there are sugar molecules, but do they really hit the wall with more force than water molecules do? Textbooks say that the translational energy of each individual molecule is the same on average, no matter what types of molecules they are.

A: Yes, that's the spirit. It's important to go beyond a surface-level understanding and think about things deeply. You have to think about this problem in a different way using numbers of possible arrangements.
H: I'm getting more and more confused….
A: Let's go back to the pressure of gases and start from there. We'll divide the gas container into compartments that can each contain only one molecule. To make the calculations easier, we'll consider a container that has just 3 compartments. How many different ways are there to arrange 3 molecules into those compartments?
H: That's easy. Assuming the molecules are indistinguishable, there would be only one way to put 3 molecules into 3 compartments (Fig. 2b).
A: Okay, how many ways are there to arrange 3 molecules in a container with 4 or 5 compartments?
E: If there are 4 compartments, then you choose one compartment to leave empty, so there are 4 ways. If you have 5 compartments, uh, if you use a combination, you get ${}_5C_3 = 10$ ways. But what's that got to do with pressure?
A: The more compartments there are, i.e., the more space there is, the more possible arrangements of molecules there are. These individual arrangements emerge with equal probabilities. In other words, macrostates (such as volume) are more likely to emerge when there are a large number of possible arrangements for composing that state. Gases can assume a larger number of possible arrangements in a wider space, so this tendency is the pressure of gases attempting to expand [4].
H: Umm, I feel like I've been deceived.
E: Okay, now explain the semipermeable membrane stuff from earlier.
A: Take another look at Fig. 2a. The density of the water molecules is the same on both sides, but the solute molecules are distributed in a wider area on the right side. That means there're a higher number of possible arrangements there, so the state changes on that side.
H: Aah, now I feel even more deceived.
A: This view[1] often comes in pretty handy. Try chewing on it for a while.

The Shapes of Macromolecules

E: Hey, artificial cell, I'm surprised you have that much intellect even though your composition is so simple.
A: Part of it is because I need it for my performance here (heh). Okay, next, let's talk about how the shapes of proteins, DNA, and other macromolecules are involved with osmotic pressure. First, just to make sure, you know that proteins and DNA are chains of unit molecules (monomers), right? They're flexible, long ropes, but

[1] The concept of entropy is taught in thermodynamics and statistical thermodynamics lectures at university.

instead of being stretched out, they're crumpled up. Maybe you can imagine them as being like balls of yarn.

H: Well, I certainly know that much. Proteins have side chains that are strings of 20 different amino acids linked together, and amino acids pull each other in and repel each other, so strings of the same sequence of amino acids will always be folded into the same shape.

A: That's right. But we won't get into that now because we're talking about osmotic pressure, which is more basic than that. Instead, let's consider a long DNA chain. I said it's like a ball of yarn, but because of thermal motion, it somewhat spreads out and unsteadily changes into all kinds of shapes (Fig. 3a, left). In the environment inside the cell, there are lots of proteins and other macromolecules all around. The solute proteins here are decently big (about 10 nm), so sterically, they can't get inside the ball of DNA. So, inferring from the explanation of osmotic pressure a little earlier, what do you think happens next?

E: Let me see. There's no solute inside the ball of yarn, but there is solute outside it. So water goes out of the ball of yarn, and it shrinks?

A: Correctamundo!

H: DNA itself becomes a semipermeable membrane in relation to solute, doesn't it (Fig. 3a, right) [5]? My preconception was simply that osmotic pressure = semipermeable membrane, but now my thinking has shifted.

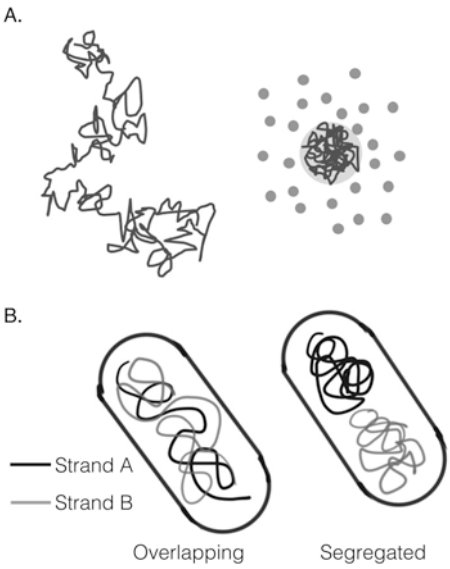

Fig. 3 (**a**) When a relatively spread-out macromolecule strand (left) in a diluted solution is surrounded by a high number of relatively large solute molecules, the effect of osmotic pressure (the excluded volume effect) will cause the macromolecule to condense (right). Specifically, the solvent area (gray) on the inside of the macromolecule, where solvent cannot enter, will decrease. (**b**) The number of possible arrangements for the macromolecule strands is higher when the strands are segregated rather than overlapping

A: Keep in mind that we haven't presumed anything about attraction between molecules in this effect of the DNA ball shrinking, i.e., being compressed. In other words, the average shape of DNA changes based solely on the number of possible arrangements of molecules. Okay, next, let's consider a situation in which two long DNA strands are confined in a closed space. Do you think the DNA strands will get tangled up (Fig. 3b)?

H: Intuitively, I'd think they would inevitably get tangled…But there's a whole 2 meters of DNA in my own nucleus, and if it got tangled, it probably wouldn't function very well. So, as to your question, I'll say that the DNA strands don't get tangled up.

A: Let's think about this in terms of numbers of possible arrangements. Two parts of a DNA strand cannot occupy the same space. If strand B tries to enter a space where strand A is already present, strand B can't get in because strand A already occupies the space, so the number of possible arrangements decreases accordingly. There's a greater number of possible arrangements for a state where the two strands of DNA don't spatially overlap, so they end up in separate spaces [4].

E: So, are you saying that in my body, the two strands of copied genomic DNA might wind up apart from each other in the split-off daughter cell to increase the number of possible arrangements?

A: Also, your DNA is about 1.6 mm (4.6 million base pairs) long, and it's contained within a very narrow cylinder that's 1 μm in diameter and a few microns long. Because of that, segregation of the strands is even more pronounced in your case [6].

H: What's more, proteins are tightly packed around the DNA inside the cell.

A: That crowding effect should also be related to the behavior of DNA. It's still a subject for future research.

Deformation of the Membrane

A: Moving on, let's talk about how the number of possible arrangements and osmotic pressure affect the shape of the cell membrane. Of course, Human Cell has a cytoskeleton, and *E. coli* has a cell wall, and these give you two a proper shape. But if you didn't have those things and instead had only a lipid bilayer membrane, what shapes would you take?

H: In the part of a biology textbook on osmotic pressure, a picture shows how a red blood cell, which normally has a discoid shape that's slightly sunken in the middle, will have a structure with prickly projections if the external osmotic pressure rises and water goes out of the cell.

E: If water goes out of the cell membrane pouch, or if the surface area of the membrane gets bigger while the volume of cytoplasm stays the same, the soft membrane can take on all kinds of shapes [7].

A: Recent experiments with artificial cells have shown that if there's a degree of freedom in membrane deformation and the inside of the pouch is crowded with

macromolecules and whatnots, it will deform into an hourglass shape, the same as what happens during cell division [8, 9]. Basically, even with an extremely simple artificial cell that's made up of only a membrane and internal macromolecules, the membrane undergoes a deformation similar to what happens during cell division.

E: That's related to how bacteria can divide even if they lose their cell walls!

A: That's right. Have a look at this picture (Fig. 4). The solid line represents the cell membrane, and the inside is packed with proteins and other macromolecules as solute. The center of solute molecules of a certain size can't get any closer than their own radius to the membrane, so a solute-free area is formed in the vicinity of the membrane. This is called the excluded volume. Using the osmotic pressure analogy, the excluded volume (gray) on the inside of the membrane tends to decrease.

H: To connect this to Fig. 2a, the excluded volume is the area in the figure that has only solvent, while the area with proteins is the solution, correct? So, we should

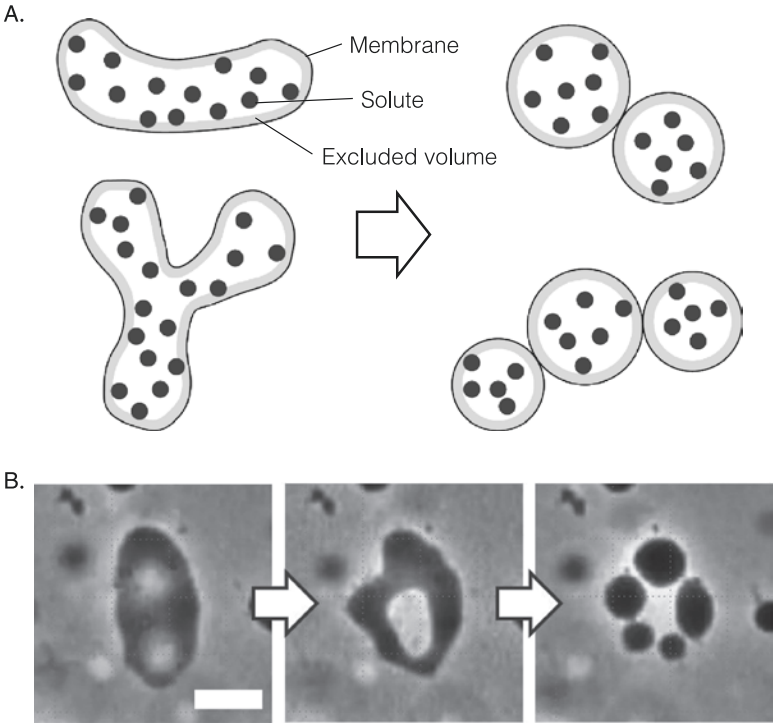

Fig. 4 (**a**) Deformation of the membrane due to the excluded volume effect. The gray area in the vicinity of the inside of the membrane is the excluded volume (the area with only solvent). The membrane deforms as if undergoing cell division, and an increase in positive curvature causes a decrease in the excluded volume. (**b**) Microscope image of an artificial lipid membrane deforming as if undergoing division. (Image taken by the Suzuki laboratory). The scale bar represents 5 μm

imagine a decrease in excluded volume as water trying to go out of the area that has only solvent, yes?

A: So, how is that accomplished? Over a short period of time, you can ignore the entrance and exit of water and substances through the membrane. The only change is in the shape of the membrane. Then, as the inside of the membrane bends and the curvature increases, the excluded volume inside the membrane decreases, though only slightly.

H: Now that you've said that, I feel that this would indeed happen.

A: For instance, picture a thin plastic board (the lipid membrane) with a sponge sheet (the excluded volume) stuck onto one side. If the sponge is on the inside, when the plastic board bends, the sponge will be compressed and its volume will shrink, but only slightly. Basically, the pouch of a lipid bilayer membrane with a relatively large surface area is soft, which allows it to take on a variety of shapes; but when it's packed with macromolecules, the membrane tries to bend inward under the excluded volume effect, so the membrane is deformed as if going through cell division. Consequently vesicles change their shapes into a dumbbell or a pearl chain or something like that.

E: Umm, I see. I have been wondering how primitive cells could have divided, but now that you've told me all of this, it doesn't seem that it was that hard.

Single Molecularity of Genome

H: The topic of this book is how molecular minority and single molecularity are involved in the characteristics of life. To conclude this conversation, let's turn to that subject and summarize things.

E: It's been said that having one or two copies of genomic DNA as design information in the cell is important for the emergence of individuality and diversity, right? If the copy number is high, a sudden mutation in a gene will get averaged out by all the other copies of that gene, and the mutated characteristic won't emerge. If that happens, all the cells will be average; if the environment changes and the cells can't adapt, they'll die. Individuality and diversity are important for the survival of a species.

H: It's okay in principle for genes to be on scattered fragments of DNA; but in fact, all genes are on a long strand of DNA. If genes are connected in a single DNA strand, then, you'll have two accurate copies of all genes when you copy it once. If genes were scattered DNA fragments, the copy numbers would likely be unbalanced.

A: In the context of today's conversation, I think the physical size of a long strand of genomic DNA helps the copied DNA to be well distributed when the cell divides. Early in the discussion, I introduced the theory that macromolecules excluding each other might increase the number of their possible arrangements and help them to be distributed neatly into daughter cells (vesicles), right? Also, experiments with artificial cell membranes have gradually revealed that when

you make the inside of the membrane pouch more crowded, interaction emerges between the excluded volume effect of DNA molecules and the membrane deformation. Genomic DNA is more than just a simple carrier of genetic information; I get the sense that its gigantic size as a macromolecule might produce cellular characteristics.

H: A single substance having diverse properties is important in the mechanisms of living things, isn't it? When humans make machines, it seems that they design a single part or unit to endow the machine with a single function. I'd like to teach humans the keywords of diversity and redundancy.

E: That was interesting. Thanks for the conversation, Artificial Cell.

References

1. Alberts, B., et al. (2007). *Molecular biology of the cell* (K. Nakamura, Trans., 5th Ed.). Garland Science, New York
2. Sadava, D. E., et al. (2013). *Life: The science of biology* (10th ed.). New York: Macmillan.
3. Errington, J. (2013). L-form bacteria, cell walls and the origins of life. *Open Biology, 3*, 120143.
4. Dill, K., & Bromberg, S. (2010). *Molecular driving forces: Statistical thermodynamics in biology, chemistry, physics, and nanoscience*. New York: Garland Science.
5. Vasilevskaya, V. V., et al. (1995). Collapse of single DNA molecule in poly(ethylene glycol) solutions. *The Journal of Chemical Physics, 102*, 6595.
6. Jun, et al. (2006). Entropy-driven spatial organization of highly confined polymers: Lessons for the bacterial chromosome. *Proceedings of the National Academy of Sciences of the United States of America, 103*, 12388.
7. Boal, D. (2011). *Mechanics of the cell* (2nd ed.). Cambridge: Cambridge University Press.
8. Natsume, et al. (2010). Shape deformation of giant vesicles encapsulating charged colloidal particles. *Soft Matter, 6*, 5359.
9. Terasawa, et al. (2012). Coupling of the fusion and budding of giant phospholipid vesicles containing macromolecules. *Proceedings of the National Academy of Sciences of the United States of America, 109*, 5942.

Getting to Know the Functions of Small Numbers

Motoshi Kaya

Many types of proteins are at work inside our bodies. These proteins include so-called molecular motors, which harness chemical energy from sources such as ATP to generate force and torque. For example, molecular motors called myosins generate force that causes muscles, the heart, and blood vessels to contract. Failure of these molecular motors to function normally is known to trigger heart and muscle disorders; thus, molecular motors are crucial for maintaining normal function in our bodies. This chapter is in the form of a conversation between two students: the individualistic Koki Beoka, who loves weight training; and an upperclassman named Tsukasa. Their conversation will help you to understand how myosins function when they congregate into a group, as well as the biological significance of these functions.

How Do Muscles Contract?

Koki: I've been doing weight training four times a week lately, and I've gotten a lot stronger. That proves I've got more muscle now, doesn't it? Heh hehheh.

Tsukasa: There are likely to be two possible reasons for that. One is that you've got better at using your muscles. In other words, coordination among your muscles has been optimized to allow you to use them in the best way possible for lifting weights. Second, as you said, you've got more muscle.

Koki: Okay, so you're saying I've improved the way I use my muscles (coordination). But having more muscle means that I have more of the proteins that make up muscle, right?

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Tsukasa: Right.
Koki: This is a hardcore bodybuilding question, but what kinds of proteins are they?
Tsukasa: The proteins at the root of muscle contraction are called myosins and actin. Have you ever heard of them?
Koki: I may have learned about them in my high school science classes, but I don't remember them at all. How do they contract muscles?
Tsukasa: Myosins are proteins that have two heads shaped like golf drivers; they're made up of about 300 molecules that come together to form a filament. Actin, on the other hand, forms filaments in which globular molecules connect with each other to make strands, and pairs of those strands are twisted into double helices. The filaments are lined up in parallel, layered together, and packed into muscles (Fig. 1a). Every myosin molecule faces the center of the myosin filament, so when they interact with actin, they pull actin towards the center, which makes the muscle contracts to exert force (Fig. 1b).
Koki: Whoa, that's pretty complicated, bro. Is it similar to tug of war?
Tsukasa: Exactly. That's an easy way to understand it. I guess it's just like myosins are the people in a line pulling the rope, and actin is the rope.
Koki: Okay, I can picture that. But, you know, when you play tug of war, everyone calls out to get their timing together and use all their strength, but I guess myosins can't do that, huh. It's not like proteins can communicate with each other. So how does that work, bro?
Tsukasa: I thought you might ask that. Things are going to get a little complicated. Is that okay?
Koki: Dude, no problem.

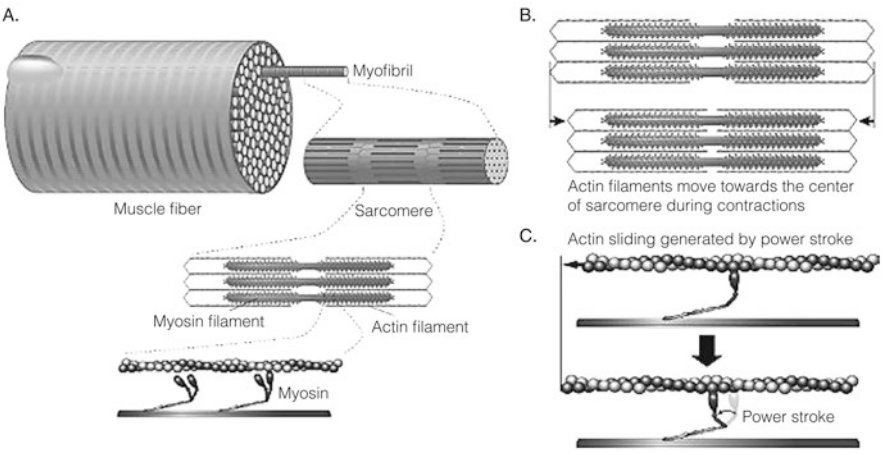

Fig. 1 Muscle contraction. (**a**) Hierarchical structure of muscle from myosin and actin to muscle fiber. (**b**) Muscle contraction is generated by myosin and actin filaments sliding past one another. (**c**) Actin sliding is generated by power stroke of myosin molecule

Is There a Mechanism That Makes Muscles Contract Efficiently?

Tsukasa: In that case, I'll tell you about the cutting-edge muscle research. Can you follow along?

Koki: Yeah, bro. You don't need to treat me like I'm stupid.

Tsukasa: First of all, I'll explain some basics so that you can get an idea of the world in which proteins do their thing. Most proteins, including myosins, are several to tens of nanometers in size. By the way, 1 nm is one billionth of a meter, so that gives you an idea of how small that is. That's small enough for proteins to collide with water molecules and shake. Long story short, it's a world in which water molecules and other proteins collide and shake constantly. You and I can walk straight ahead, even in the middle of a crowd; but in the molecular world, molecules are always bumping into each other and shaking.

Koki: What? It's hard to picture that world, but I guess it's a world in which stuff can't just move like we do, huh, bro?

Tsukasa: Right. Myosins and actin interact with each other in that world to exert force; it's pretty hard to try to move with the precision of a machine. So, you need to keep in mind that the functions of proteins that I'm going to explain emerge from a mechanism by which things are probabilistically prone to happen.

Koki: Whaa? Okay… (scratches head) So, what exactly is going on when myosins interact with actins to produce force?

Tsukasa: It's kind of hard to explain, but try to picture the myosin filament as being tethered to the ground. Myosin heads, which are shaped like golf drivers as I told you earlier, fly around all over the place, and the tips of the drivers bind onto actin filaments. The tip of the myosin head acts as a shaft, and the shaft of the golf club rotates. This is called a power stroke. Assuming the myosin filament doesn't move, the power stroke will make the actin move horizontally (Fig. 1c). The angle of rotation is about 60–70°, and the shaft is about 8 nm long, so you can predict that actin will move about 7 nm forward. But actually, because of molecular collisions, the myosin head shakes a lot as it binds to actin, so it can't always bind to the same site. That means that sometimes, actin moves either more than or less than the predicted distance. That's how myosin molecules interact with actin to produce muscle contraction. By the way, in a muscle, a single actin filament should be able to interact with about 70 myosin molecules, so if you use the tug-of-war image we were talking about earlier, there are 70 people pulling a single rope.

Koki: Ohh, I gotcha. So the 70 myosin tug-of-war dudes can't call out in sync to pull the actin together like in real-life tug of war, right, bro? And when you think about collisions between molecules, it sounds like it'd be even more of a mess than tug of- war with preschool kids. Plus, maybe there'd be times when no one is pulling and stuff like that. But even with all that, I can easily bench press

100 kg, bro. It's weird that my massive muscles are packed with so many of those sloppy myosin molecules.

Tsukasa: Wow, talk about casual bragging. Meh, whatever. From what I've told you so far, you can picture that each individual myosin molecule binds haphazardly and arbitrarily to actin to produce force. But thanks to recent studies, we know that that's not the case.

Koki: Wait, what are you saying? Are you telling me myosin molecules can communicate with each other?

Tsukasa: No, that's impossible, but it does seem like myosins are in sync with each other on their timing for producing force. Not only that, but this synchronization happens more easily when there's a big load on the muscle.

Koki: That's some crazy stuff, bro! So you mean that when myosins are going up against a good team in tug of war, they work better together?

Tsukasa: Exactly! See, you're getting it!

Koki: I told you that you don't need to treat me like I'm stupid, bro. Ooh, this is a good song. Do you like it?

Tsukasa: Huh?

Koki: Sorry, it's a good background music…I got distracted by something off-topic.

Tsukasa: (Wow, this guy's a piece of work.) Meh, anyway, if the tug-of-war opponent of myosins is good, then they work together and synchronize their timing for producing force. Now, for the problem of how these myosins work together without being able to talk.

Koki: Yeah! I don't get it at all.

Tsukasa: The answer is pretty difficult…

Koki: Nah, it's fine. Explain away. I'll ignore the background music.

Tsukasa: To explain it, I'll have to introduce a little biochemistry. First of all, myosin can detach from actin by binding to ATP. You know about rigor mortis, right? When someone dies, the ATP in their muscles is depleted, almost all of the myosins stay bound to actin and can't detach, and the muscles end up getting stiff. But with you and me, ATP is constantly being produced, so myosins can bind to ATP to detach from actin. The detached myosins hydrolyze ATP into ADP and phosphate. Then, the myosins bind to actin again; after that, the phosphate leaves, and only ADP is bound to the myosin. This is the state in which the myosin is when it uses the power stroke I explained earlier to move actin and produce force. When the power stroke ends, the ADP also detaches from the myosin; then, new ATP binds to the myosin, and it detaches from the actin. That wraps up a full cycle of ATP hydrolysis-mediated myosin–actin binding and release (Fig. 2). This whole cycle probably takes about 0.03 s.

Koki: Are you saying that myosin goes through a bunch of fast cycles that last 0.03 s in which it sticks to and detaches from actin, bro? That's hella fast. So, instead of constantly pulling on the rope like we do in tug of war, they pull it, let it go, pull it again, yada yada yada, right?

Tsukasa: Right. When we're playing tug of war, we can move together as we pull the rope; but in muscles, the myosin filaments are anchored and can't move. So

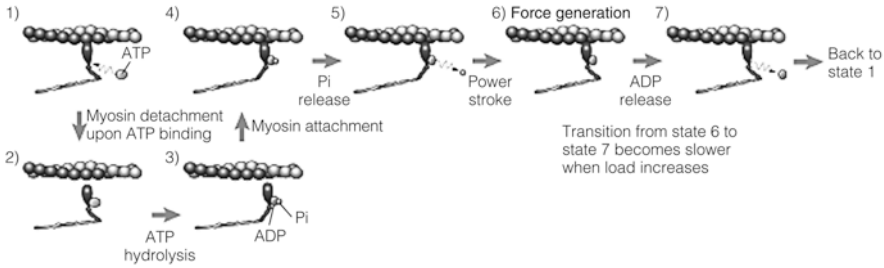

Fig. 2 Cyclic interaction of myosin with actin. Myosin detaches from actin upon ATP biding (state 1–2), attaches with actin after ATP hydrolysis (state 3–4) and executes power stroke to cause an actin sliding during force generations (state 5–6)

each myosin molecule sticks to actin and then detaches, which results in only actin being pulled towards the center of the muscle without the myosins changing their positions.

Koki: So that means that while these myosins are detached from actin and can't produce force, other myosins stick to the actin and produce force, right, bro? Earlier, you were saying that about 70 myosin molecules can interact with one strand of actin, but what percentage of those myosin molecules are bound to actin?

Tsukasa: Oh, you touched on another good point. About 10% of them, so about 7 out of 70 are producing force. Here comes the interesting part: myosins have a special property for producing greater force, a sensor that detects loads.

Koki: Huh, a sensor in proteins?

Tsukasa: It's a little bit of an exaggeration to call it a sensor, but to explain it, you need to understand the ATP hydrolysis cycle I just talked about. A power stroke results in ADP binding to myosin when it produces force; but when there's a load on the myosin, the structure of the head changes, and the pocket in which ADP binds to the myosin shrinks, which makes it hard for the ADP to slip out. What do you think happens then?

Koki: When there's a load on the myosin and ADP can't get away from it, you mean? Umm, if the ADP can't get released, then ATP can't bind to myosin, so that makes it hard for myosin to detach from actin, right? Oh, I got it! Myosin stays bound to the actin for a long time, which means that more myosin molecules end up bound to the actin! Now I totally get why you wanted to explain the ATP hydrolysis cycle.

Tsukasa: Yes, that's right! Nicely done. Loading warps the shape of the myosin head, making it difficult for ADP to detach, which means that myosin stays bound to actin for longer and that more myosin molecules bind to actin. It's a really good system, isn't it?

Koki: Totally. But besides what you were just saying about there being more molecules, you were talking about synchronizing the timing of force production. What happens there?

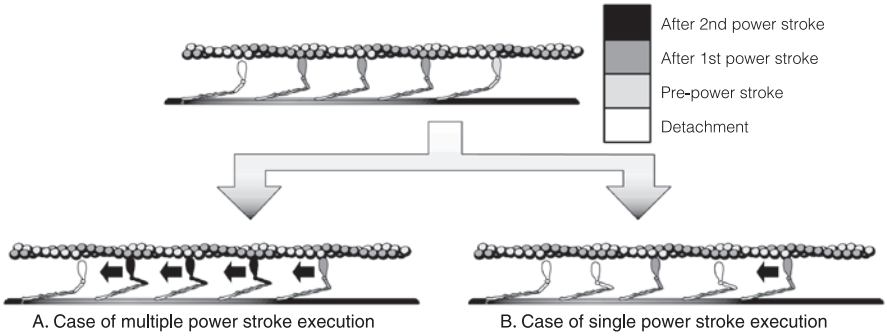

Fig. 3 Effect of the number of power stroke executions on collective force generations in myosin motor ensemble. (**a**) In case of multiple power stroke execution, a chance of power stroke synchronization increases. In the top panel, the pre-power stroke myosin (light shaded color) executes power stroke, resulting the sliding of actin filament as shown in the bottom panel. The sliding of actin filament enhances a chance of the second power stroke execution for myosins, which already complete the first power stroke (dark shaded color in top panel). Consequently, the power stroke executions of these myosins are nearly synchronized (black color in bottom panel). (**b**) In case of single power stroke execution, myosins, which complete the first power stroke (dark shaded color in top panel), either remain attached (dark shaded color in bottom panel) or detach from actin filament (white color in bottom panel). Hence, a chance of power stroke synchronization substantially reduces

Tsukasa: Yeah, recent studies have made this clearer; instead of just one power stroke, myosin may make several power strokes before it detaches from actin. As for what that has to do with the synchronization that happens when force is produced, it's actually a matter of probability theory.

Koki: Uh, I totally don't get what you're talking about.

Tsukasa: That's what I thought. Basically, when a load acts on myosin in a state in which it can make multiple power strokes in a row, it makes it hard for myosin to move, as I just explained, and it disables power strokes. The thinking behind this is that if there are more myosin molecules in this state, then probabilistically, they will synchronize and be able to make power strokes more easily (Fig. 3). Although the chance of myosin being able to make only one power stroke is not zero, it's probabilistically unlikely. The reason for this is simple: when myosin completes a power stroke, it can't perform another one if it doesn't detach from actin. So, the chances that the myosin molecules will synchronize to perform power strokes are extremely low. Alternatively, if there's a chance for multiple power strokes, then if one power stroke happens, the next power stroke will come, so there's a chance for synchronization.

Koki: Got it. So basically, there are two characteristics: the delayed detachment of ADP that happens because loading warps the structure of the myosin head, and power strokes having multiple levels, meaning they don't just increase the number of force-producing myosin molecules as loading increases, they also lead to a higher chance of synchronized production of force among the molecules. If these characteristics are in place, then probabilistically, synchronized force

production will happen among myosin molecules without the molecules communicating with each other. Is that the basic idea, bro?

Tsukasa: Exactly! According to recent studies, in small groups of about 20 molecules, 3–4 molecules will synchronize to produce force. By the way, even in a small muscle with a radius of about 1 cm, there are more than a trillion myosin molecules. Actual muscles are made up of a nearly infinite number of molecules, but what's really interesting is that proper cooperative functioning between molecules happens even when there are only a few molecules. As I said at the beginning of our discussion, the functions of molecules themselves are pretty far from a precision machine, but when just a small number (such as 20) of molecules gather, then probabilistically, individual functions (in this case, cooperativity in force generation) will develop. These unique functions are thought to form from small numbers of molecules not just in muscles but in lots of other biological functions; this emergence of functions from small numbers of molecules is crucial for understanding biological functions. This means that with tens, thousands, or tens of thousands of molecules, other new group functions should emerge. Figuring this out is going to be important from here on out.

Koki: Aw dude, that's some hella big talk. I had no idea that this kind of stuff was going on inside my muscles. It's mind-blowing. But we've been using our heads too much. Why don't we use our muscles now? Let's focus on muscle training. For my next set, I'm going to try to bench press 140 kg, so spot me twice, please.

Tsukasa: Uh, okay. (Geez, and we just had a great conversation. This guy really is a piece of work.)

Working in Small Numbers: The Behaviors That Emerge When Small Numbers of Bionanomachines Team Up

Junichiro Yajima

Bionanomachines are very small, light machines made of proteins of approximately tens of nanometers (10^{-9} m) in length and weighing approximately 10^{-19} g. A single bionanomachine (1 molecule) has a small output at the level of piconewtons (10^{-12} N), and its input is at the level of thermal energy; it functions in an environment that is constantly exposed to thermal noise. Machines made by humans differ greatly in terms of materials, size, and energy input and output.

Let's listen in on a family conversation about these extremely small, incredibly lightweight bionanomachines.

The World in Which Bionanomachines Work: Is It Crowded?

Rachel: Daddy, are you watching "Fantastic Voyage"[1] *again*? You never get tired of it, do you? I've seen it so many times that it's pretty much burned into my memory. People shrink to a really tiny size and go inside someone else's body to treat him, blah blah blah.

Anna: I wonder when we'll be able to actually do that. You know, be showered in a special light that shrinks you so you can swim inside the body and inside cells. Or take a pill that shrinks you or something like that. I found a comic book about that in the library at school.

Father: Unfortunately, that's totally impossible with current science and technology. To shrink someone that small, you'd also have to shrink all of the cells (already micrometers in size) that make up the body, plus all of the proteins and

[1] A strongly fantasy-tinged 1996 American sci-fi spy movie. I first saw it when it aired on Japanese TV when I was in elementary school, and it made quite an impression on me.

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DNA (nanometers in size) in the cells, *and* all of the atoms (angstroms in size) that compose them, so you shouldn't think of it as anything more than sci-fi. But whether any of those will ever be possible depends on how hard scientists work from here on out…after all, scientists are people who will exhaust every possibility to try to make something a reality, even if it seems unrealistic, as long as there's no proof that it's absolutely impossible. Anyway, I've loved that movie ever since I was a kid, but I wouldn't want to be shrunk down and swim inside a cell. Can the two of you even imagine what kind of a world the inside of a cell is? I think it's completely different from the world you live in.

Rachel: What kind of world is it? The cells I observed in science class seemed hollow. Anna, did you already observe cells in elementary school? The drawings of cells in my high school textbook only show the major cellular organelles, and the inside seems hollow, so it seems that you could swim around pretty freely in there. It might be fun.

Anna: The giant pool I went to during summer vacation was super-duper crowded, and I couldn't really swim around in it. As you said, it might be easier to swim inside a cell.

Father: Actually, the inside of a cell is more crowded than you're imagining. It's filled to the brim with proteins, nucleic acids, sugars, and stuff like that. For example, there are apparently 100,000 molecules of mRNA, which copies genetic information; and one million ribosomes, the factories that make proteins in cells [1]. There are bionanomachines called kinesins that carry stuff inside the cell; they serve as transport machines that bump into things as they move along, like they're bobbing through a crowded pool (Fig. 1). When the two of you observed cells at school, they probably looked hollow to you. While they may look hollow, that world is made up of more than just what you can see. If you use a high-resolution microscope, you'll be able to see finer structures. If you don't doubt what you see or change the way you look at things, you might overlook the true nature of things or miss out on finding something new.

Do Bionanomachines Have a Brain?

Anna: Huh. Speaking of which, cells are different from pools because skin blocks light from getting to cells, so it must be dark inside them. Maybe it's like the bottom of the ocean.

Father: It probably is dark. Bionanomachines serve their functions in that darkness. Of course, cells aren't equipped with modern conveniences like lights. Bionanomachines don't rely on vision for judgment, since they essentially don't have eyes.

Anna: Then how do they know what direction to go in and stuff?

Father: It's decided by the interaction between the surface molecules that make up the bionanomachine and the surface molecules of the rail it's grounded on. Weak interactions between molecules called electrostatic force, hydrogen bonds, and

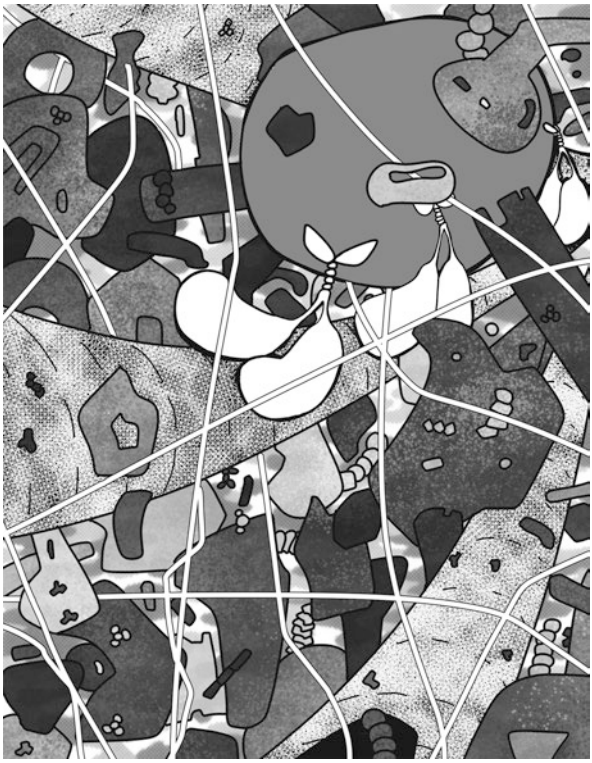

Fig. 1 Is it crowded inside a cell?

hydrophobic bonds are important, but the extent of their actions is limited to a range of a few nanometers. It's as if every step is a leap of faith.

Rachel: So, it's like they have to rely on feeling stuff around them and making sure they know where their feet are? Like being blindfolded and moving across a tightrope carefully one step at a time?

Father: Right. If they don't feel around, they won't know where they are. But bionanomachines don't have intelligence, so they're not even capable of the kind of simple anticipation that humans do unconsciously; they don't walk along while thinking "Next, I'll step here" or "I'm trying to get over there". They just try to step in whatever direction, and they take their next step wherever they happen to be able to do so. Sometimes, their next step fails, and they end up going back to where they were before; and sometimes, they go off the rail. Bionanomachines are super-light, so gravity doesn't concern them. So, concepts like up, down, upside-down, and falling don't apply to them; if they take a misstep, they just end up flying somewhere else.

Anna: If they take a misstep, they don't fall, they fly? They can fly?

Father: These bionanomachines don't have structures like engines or wings that let them fly like an airplane. They're in a watery solution, so there's an insanely huge number of water molecules around them. The water molecules move on

average at about the speed of a bullet, and they slam into the bionanomachines from all directions; the impact from those collisions is how the bionanomachines move. The collisions happen randomly, so it's impossible to predict which direction the bionanomachines will fly in. This kind of random movement is called Brownian (thermal) motion [2].

Anna: Brownian motion sounds like it hurts. It's like a really sloppy machine. It looks really random, but I guess it actually works well. It's kind of a weird world.

Father: It's hard to understand intuitively, I know. On top of that, when these super-tiny, super-light bionanomachines swim, inertia doesn't work at all, and viscosity rules. Basically, picture them swimming through something sticky, like syrup.

Anna: Wow! So just being so small and light means the way they swim is totally different from the way we swim in a pool. Bionanomachines have to survive in a really harsh environment, huh? It's a lot tougher than I thought. What kinds of stuff bionanomachines actually do in cells?

Behaviors Born Out of Teamwork

Father: For example, bionanomachines called kinesins give vesicles piggyback rides to their destinations, and they split chromosomes in two when the cell divides. Without these machines, the brain can't do anything, and cells can't multiply. Each of these bionanomachines can adeptly function on its own but when a few of them get together, they sometimes behave in ways we couldn't even imagine. The reason we can't even imagine these behaviors is that there are non-linear relationships hidden in them. If even one molecule starts acting differently, then when several individual molecules combine, it can be impossible to intuit how they'll behave.

There's a certain kinesin [3] that moves along proteins called microtubules in a set direction when it's a lone molecule; but when several molecules of them join up and work together, they move in the opposite direction. No one really understands the molecular mechanism of how it moves in opposite directions based only on whether there are lots of kinesin molecules or a small number of them, but you have to wonder what kind of gimmick could be at work when the combination of a bunch of individual factors makes them act totally differently as a whole.

Besides kinesins, there are bionanomachines called dyneins that have interesting motor characteristics related to the number of molecules. When scientists isolated dyneins and microtubules from inside cells and observed them with a microscope that can measure their movement in a three-dimensional space [4], here's what they found: when a dynein molecule is alone, it just moves straight along a rod-shaped microtubule; but when several dynein molecules work together, they move along the microtubule in circles. They're equipped with randomness that lets them rotate clockwise, counterclockwise, or not at all. It used to be thought that bionanomachines only had a single function that they

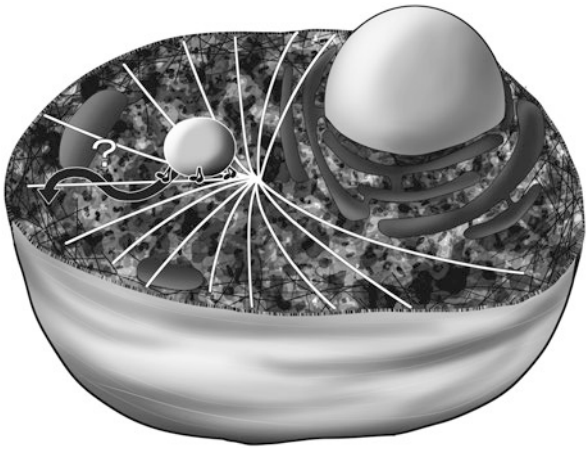

Fig. 2 Do teams of small numbers of molecules perform rotational motion? (Illustrations: Kyohei Matsuda)

performed like their lives depended on it, but it seems as though they have the flexibility to change their behaviors based on the number of molecules in order to adapt to their environment (Fig. 2).

Rachel: Whoa. It's like 1 + 1 doesn't just equal 2; it can also be 5 or −3 depending on the situation.

Father: Great point. The molecular mechanism of behaviors of small numbers of molecules is being researched around the world, but no one's figured it out yet. I wonder what the trick is: do machines work together and get in each other's way depending on where they are, or do they pull and push each other in a kind of mechanical communication? Like I said earlier, bionanomachines don't have intelligence, so they just move wherever. That movement affects other machines, and they do things together that they couldn't do individually. I think it's important for humans to work the same way bionanomachines do. If different people's actions and ideas work on each other, it might lead to ideas that never would have occurred to you on your own and to totally different behaviors.

Mother: Alright, why don't we all work together to make dinner now so we can turn out something good? But based on what you're talking about, it might turn out badly.

Father: …

References

1. Milo, R., & Phillips, R. (2015). *Cell biology by the numbers*. New York: Garland Science.
2. Yonezawa, F. (1986). Brownian motion (Butsurigaku One Point 27). Kyoritsu Shuppan.
3. Roostalu, J., Hentrich, C., et al. (2011). Directional switching of the kinesin Cin8 through motor coupling. *Science, 332*, 94–99.
4. Yajima, J., Mizutani, K., et al. (2008). A torque component in mitotic kinesin Eg5 revealed by three-dimensional tracking. *Nature Structural & Molecular Biology, 15*, 1119–1121.

Thinking Small Numbers: When, Where, and How Many Molecules There Are in the Cell

Yuichi Taniguchi

The cell is the basic unit of life. Wriggling around inside the cell are molecules with various roles; when these molecules work well together, all sorts of functions emerge. Interestingly, these molecules are surprisingly small in numbers. As a result, behavior of a minority molecule can sometimes change the function of an entire cell and therefore potentially dictate the fate of an entire organism. Here, through a conversation between a high school student (16 years old) who has recently begun to take an interest in life sciences and her biology teacher (35 years old), let us learn about the "sociality" of cells formed by these many molecules.

Student: Our bodies are made of cells of about 10 microns in size, right?
Teacher: Right.
Student: But cells are really small. I can't get a real idea of what is going on at the level of individual cells.
Teacher: I understand how you feel. In that case, why don't we try thinking in terms of a single "factory" so you can get a more tangible sense of the phenomena in a cell?
Student: Yes, sir. Please explain.

The Cell: A Multipurpose Factory Facility

Teacher: Cells are easy to understand if you think of them as multipurpose factories that produce all kinds of products (Fig. 1).

First of all, the factory has a boss, the manager (genomic DNA), who gives out instructions to make all sorts of products. When instructions are given, first, a writ-

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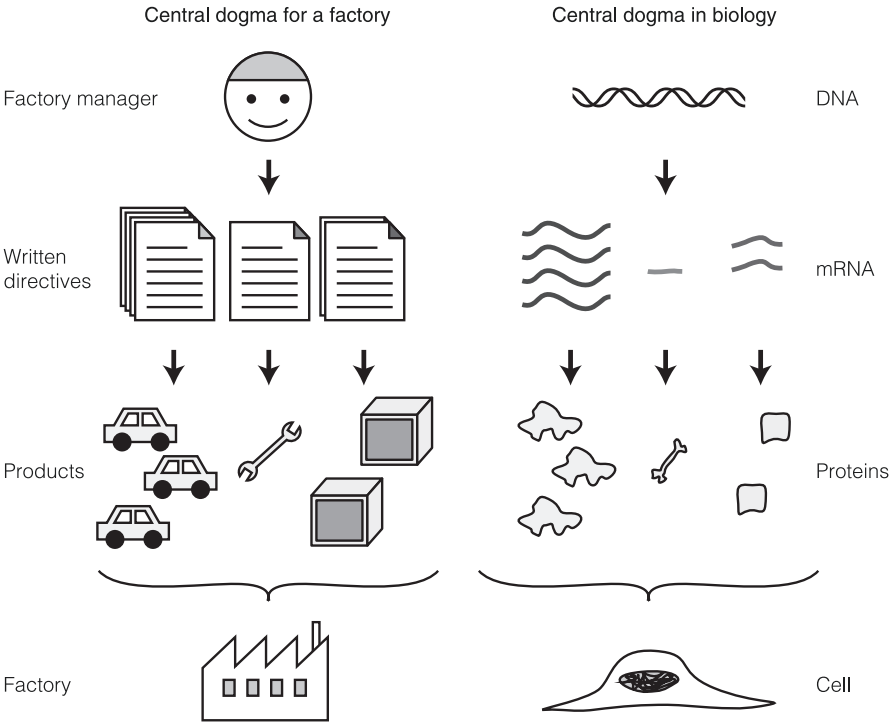

Fig. 1 Comparisons between a factory and a cell

ten directive (mRNA) is issued. When it gets to the production division, they begin turning out products (proteins). This flow of manager → written directive → product is the core process (central dogma) of the factory.

Student: I see. So there are a lot of proper rules for production.

Teacher: Right. But compared to actual factories, production in the cell is pretty sloppy.

Student: It is?

Teacher: First of all, written directives usually get issued as the manager thinks of them, so the timing is always random.

Teacher: Basically, like rolling a die, there might be only written directive issued to produce a certain product one day, and there might be six written directives issued the next day. Some days, there might even be no directive.

Student: I see. But even actual factories might have bosses who act like dictators in that way.

Teacher: Maybe so. But another thing that's different from an actual factory is that the workers in the production division are also sloppy. When they get a directive, they randomly turn out however many units of a product they feel like making that day. On top of that, the supervisor of the directives is sloppy too; he loses a certain percentage of written directives every day. If a written directive is lost, subsequent production of that product stops.

Student: That sure is sloppy. Does the factory run with all of that going on?

Teacher: It actually runs surprisingly well. The factory has a collection of know-how (genetic information optimized by evolution) accumulated over many years, and there are all sorts of rules in place so that production can run even with sloppy workers.

Student: What kinds of rules?

Teacher: For example, when an important product is going to be made, instead of just one written directive ordering production of a large number of units, a whole bunch of directives ordering the production of a certain number of units are all issued at once (transcriptional bursting). This way, even if the production division makes different numbers of units with each directive, if there are lots of directives, then the total amount of production when adding up all the numbers in the directives will be just about set. Also, think about what happens if the supervisor loses a written directive; if there were only one directive, the production line would come to a screeching halt. But if there are lots of directives and one is lost, there are still plenty of others left, so a set production volume can still be ensured.

Student: So it's like how if you bug someone by telling them the same thing over and over, they'll eventually listen to you. That's so like a living thing.

Teacher: Man-made machines like computers require absolute compliance with every single order, but cells are quite different in that regard. Maybe this is where the crucial differences between machines and life come from.

Production Strategies in an Industrial Group

Student: I guess there are all kinds of species in nature, but do they have different rules?

Teacher: The basic flow of manager → written directive → product is the same, but there's a wide variety of underlying rules as they're different for each species. Now then, let's think about differences in the rules among species as differences in the rules among industrial groups.

Student: Yes, sir. Please explain.

Teacher: First of all, the factory I was talking about earlier belongs to an industrial group (a species), and it runs its production according to a set corporate philosophy. Every factory in an industrial group has a quota where they're tasked with adding new factories that are copies of the present factory (cell division) within a certain period of time. Also, to cut down on costs, these additions are made almost entirely with products made by the original factory.

Student: These rules sound pretty harsh.

Teacher: They are. But they're necessary to beat other industrial groups in the battle for survival. If an industrial group didn't have these rules, they'd be numerically swallowed up by other industrial groups (natural selection).

Student: I see. You're saying that only committed industries can survive.

Teacher: An industrial group has all sorts of characteristics. For example, depending on the industry level of maturity (degree of evolution), it's classified as either a developing industry (bacteria or other prokaryote cells) or an advanced industry (plants, humans, or other eukaryotic cells).

Student: How are they different?

Teacher: There are differences in things like how thoroughly the production process is checked, the cyclability of the production process, and cooperativity among factories. For example, in developing industries, the number of managers in each factory (the genome copy number) is 1, whereas every factory in an advanced industry has multiple managers (in the case of humans, 2); that way, even if one manager gets sick (genomic mutation), it minimizes the effect on the factory production.

Student: So it's like a commercial airliner that always has more than one pilot.

Teacher: Also, the addition of new factories is a major event for a factory. Advanced industries set up their production phases (cell cycles) to be in sync with the timing of an addition so that all products and materials are completed in time. By the way, the production phase is mainly divided into four phases. Phases 1 and 3 (the G1 and G2 (growth) phases of the cell cycle) are for production activities to make the factory bigger; whereas phase 2 (the S (synthesis) phase) is about appointing a manager for the new factory (DNA replication); and in phase 4 (the M (mitosis) phase), the new factory is built (cell division).

Student: I see. So the factory sets up a cycle and pulls together to increase its turnout of the units needed at that moment with the goal of building an addition, is that right? I guess advanced industries have more advanced teamwork.

The Lineup of Products and Numbers of Units

Student: So the factories we've been talking about are multipurpose factories that turn out all kinds of products. How many types of products can a factory make, and how many units can they make?

Teacher: It varies quite a bit depending on the industrial group, but they say that famous developing industry B Corp. (*E. coli*) produces approximately 1 million units of about 4,000 types of products; while famous advanced industry H Corp. (humans) produces approximately 100 billion units of about 100,000 types of products.

Student: Whoa. That's a whole lot of different types of products.

Teacher: It definitely is a lot compared to actual factories. But considering that cells have to make new factories almost solely with products made in-house, it may be that they have no choice but to turn out so many types of products.

Student: How many units of the same product do they make?

Teacher: Oh wow, it's all over the place. Let's take B Corp. as an example. For high-demand products, they turn out about 100,000 units; but for other kinds of products, depending on the factory, they might not make even a single unit. The median number of units across all types of products is about 10.

Student: I see. I had no idea they did that kind of small-lot production. What are the types of products that they turn out in large quantities?

Teacher: Stuff like the production machines (ribosomes) that turn out all kinds of products, the printer for the written directives (RNA polymerase), and the parts for constructing the factory building (actin, microtubules, etc.). On the one hand, products that are necessary for factory additions (essential proteins) tend to get made in especially large numbers. On the other hand, they don't turn out many units of products with unclear effects, products that normally don't get used much, or products for a house or a factory that can get by with just one unit.

Student: Then I'd imagine that a corporate strategy would be really important because it'd determine what products to make and how many of them to make with certain resources and materials (sugars, amino acids, lipids, etc.). How does that stuff get decided?

Teacher: What products to make and how many of them to make are decided through the long history of the industrial group. Every factory manager randomly revises the policy for how many units of a product to make (genetic mutations). But these revisions usually don't go well, and the factory starts going to the dogs. But on extremely rare occasions, these policy revisions will end up making the factory run better. When that happens, the factory can build lots more new factories. After a long while, the number of copies of that factory will account for a large percentage of its industrial group; in other words, the entire industrial group policy ends up changing (evolution as a species).

Student: It must be annoying for the people in the factory that most of the policy revisions don't work out well. But I guess those kinds of ventures are necessary for the survival of the whole industrial group.

Teacher: Exactly. In the same way, improvement of existing products and development of new products come about as a result of factory managers' adventurous policy revisions and the sacrifices of many factories.

The Effects of "Fickleness"

Student: So the factories we've been talking about are different from the factories we see around us because their production activity is based on "fickleness", isn't it? How does this fickleness affect the way the factory runs?

Teacher: Good question. Let's consider an actual production process as an example.

Student: Okay.

Teacher: As a contrast to fickle (probabilistic) production, let's consider assembly line (deterministic) production. With the fickle method, a random number of units are made over a fixed interval of time. By contrast, with the assembly line method, the number of units made is always the same. Running production on a strict schedule requires extra effort (energy), but the advantage is that you'll always get the same number of units.

Student: So it's a double-edged sword.

Teacher: However, the fickle method obeys a statistical law (Poisson's law) in which the more the production is increased, the smaller the variation will be in the number of units produced over each interval; specifically, the variation in the number of units produced over that interval will be inversely proportional to the square root of the number of units.

Student: I see. So the effect of fickleness is mitigated by large orders.

Teacher: Right. Thanks to that property, it's possible to make a fairly steady number of units per interval for popular products made in large numbers. By contrast, with products for serious enthusiasts, which are made in small numbers, the number of units produced is directly tied to the fickleness of the factory manager.

Student: I see. That makes sense. So to look at it another way, are you saying that the "character" of each factory only appears in products made in small numbers?

Teacher: Based only on what we're talking about now, yes, that would be definitely true. But in reality, there are pretty big differences among factories in the numbers of units of products made in large numbers, too.

Student: Why is that?

Teacher: Because the fickleness adds up. I told you that in a factory, the production machines (ribosomes) and the written directive printer (RNA polymerase) are manufactured in advance, right?

Student: Right.

Teacher: Let's say that a certain factory, on a whim, mass-produces machines involved in the production of all products (global factors). Then, the next time fickleness occurs, that factory will be able to produce way more machines than other factories. If this positive spiral (positive feedback) continues, the gap between elite-level, high production capacity factories and lousy factories will get bigger and bigger, which gives factories distinct characters (cellular individuality).

Student: So even though it starts out as a copy factory with the same manager (DNA), a big gap will open up between the original and the copy depending on whether well-timed fickleness happens? It's kind of sad.

Student: Still, it's interesting that a system would let "fickle" production happen.

Teacher: It is. At a glance, it seems inefficient, but over a long period of time, you'll definitely be able to increase the set amount of production. On top of that, by not putting strict controls on the timing of production, you save the energy you would have otherwise spent.

Student : I guess you could call it the ultimate flextime system.

Teacher: But on the other hand, highly evolved systems conduct production activities with a cycle tailored to the production phases and with synchronized expression of various products.

Student: So "fickleness" is beneficial for cells when they're working alone, but it hurts when they work together. That's something we can understand from human society too.

Determining Small Numbers: How the Number of Flagella Is Determined

Seiji Kojima

The word "bacteria" may conjure a negative image of germs that cause diseases. However, the image of bacteria swimming lithely using flagella, their motility organs, is beautiful—even moving. Also, bacteria should not be looked down on as simple organisms merely because they are unicellular. Their simplicity bestows them with mechanisms that allow them to live as efficiently as possible. For example, they can set up apparatuses made of proteins at appropriate sites, and only as many as they need. As one such example, this chapter will discuss the motility device called the flagellum, which bacteria use to swim through aqueous media. Let's take a look into this world via a conversation between a university professor engaged in research on flagella and a third-year student in the department who is unsure of which lab to choose.

The Formation of Cellular Apparatuses and the Importance of Their Numbers

Professor: Hello. You're going to be a senior next year, so have you pretty much decided which lab you want to be in?

Student: Hello, Professor. No, I haven't decided yet. I entered the division of biological science because I've been interested in how our bodies are made ever since I was a kid, but my interest is too vague, and I still haven't found anything to help me to decide which specific topic I want to focus on for my graduation research.

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Professor: In that case, let's try sorting out your interests and simplifying them. What you want to learn about is, essentially, how the bodies of living things are formed. Is that right?

Student: Yes, sir.

Professor: As you know, if you look at it in detail, our bodies are made up of cells, which contain all sorts of apparatuses that work to maintain life. So, to understand the mechanisms that make up the body, you first need to learn how cells, the smallest units of the body, are formed; in other words, you need to learn how the individual apparatuses inside cells are produced. The crucial part here is that these apparatuses are set up at the appropriate sites and only the number of apparatuses necessary.

Student: Huh? Are you saying that sites and numbers are crucial?

Professor: What I'm saying is that if the apparatuses are set up at strange sites, or if there are more or fewer apparatuses than necessary, the overall function of the cell will suffer, which will trigger illness. Over the long course of evolution, biological activity has been honed to be conducted as efficiently as possible; wouldn't it then make sense that the appropriate number of apparatuses would be set up at specific sites? We study bacteria, so let's think about a bacterium—a life form consisting of a single cell. A bacterium has only the bare minimum of things it needs to live, so it's thoroughly stripped of anything it doesn't need; and the types of apparatuses produced, the number produced, and their positions are all strictly determined.

Student: I see. I hadn't thought about it like that before.

Professor: So instead of your area of interest, "the bodies of living things", try thinking about "cellular apparatuses". In studies on the mechanisms of how cellular apparatuses are formed, you don't have to bother with using complex multicellular organisms; you can do plenty of research on simple bacterial cells instead. What I mean is that you can tie in your interest to my area of research: flagella, the motility apparatuses of bacteria. That way, we can change your first question to "How are flagella made?" Perhaps I'm being a little pushy, but what do you think?

Student: Hmm, I think you've kind of convinced me. But I've only ever been interested in biological mechanisms in higher organisms. I've never dealt with bacteria, but if I can decide what topic I want to learn about, I know now that I can use bacteria to figure it out.

Professor: Exactly. If you want to learn about how the body grows after birth, you have to study complex organisms (like mammals), but depending on the question you want to answer, there's plenty of research you can do on unicellular bacteria. Besides, bacteria are well suited to research in many ways. They grow quickly, and instead of using conventional biology, chemistry, or physics alone, you can use novel techniques that combine them all. There are lots of researchers who start out by using bacteria to learn the basics of life sciences and then go on to study biological phenomena in higher organisms.

Student: Now I'm really interested. Could you go a little more into detail?

Flagella: The Motility Apparatuses of Bacterial Cells and the Control of Their Numbers

Professor: Alright! Now you're sinking your teeth into this! By the way, have you ever seen bacterial cells swim?

Student: No. But I really don't want to…it seems gross.

Professor: It isn't. I was blown away the first time I saw with my own eyes a bacterium rotating its flagellum to swim. It was really cute. Well, anyway, our lab studies a marine bacterium called *Vibrio*. They have a single flagellum at the end (the pole) of the cell (Fig. 1a). The flagellum is made of a spiral-shaped part called the filament outside the cell; a part called the hook, which acts as a joint; and a rotary motor, which is embedded in the inner cell membrane, at the base of the flagellum. When the spiral-shaped fiber is rotated like a propeller by the motor, it gives the bacterium the propulsion that enables it to swim. What's unique about the flagellar motor is that they use energy from ions flowing into the cell (the electrochemical gradient of ions formed inside and outside the cell membrane) as a power source [1].

Student: So the flagellum is a motor apparatus. And there's only one of them produced at the pole of the cell for some reason. It certainly is odd. Can it not have more flagella?

Professor: Good question. You would think that if there were lots of flagella growing at the pole, there would be more power, and the bacterium could swim faster. But that's not how it actually works. If there were multiple flagella, the motor for each one would rotate freely, the flagellar filaments would get tangled, and the bacterium wouldn't be able to swim well (Fig. 1a).

Student: So, there has to be only one flagellum for the bacterium to swim well. If it can't swim well, it can't get to nutrients or get away from things it hates, so it's a matter of life and death, isn't it? That's why it has a mechanism that forms only one flagellum. Is that what you're trying to determine?

Professor: Exactly. Actually, the main theme of my research is the mechanism of flagellar motor rotation. I didn't pay much attention to the number of flagella at first.

Mutants with Abnormal Flagellar Motors

Student: Wow. But motors made in living things work completely differently from man-made motors, so it's an interesting research topic.

Professor: It sure is (heh). *Vibrio* can swim especially fast and have amazing performance; their motor, which is their engine, reaches a rotational rate of 1700 revolutions per second.

Student: 1700 revolutions per second? I can't even picture that, but how does it rotate so fast?

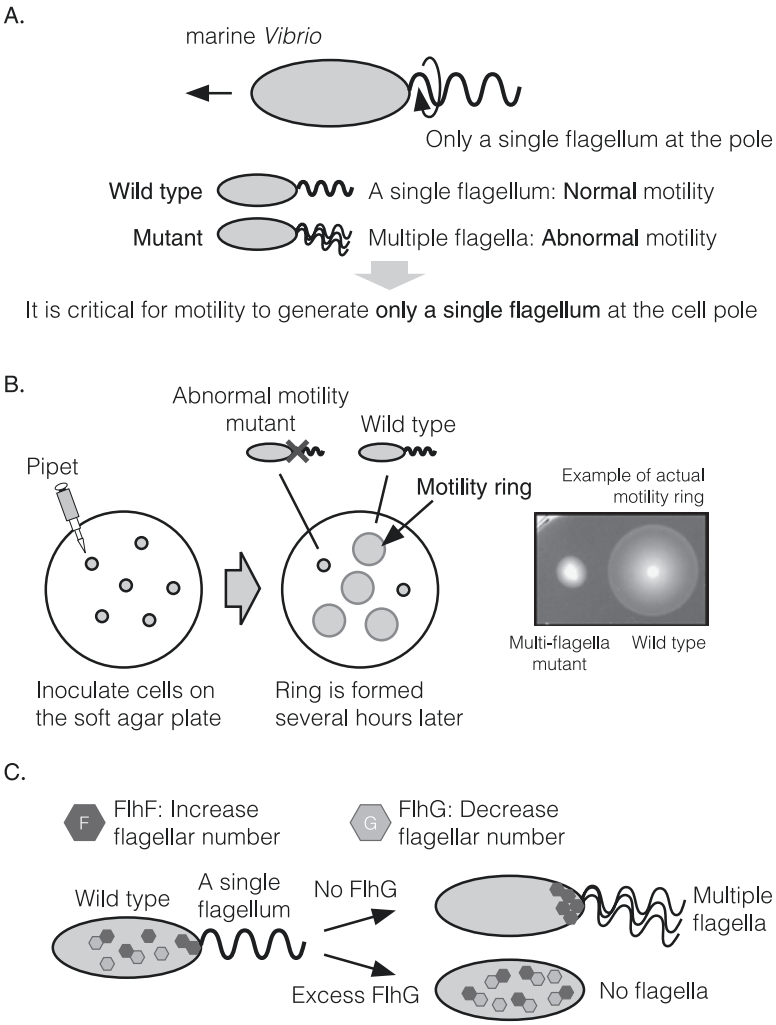

Fig. 1 Marine *Vibrio* has only one polar flagellum. (**a**) *Vibrio* swims in seawater by rotating its single polar flagellum. If *Vibrio* has multiple flagella, they become entangled, rendering *Vibrio* unable to swim well. (**b**) When *Vibrio* is grown in soft agar medium, wild-type strains repeatedly divide as they move toward the edge in search of nutrients, forming motility rings. Strains that swim abnormally cannot move in the agar medium and therefore do not form rings. The right panel is a photo of a motility ring. (**c**) The polar flagellar number regulation model published in 2008. FlhF acts to increase the number of flagella, while FlhG acts to decrease it. In the absence of FlhG, large numbers of FlhF congregate at the cell pole, resulting in the formation of multiple flagella. However, when there is an excessive amount of FlhG, FlhG binds to FlhF and remains in the cytoplasm; consequently, FlhF is absent, and no flagellum is formed. Wild-type bacteria have a specific balance between FlhF and FlhG; there are only as many FlhF molecules as necessary at the pole, and only one flagellum is formed

Professor: Actually, we don't know yet. Part of it might be because it uses sodium ions that are usually present in its marine environment. Oops, I did get a little sidetracked here. The reason I started studying the number of flagella is because among *Vibrio* that couldn't swim well due to some abnormalities in their motor system (they are called mutants; normal bacteria are called wild type), I found some bacteria that had several flagella growing at their poles.

Student: Now that the subject has suddenly changed, I'm lost. How did you find abnormal *Vibrio* that can't swim well in the first place?

Professor: By applying a chemical substance called a mutagen. *Vibrio* incorporate the mutagen into their bodies, and it sticks to their DNA, i.e., their genes. When that happens, the properties of the DNA change, and abnormalities occur in the genes that get passed on to their offspring.

Student: Wow, mutagens are scary.

Professor: They are. They can affect our own bodies, too, not just *Vibrio*, so you have to be careful when you handle mutagens in the lab.

Student: What happens when there are abnormalities in genes?

Professor: You know that proteins are made based on information from genes, right? Proteins serve all kinds of functions in cells; they also make up the parts of the apparatuses I told you about in the beginning. In other words, when there are abnormalities in genes, they make defective apparatuses.

Student: No kidding. So when there are abnormalities in genes, defective proteins get made, and the cell ends up with a broken-down flagellum.

Professor: That's right.

Student: But when you think about it, it's not like mutagens only mess up a specific gene; they act randomly on lots of genes, don't they? Wouldn't that result in cells with all kinds of defects? How do you single out *Vibrio* with defective flagella?

Professor: You just touched on something interesting. Actually, there's a very simple method for doing it.

Student: Can even a beginner like me do it?

Professor: Absolutely. You just "plant" the mutagen-treated bacteria in a soft agar medium, put it somewhere warm, and wait. Then, you see whether there are any motility rings (Fig. 1b) a few hours later.

Student: What's a motility ring?

Professor: You harden the nutrient-containing medium (which is like soup) to a consistency like gelatin. The agar medium contains water and is soft, so when you inoculate bacteria in it, the ones that can swim will constantly divide and multiply as they move around in search of nutrients. They spread out from where you first inoculated them toward the edge, and you get rings formed by groups of moving bacteria (Fig. 1b). The rings are pretty neat and tidy.

Student: Ooh, wow. So if bacteria have abnormal flagellar motion that prevents them from swimming, will the rings be smaller?

Professor: Exactly. Simple and easy to understand, isn't it?

Student: It seems that you could even do it in undergraduate practical training.

Professor: Yeah, I'll try to teach this next year. If you find bacteria that can't swim well, the next step is to observe the cell under a microscope to figure out why

they can't swim. Many mutants either don't have flagella or have something wrong with the rotation of their motors, but I found mutants that had a whole lot of flagella growing at the poles of their cells.

Student: That must have thrown you for a loop.

Professor: Yeah, I was really surprised at first.

FlhF and FlhG Regulate the Number of Flagella

Student: Which genes developed abnormalities?

Professor: Actually, right around that time, a research group in United States that was using another species of bacteria reported that two genes called *flhF* and *flhG*, which are next to each other, are involved in regulating the number of flagella, so we thought there might be something wrong with these genes and decided to look into them.

Student: So you had potential suspects. In that case, I guess you found out the results pretty quickly.

Professor: We did. We ended up finding a nonsense mutation (an abnormality where synthesis of a protein stops midway through) in the *flhG* gene [2]. In other words, the FlhG protein wasn't being produced correctly.

Student: Ooh, bingo! If FlhG isn't made correctly, then there will be more flagella, so FlhG acts to reduce the number of flagella, right (Fig. 1c)? What does FlhF do?

Professor: You're a sharp one. As you said, FlhG negatively regulates the number of flagella. On the contrary, we learned that if you produce bacteria with the neighboring *flhF* gene removed, flagella hardly grow at all. In other words, we learned that without FlhF protein, no flagellum is produced, so FlhF must act to increase the number of flagella (Fig. 1c).

Student: Okay, so on the flip side, if you make the cell to produce a lot of proteins, does that mean a lot of FlhF would result in more flagella, and a lot of FlhG would result in no flagellum?

Professor: Exactly. So, what do you think we looked into next?

Student: I'm wondering where in the cell FlhF and FlhG act. If they make polar flagella grow, I'd assume they act at the pole of the cell.

Professor: You're good. We linked the fluorescent protein GFP from the jellyfish *Aequorea victoria* to FlhF or FlhG in cells and observed them with a fluorescence microscope. This makes FlhF and FlhG light up, so you can see where they are in the cell. When we did that, we saw bright spots at the pole where there was lots of FlhF-GFP; on the flip side, we only observed congregations of FlhG-GFP at the pole in about half of the cells. In the other half, the whole cell glowed faintly, which meant that FlhG-GFP was diffused throughout the cell.

Student: So FlhF was at the pole, huh? Does that mean that the number of FlhF at the pole is the key to the number of flagella? If the number of polar flagella increases when there's no FlhG, does that mean there's a lot of FlhF at the pole?

Professor: Good question. In strains with no FlhG, there was definitely a lot of FlhF-GFP at the pole glowing brightly. Also, we learned from another experiment that FlhG binds to FlhF. So, we came up with a model in which FlhG binds to FlhF and is retained in the cell, which results in an appropriate number of FlhF molecules at the pole and exactly one flagellum (Fig. 1c) We published a paper about it [3].

Student: It's all coming into focus now. FlhF works at the pole and increases the number of flagella, while FlhG works in the cytoplasm and reduces the number of flagella. Do I have that right?

Professor: Taking in the results of the latest studies, it's actually a bit more complicated than that, but, well, I guess you could say that's the general idea.

In Conclusion

Student: I've learned today that in order to make a single flagellum, two proteins called FlhF and FlhG work together, and it's important to have only as much FlhF as necessary in the target site. Now, I want to know more about how it all works. If I make it the theme of my graduation research, I guess I can find out for myself.

Professor: That's right. I'm glad you've taken an interest in it. As I explained to you today, having the appropriate numbers of proteins in appropriate locations is absolutely crucial in biological functions. Even a slight loss of balance in numbers can result in abnormalities, especially when small numbers work best. I'd like to shed light on this problem of numbers using bacterial flagella. Researching bacteria may sound outdated, but their small bodies contain all the crucial mechanisms they need to live. Also, like I told you at the beginning, you can use all sorts of techniques with bacteria. Besides, whatever else I might tell you, bacteria are truly beautiful when they swim. You should give some thought to joining our lab.

References

1. Terashima, H., Kojima, S., et al. (2008). Flagellar motility in bacteria: Structure and function of flagellar motor. *International Review of Cell and Molecular Biology, 270*, 39–85.
2. Kusumoto, A., Kamisaka, K., et al. (2006). Regulation of polar flagellar number by the *flhF* and *flhG* genes in *Vibrio alginolyticus*. *Journal of Biochemistry, 139*, 113–121.
3. Kusumoto, A., Shinohara, A., et al. (2008). Collaboration of FlhF and FlhG to regulate polar-flagella number and localization in *Vibrio alginolyticus*. *Microbiology, 154*, 1390–1399.

Controlling Production with Small Numbers: Precision Apparatuses Made of Proteins at Work in Bacteria

Katsumi Imada

Many bacteria that cause disease in humans and animals, such as salmonella, pathogenic *Escherichia coli* O157, and *Vibrio cholerae*, move by rotating their flagella, which are fibrous locomotor apparatuses growing from their bodies, resembling screws. Though the thickness of a flagellum is only 1/4000 that of a hair, an enlarged view with an electron microscope reveals that a flagellum has a machine-like appearance. A flagellum is a complex device made of a combination of roughly 30 types of protein molecules. Parts can contain as few as 1–5 protein molecules or as many as tens of thousands. Cells have various infinitesimally small complex apparatuses which, like flagella, are composed of an aggregation of protein molecules and support biological activity. When these devices are needed, only the exact number needed is made, thus enabling them to function well. The production of these apparatuses is regulated by proteins; in the case of flagella, production is regulated by a small number of proteins called export chaperones. Here, we examine how bacterial flagella are made through a conversation between Shun, a high school student who unfortunately contracted food poisoning, and Maiko, the school nurse.

Bacterial Flagella

Maiko: I'm sorry about that food poisoning you got on the field trip the other day. Are you feeling better now?

Shun: I had non-stop diarrhea, I ran a high fever, my stomach hurt, it was something awful, but I'm okay now. The doctor at the hospital said it was caused by salmonella.

Maiko: Salmonella? There's an electron microscope image of it on that poster (Fig. 1).

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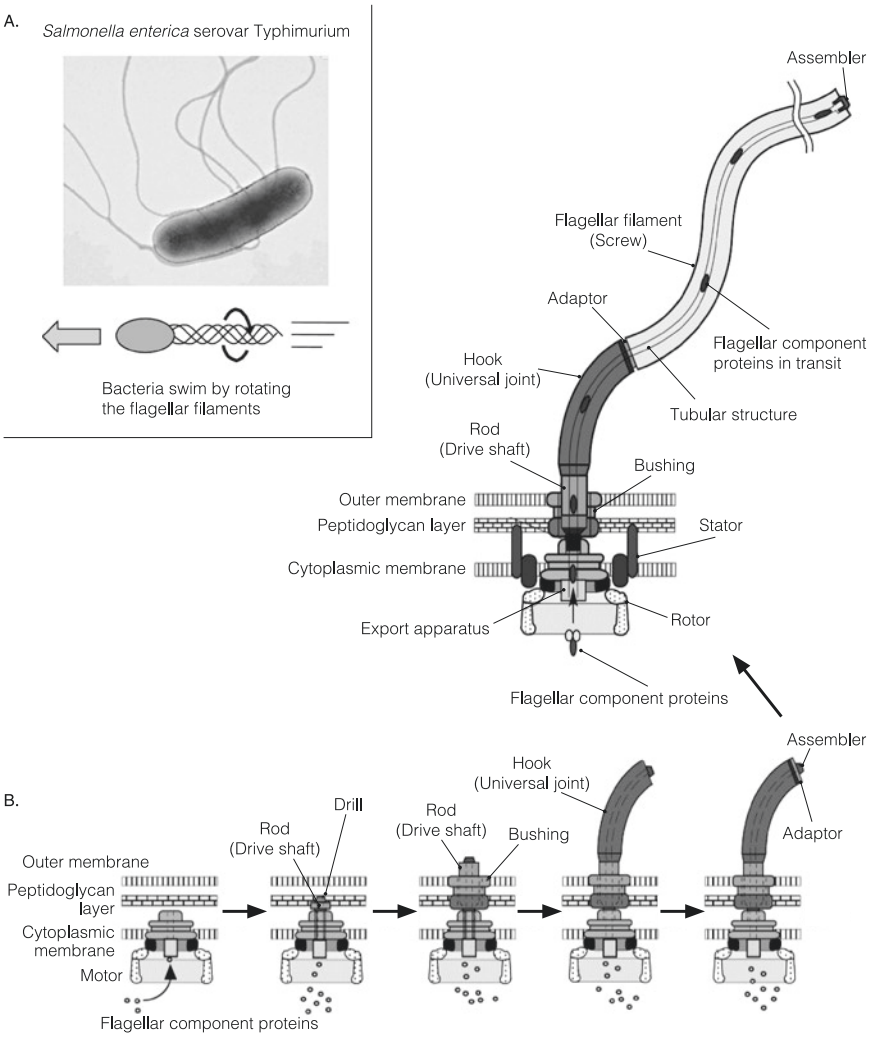

Fig. 1 (**a**) Swimming of *Salmonella*. (**b**) Structure and the construction pathway of the bacterial flagellum

Shun: Huh, so this is what did it, eh? There're some hairs growing around its body. They look nasty.

Maiko: Those hairs are called flagella. They're not like the hairs on your head. They move.

Shun: Move?

Maiko: Bacteria such as salmonella and *Escherichia coli* rotate those hairs to swim. If they swim to the cells in your gut, they cling to them, multiply, and can cause diarrhea.

Shun: Rotate?
Maiko: Right. The base of the hair has a motor made of proteins. Bacteria swim by rotating the hairs at a speed of about 300 revolutions per second (Fig. 1).
Shun: They rotate their hairs with motors? What? And they can swim just by rotating their hairs?
Maiko: They're not really hairs. They're like complex machines. Every part has a proper function, and the hair works like the propeller on a boat. A really tiny propeller.
Shun: But we're talking about a living thing, aren't we? How the heck does a living thing have a propeller?
Maiko: There's more to it than that. It's got a sturdy rotating shaft connected to the motor; a part that works as a soft joint; a part that works as a long, hard propeller; and a bearing. It looks like a machine designed by humans (Fig. 1).
Shun: I didn't know bacteria had stuff like that in them. By the way, what is it made of?
Maiko: The hair, the motor, everything is made of proteins. You need about 50 types of proteins to make the flagellum, and they're all made by the bacterium. A flagellum is about 20 micrometers long (1/50 of a millimeter). I think it takes a little over an hour to grow that long from scratch.

The Flagellum Grows at the Tip

Shun: Whoa, it grows. Like hair, I guess.
Maiko: The hair on your head grows from the root, but a flagellum grows by having "protein parts" stick to the tip. It's like building a skyscraper or a tower. The protein parts get carried to the top to assemble the flagellum.
Shun: Huh. But how does it get the parts to the top?
Maiko: The inside of the flagellum is hollow from the root to the tip, like a straw. The protein parts go through the straw to the tip and get piled up in order (Fig. 1).
Shun: Whoa, they go through the inside, huh? Okay, so how do the parts get inside the straw?
Maiko: At the base of the flagellum, in the center of the motor buried in the cell membrane, there's an export apparatus that sends the protein parts into the straw.
Shun: Export apparatus?
Maiko: Right. And it's made of proteins. It picks the protein parts that are made by the bacterium and sends them out in order.
Shun: There's a set order? Whoa.
Maiko: The first part that gets sent is the rod proteins. The rod is the rotating shaft and is built on top of the motor by stacking up the rod proteins in a spiral shape (Fig. 1). The rod is made of about 60 proteins of 5 different types.
Shun: So the shaft goes through the cell wall. Isn't the cell wall getting in the way?

Maiko: Right, right. The tip of the rod has protein molecules acting as a drill that opens a hole in the cell wall so that the flagellum can grow outside the bacterium (Fig. 1).
Shun: A drill? It's like construction work, isn't it?

The Length of the Hook Is Determined by a Molecular Ruler

Shun: So what happens next?
Maiko: Once the hard and sturdy rod is built, a "hook" is built on its tip; the hook is a soft, bendable joint, like a rubber tube (Fig. 1). The hook extends and shrinks as it rotates, so it can rotate the flagellum properly even if the flagellar filament faces a different direction.
Shun: So the hook is made of proteins too. Everything is made of proteins but their properties are all so different.
Maiko: The hook is made of about 120 protein molecules of the same type piled up on the tip of the shaft in a spiral shape. It extends to a length of about 55 nanometers (1 nanometer is 1/100 of a millimeter).
Shun: Is it always about 55 nanometers long?
Maiko: It is. The length is important, and it has its significance. If the hook is too short, it isn't flexible enough and can't rotate properly when the flagellum bends. If the hook is too long, it gets limply, and the flagellum can't function as a proper propeller. So, as the hook is being made, its length gets measured every now and then to make sure it stops growing at the perfect length.
Shun: Wait, its length gets measured? By whom? How?
Maiko: There's a protein that acts as a ruler. Well, more like a measuring tape than a ruler, I suppose. While the hook is being made, a ruler protein is regularly sent to measure the length of the hook. The ruler protein has a domain that extends like a measuring tape and a spherical domain. The extending part of the ruler protein goes through a hole that's right in the middle. It goes further into the hole, and when its fully extended tip reaches the tip of the hook, it measures whether the hook is just the right length based on where its spherical domain is (Fig. 2).
Shun: What happens when the hook is the right length?
Maiko: When the hook is about 55 nanometers long, the spherical domain of the ruler protein comes into contact with the export apparatus at the root, and the spherical domain flips the export apparatus switch. When that happens, the protein parts for the hook and rod, which were transported until then, can't get through anymore, and the only proteins that get transported are the ones that make the rest of the flagellum, which couldn't get through before. That's why the length of the hook is about 55 nanometers (Fig. 2).
Shun: The measuring tape is a weird protein, huh? So, what happens when the measuring tape is absent?
Maiko: The switch doesn't get flipped, so the hook gets longer and longer and becomes this weird, flaccid, bent object called a polyhook.

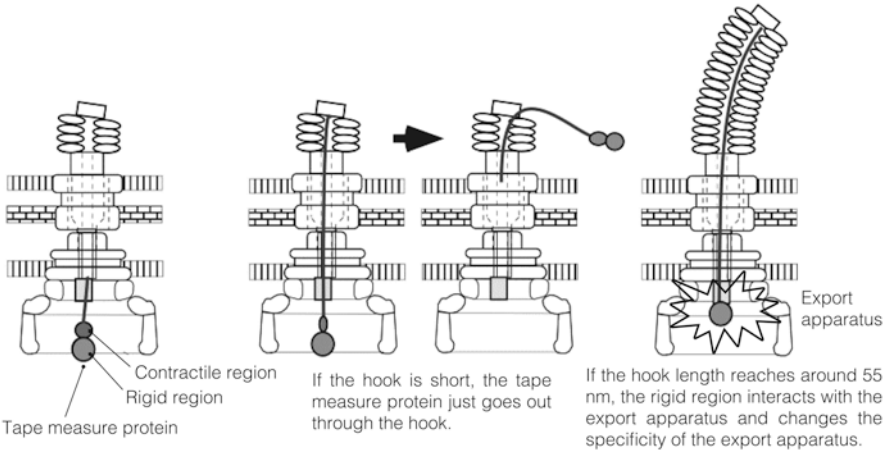

Fig. 2 The control mechanism of the Hook length

Shun: If it's all flaccid, then I guess it can't swim right.

Maiko: On the flip side, if there's too much measuring tape, it'll measure the length of the hook constantly, and it could end up flipping the switch before it's supposed to, before the hook gets to 55 nanometers. If that happens, the hook will be too short, and it won't be able to move properly. So the amount of measuring tape is important.

How a Long Flagellar Filament Is Made

Shun: What happens after the hook is made?

Maiko: Next, a 20-micrometer flagellar filament is finally made, but before that, there are two other things that are made on top of the hook: an adapter that connects the soft hook and the hard propeller, and an assembly apparatus that helps the flagellar filament grow at the tip (Fig. 1). The adapter is made of 11 molecules, each of two types of proteins, and the assembly apparatus at the tip is made of 5 molecules of a single type of protein. When the adapter and the assembly apparatus are mounted on the tip of the hook, the preparations for making the flagellar filament are complete.

Shun: The flagellar filament sure is long. How many types of proteins is it made of?

Maiko: The flagellar filament, which acts as a propeller, is made of just one type of protein, called flagellin. The flagellin sent to the tip is inserted directly below the assembly device. Tens of thousands of flagellin molecules are used for each flagellum. So, at the stage when the flagellar filament is made, a whole bunch of flagellin molecules get sent in one shot.

Shun: So, when you say they all get sent in one shot, are you saying that once the hook is finished, flagellin molecules start getting made?

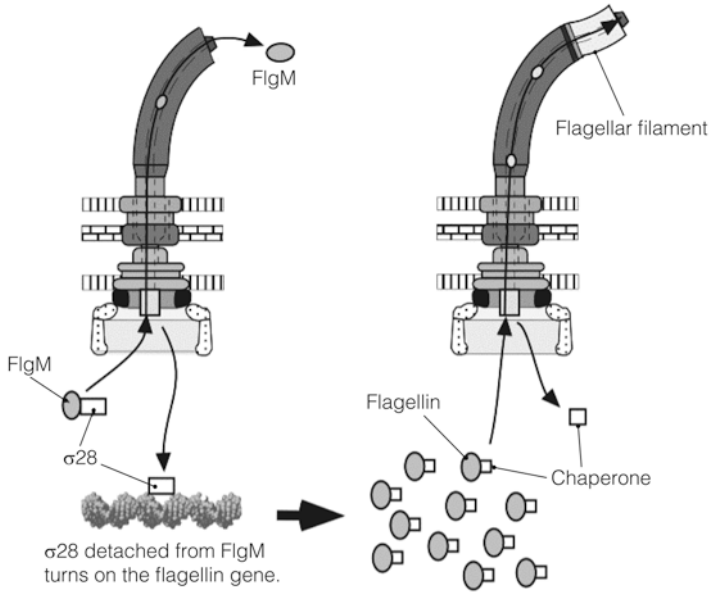

Fig. 3 The control mechanism of the flagellar filament formation

Maiko: Right. Proteins such as flagellin that are placed at the end of the hook aren't made until after the hook is made. Until the hook is finished, the switch for the gene that codes for flagellin is turned off; once the hook is finished, the switch is turned on.

Shun: How does the switch get turned on?

Maiko: The switch for gene expression is turned on by a protein called sigma 28. Normally, it's paired up with a protein called FlgM and just sits there quietly, not doing anything. But when the hook is finished and the export apparatus switch is flipped, FlgM moves out through the export apparatus, with sigma 28 left behind. Then, sigma 28 turns on the gene switch, and a bunch of flagellin molecules are being made (Fig. 3).

How Flagellum Production Is Stopped

Shun: Okay, I get how all those flagellin molecules are being made, but how does the process get stopped?

Maiko: Good question. Of course, there's a mechanism that stops the production process. It's done by proteins called export chaperones. Normally, they're paired up with a protein that composes the rest of the flagellum. It's just similar to FlgM and sigma 28.

Shun: Is it important for it to be paired up?

Maiko: It is. Inside the cell, it's paired up, but when its partner is exported out, the chaperone is left behind inside the cell and does all sorts of things (Fig. 3), the same as sigma 28.

Shun: "All sorts of things"? Like what?

Maiko: For example, a chaperone pairs up with an adapter protein and gives an order to produce a bunch of FlgM. If the order is given while FlgM is outside the bacterial cell, the switch for producing flagellin stays turned on. Then, a bunch of flagellin is made later and gets paired up with sigma 28, which turns off the switch and keeps the balance.

Shun: Gotcha. So there's a proper system for turning off the switch.

Maiko: Also, the chaperone that pairs up with flagellin has a function that makes it hard for FlgM to be sent out of the cell. So, as flagellin is continuously exported, more and more chaperones build up inside the cell, which makes it harder and harder for FlgM to be exported. When that happens, FlgM immediately pairs up with any unpaired sigma 28. Then, as lots of flagellin is exported, flagellin production drops rapidly. If a lot of flagellin has been exported, it means that the flagellar filament is already long enough, so flagellin isn't needed anymore.

Controlling the Number of Flagella

Shun: That's a good system. So, I get how flagellin production gets stopped, but what about the hook? If the parts for the hook can't be exported, wouldn't that mean they accumulate in the bacterium? Isn't that bad?

Maiko: Export chaperones do a good job of regulating that, too. The chaperones paired up with the assembly apparatus proteins can turn off the switch for making the protein parts for everything up to the hook. It's a protein called FliT; when its partner is exported, FliT remaining in the cell turns off the switch (Fig. 4). The presence of unpaired FliT means that the hook has been finished, and the export device switch has been flipped. If everything up to the hook is finished, then the hook parts aren't needed anymore, so the switch is turned off to avoid unnecessary production.

Shun: That's an interesting system. The amount of chaperones left in the cell tells you how much of the flagellum has been made. It seems like everything would be pretty messed up if there were no chaperones.

Maiko: Exactly. Bacteria would be in a bad state. Without FliT, bacteria wouldn't be able to stop flagellum production, and they'd be a hairy mess of nothing but flagella (Fig. 4).

Shun: How many flagella do bacteria usually grow?

Maiko: I think salmonella have around 5 to 10 flagella. With that number, the flagella can entwine with each other nicely to form a thick bundle that works as a big propeller, and the bacterium is able to swim really fast.

Shun: So what happens if there are lots of hairs?

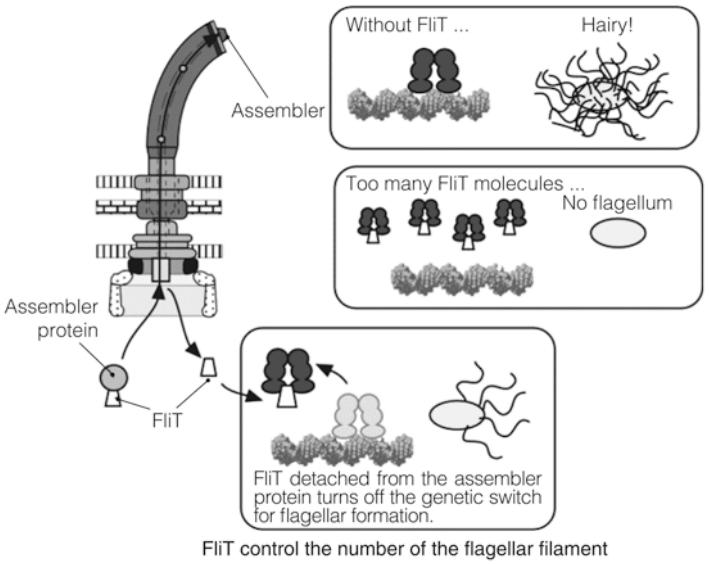

Fig. 4 The control mechanism of the number of the flagellum

Maiko: If there are too many hairs, they get tangled into a big mess, and the bacterium won't be able to swim well. On the flip side, if there's too much FliT, flagella can barely grow at all; the bacterium would be slippery and unable to swim. It's really important for the bacterium to have just a little bit of FliT so it can survive (Fig. 4).

Shun: Okay, so if that function of FliT is stopped, a bunch of hairs would grow, right? That's good info. My dad will be happy. I can make a hair-growing drug and get crazy rich!

Maiko: I told you that hair in bacteria is different from the hair on your head… bacteria get all hairy, but it probably wouldn't work on your father.

Shun: Huh. That's too bad.

Organisms that Function with Small Numbers of Molecules

Hajime Fukuoka, Yong-Suk Che, and Akihiko Ishijima

Escherichia coli and other bacteria are said to be the lowest forms of life in the biological world. Nevertheless, bacteria are magnificent organisms that can recognize the ambient environment, propagate ambient information into its body, and (in the case of most bacteria, at least…) possess the capacity to swim toward a more favorable environment; these are systems that no man-made machine could even hope to imitate. This complex machinery is constructed in an extremely small unit of space about a femtoliter. Because this organism is so small, its proteins function in far smaller numbers than seen in other organisms (on the order of about 100 protein molecules involving methylation and demethylation, and 1000 phosphorylated protein molecules work in a cell). Let us gain an understanding of the chemotaxis and motility of bacteria through the following conversation between a mother and her daughter.

A Mother–Daughter Conversation

The mother is a professor of biophysics at university. Though she loves doing experiments, she's been overwhelmed lately with other things such as meetings and reports, leaving her with no time to conduct experiments.

Her daughter is a 15-year-old high school student who, taking after her mother, has a brain for science rather than humanities. Although she doesn't have many chances to do experiments, she takes in all sorts of information from school and TV.

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Biophysics?

Daughter: Hey mom, what do you research at the university?
Mother: Biophysics.
Daughter: Biophysics? There's no class like that in high school. We do have physics and biology, though…
Mother: As you said, there are no biophysics classes until college. Try thinking about it like this: whenever you study something, there are all kinds of ways to think about it and methods you can use, right?
Daughter: Right…
Mother: For instance, to study the movements of planets, you need things like math and physics; and to study chemical reactions between one thing and another, you need chemistry, right?
Daughter: Yeah…
Mother: Well, there are all kinds of methods and ways of thinking that you can use to study life. When you look at life from the perspective of chemistry, you get biochemistry. When you think about life in terms of math, that's mathematical biology. When you examine life as physical phenomena, it's called biophysics.
Daughter: Whoa…and I thought biology and math were totally different subjects….
Mother: Yes, biological phenomena are very complex. To understand them, you have to apply all sorts of academic disciplines.

Lower Life Forms? Bacteria, Artificial Intelligence, and Brain Research

Daughter: So what exactly do you research in biophysics?
Mother: I study bacteria, especially *E. coli*.
Daughter: Whaaaat?! *E. coli*?! But it's so nasty!!!
Mother: No it isn't. It's not really so nasty.
Daughter: But I thought you were researching something cooler…like the brain, or artificial intelligence, or induced pluripotent stem cells (iPS) cells, or…
Mother: Oh dear…you've been getting too much of your knowledge on life sciences from TV…
Daughter: Well, yeah…but aren't bacteria really low-life forms? Can you really get anything out of studying them?
Mother: Well…iPS cells certainly seem immediately useful…but an *E. coli* is a worthwhile organism of research in terms of a topic like "What is life", even if isn't useful right away.
Daughter: …
Mother: You've probably already learned this in school, but you know our bodies are mostly made of proteins, right?

Daughter: Yeah, and I think proteins are made of amino acids, and there are, what, 20 types of amino acids?

Mother: Yes. Hemoglobin, which transports oxygen in the blood, is made of proteins. So are muscles. Proteins are also responsible for immunity. The important thing is that the proteins made up of these 20 amino acids are at work in every living thing on earth. So, if we can learn how proteins work, it would be a big step towards understanding life.

Daughter: Huh, so we're all the same…

Mother: Yes, including elephants and tulips. Which means…there should be principles that are common to all living things.

Mother: Have you ever heard of Jacques Monod?

Daughter: No…is he a singer?

Mother: He was a French biochemist who won the Nobel Prize in Physiology or Medicine in 1965.

Daughter: Ohh, so he was a great scientist.

Mother: He once said, "Anything found to be true of *E. coli* must also be true of elephants" [1].

Daughter: I see. It's the same thing you just said about principles that are common to living things on earth.

Mother: Right. In order to understand the common principles of living things, you can study a complex brain or a simple bacterium. Every researcher is free to study whatever they want. There's no hierarchy.

Daughter: I see, I see…

Mother: I think it's easier to understand things by studying them in the simplest forms possible. Brain research certainly does seem interesting, but I'll leave that to other researchers…

Daughter: Hmm, maybe I'll be like you…

Mother: But bacteria aren't really so simple. We actually only know a tiny portion of what there is to know about bacteria. Besides, bacteria are magnificent organisms. They leave descendants, and they move in search of delicious food. They're soooo amazing!

Daughter: I see…actually, when you think about it that way, you can't underestimate bacteria, can you…

Small Spaces

Mother: By the way, do you know how big *E. coli* is?

Daughter: Uhhh, all I know is that it's reeeally small…small enough that you can only see it with a microscope. But its exact size? Nooooo idea…

Mother: It's shaped like a capsule of about 2 micrometers long and with a diameter of about 1 micrometer. It looks just like a medicine capsule (Fig. 1).

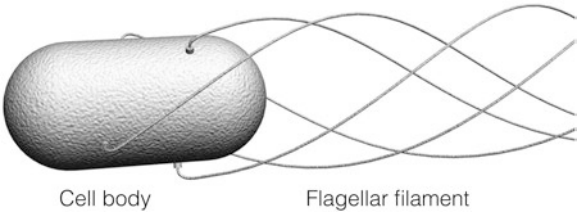

Fig. 1 *Escherichia coli*

Daughter: A micrometer is…well, 1/1000 of a meter is a millimeter, so 1/1000 of that is…a micrometer is really, really small, huh…
Mother: Okay, how many liters is it?
Daughter: Uhhhh, uhhhh, it's a capsule, so it's a sphere and a cylinder, so… (after a while) …10 to the power of minus 15 liters?
Mother: Right. The volume of its body is a very, very small unit called a femtoliter. The body has devices (machines) inside it that can examine the outside environment, use that information to consider whether the environment is suitable and move to an environment it likes.
Daughter: Seems like all those machines would be packed in really tight.
Mother: Yes, there are lots of molecules of all kinds working in a really tiny body.

Chemotaxis

Daughter: So what about bacteria do you study?
Mother: Mainly chemotaxis.
Daughter: Chemotaxis???
Mother: It's similar to the way a delicious smell almost hypnotizes you and lures you into a shop. When smell molecules from the shop get scattered into the air and reach your nose, your brain decides it's a delicious smell and orders your legs to head toward the shop, right? *E. coli* does the same thing to find places that are rich in nutrients and head towards them. It also senses other things such as temperature and pH. The opposite happens, too; *E. coli* runs away from unpleasant stimuli (Fig. 2).
Daughter: Okay, okay, I get it…but does that mean bacteria have things like noses, nerves, and motor organs in that tiny, tiny space?
Mother: Yes, but they're not exactly the same things. First, the bacterium equivalent of a nose is a receptor. It finds chemical substances in its surroundings. Its equivalent of the brain and nerves is a signal transmission system; it has a completely different mechanism from nerves, but I suppose it's similar to nerves in that it transmits information. As for a motor organ, bacteria have a flagellar motor; it's a rotary motor, which is something that's rare among organisms.
Daughter: Huh. You said they have a completely different mechanism from nerves. How does it work?

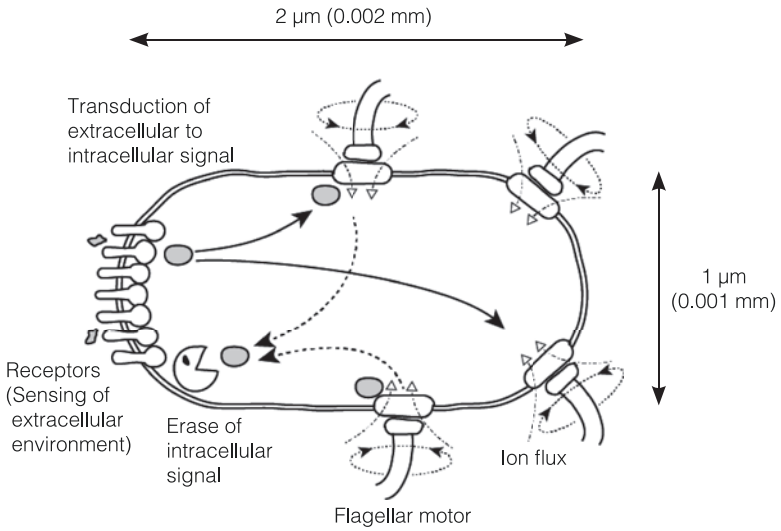

Fig. 2 Nano-world in *E. coli* cell

Mother: So, it normally takes some sort of work to carry things and transmit information, right? For instance, if you use a delivery service to send a package, you have to pay for labor and transportation costs, right?

Daughter: Definitely. It's the same with information; looking at information on a smartphone costs a communication fee, and you need battery life. And the telecom company works hard in lots of ways to make it possible.

Mother: Right. Things get carried and information gets transmitted in our own bodies too, but not for free. For oxygen to be delivered from the lungs to every part of the body, the heart has to pump blood throughout the body, and information has to be transmitted from the brain. All of that requires energy.

Daughter: Oh yeah, you're talking about adenosine triphosphate (ATP), aren't you?

Mother: Right, right. That's why in higher animals, cells differentiate into all sorts of things including nerves and blood vessels, with each type of cell playing its own role.

Daughter: But in that case…since a bacterium is single-celled, a lot of stuff has to be done in that one cell, doesn't it….

Mother: That's the interesting part. Unlike higher animals, unicellular organisms like bacteria rely on diffusive motion.

Daughter: Diffusive?

Mother: For instance, if you put a drop of ink in a tank of water, the ink gradually spreads out, right? The ink moves on its own even if you don't put any energy into it, doesn't it?

Daughter: Yeah, it does…it spreads reeeeally slowly, even though you're not doing anything to it.

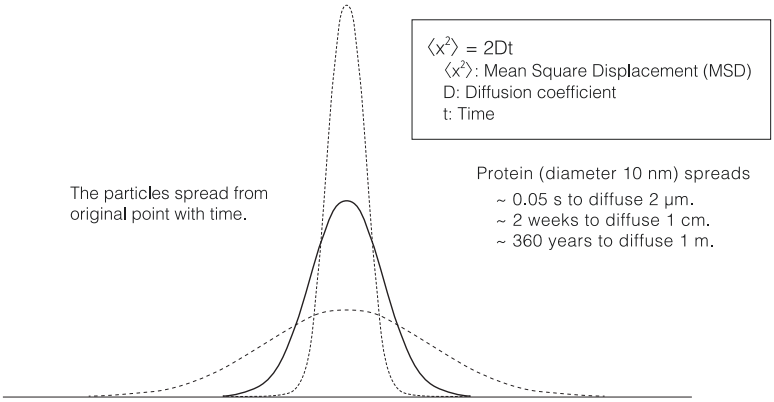

Fig. 3 How diffusion looks

Mother: It's a phenomenon called diffusion. Basically, the water molecules in the tank collide with the ink molecules at random, so the ink molecules spread out gradually. If the ink molecules use this phenomenon, they can move without using energy.

Daughter: Wow! That's awesome that they can transmit information for free! Why did higher animals go to the trouble of making complicated blood vessels and nerves?

Mother: Because that diffusion phenomenon isn't suited for moving things long distances. The mean distance that a thing moves by diffusion is proportional to the square root of the time it takes for the information to be transmitted. For instance, a signaling molecule moves from one end of a bacterium to the other in less than 1 second; but for that molecule to move 1 cm, it would take 2 weeks; and to move 1 m, it would take 360 years! (Fig. 3)

Daughter: Say what?!?! At that rate, if you took a breath to draw in oxygen within your lungs, you would die before the oxygen reaches your toes!

Mother: Exactly. So, in order to grow big, higher animals crafted a transportation system that requires energy. On the flip side, for tiny organisms like bacteria, diffusion is good enough for transporting things. Even if they made a transport device that required energy, the costs would be a waste.

Daughter: Huh. I guess size matters for living things.

Mother: But diffusion alone may not be enough to transmit information efficiently.

Daughter: It isn't? But I already had a good answer.

Mother: Well, what do you think is important for transmitting information?

Daughter: For transmitting information? Let me think…accuracy, timing…what else?

Mother: Don't you also need the sharpness of information?

Daughter:???

Mother: For instance, when a traffic light changes from red to green, how would it be if the light changed slowly?
Daughter: It'd be really inconvenient! You wouldn't know when it's okay to cross.
Mother: Exactly. It's the same with diffusion. By the diffusion, information is transmitted sharply for people close to origin of diffusion, but it takes a while for the stuff to go away.
Daughter: Uhh, I don't really get it….
Mother: Well…let's say that you just farted.
Daughter: Hey! How rude!
Mother: It's just an example. If you did, and I was a little way away, it would take a little time before I said, "P.U.!"
Daughter: I don't know if I should nod….
Mother: The fart molecules would all spread around me via diffusion and then go away, but it would happen really slowly….
Daughter: It'd smell forever! Ugh….
Mother: Right. So, simple diffusion by itself doesn't make an information transmission system.
Daughter: So, what you're saying is that to transmit the information of me having farted, I'd have to actively move the fart molecules away from me with a fan or something after they reach me.
Mother: Right, right. So, single cell organisms like bacteria need a mechanism to transmit information and another mechanism to delete it (Fig. 2). Plus, these two mechanisms have to be well-synchronized.
Daughter: Huh. Bacteria are a lot more complex than I thought.
Mother: They sure are. And there's still a lot we don't know about them.

Receptors

Daughter: Hey mom, what about their noses?
Mother: They may basically be the same as higher animals' ones. There are sites that can bind specific molecules for smell and taste, and they react according to the number of molecules they bind.
Daughter: Huh, no kidding.
Mother: But what's interesting about *E. coli* is the density of the molecules it can sense. I guess you could call it *E. coli* dynamic range.
Daughter: Dynamic range?
Mother: Maybe I should say the range of values it can sense from smallest to biggest? Bacteria can sense super low concentrations to high concentrations. To put it in really basic terms, receptors can normally only detect about a difference of one order of magnitude in strength.
Daughter: So, with taste, for example, you mean there's about a ten-fold difference in strength between "this is weak!" and "this is strong!"?

Mother: Right, right. So if taste is any weaker than the bottom of the range, you can't detect it; on the flip side, if taste is stronger than the top of the range, you'd know there's a taste, but that's all. That's how it normally works, but bacteria can detect differences in strength up to 10 to the fourth power, about 10,000-fold.

Daughter: Whoa, that's amazing…but…how?

Mother: There are lots of possible mechanisms, but cooperativity and adaptation play a big role.

Daughter: Cooperativity? Adaptation?

Mother: For example, if you work on something by yourself, you won't get very far. But if lots of people work together on the same job, it'll get finished quickly.

Daughter: Totally…but even if there are a lot of people, the job won't go well at all if they're all scattered and not organized.

Mother: Exactly. That's cooperativity. Bacteria are incredibly small, but they have about 10,000 receptor molecules. Instead of being scattered, they're all assembled in one place; they hold hands and work together, which allows them to smell their environment with dynamic range.

Daughter: Woooow, that's really fancy stuff.

Mother: So, density is really important in a reaction in a test tube; but in a cell, besides just density, the sites of the molecules, the way they congregate, their cooperativity, timing, and so on are really, really important; and what's more is that all of these factors affect each other. That's pretty complex, isn't it! On top of that, their strong cooperativity means that they all work together, so they behave as if they were a really small number of molecules.

Daughter: It's like if one person in a class at school senses smell molecules, then everyone in the class gets the sense of "wow, it stinks". But if everyone responds together, wouldn't their sense be limited to "there are smell molecules" and "there are no smell molecules"? How do bacteria produce a wide dynamic range?

Mother: That's where adaptation comes in.

Daughter: Adaptation?

Mother: You know how when you eat cake, the first bite is super sweet, but the second and third bites aren't as sweet?

Daughter: Ohhh, yeah, totally.

Mother: That's adaptation. Let's say *E. coli* senses a certain strength of smell molecules. At first, *E. coli* would react to the smell molecules; but after a while, even if smell molecules were still around, *E. coli* would reset its chemotaxis system to stop sensing the smell molecules. Then, when *E. coli* moves to an environment with fewer smell molecules or more smell molecules, it would be able to sense whether the smell molecules are strong or weak. With you and cake, after you eat cake, if you were to eat a sweeter cake, you would register it as sweet. It's the same with *E. coli*. It uses the mechanism of adaptation to change the range of what it senses in the external environment. That's how *E. coli* can sense its environment with a wide dynamic range.

The Flagellar Motor

Daughter: So you're saying that information from the receptors is transmitted within the cell by diffusion.

Mother: Right, right.

Daughter: What happens to the information in the end?

Mother: Bacteria use it to comprehend their environment in their own way and move to a more favorable environment.

Daughter: Like "the grass is always greener on the other side", huh? But to do that, they need a device to move, don't they?

Mother: Right. It's called the flagellar motor.

Daughter: Earlier, you were talking about rotary motors, but what's so special about a flagellar motor?

Mother: You're right, there are rotary motors around us everywhere we look. Car tires and smartphone vibrators are rotary motors, too. But it's rare for a rotary motor to be inside a living thing; only a few kinds of organisms have them.

Daughter: Whoa, really? But rotary motors are used in everyday life because they're convenient, right? So why don't more living things use them?

Mother: There are lots of ideas about that. For instance, to make a rotary motor, you need a shaft and bearings. And you need to minimize friction. It may be hard to make a rotary machine like that with proteins. Here's another idea: for a car to move smoothly, it needs a proper flat road, right? But it seems that it would be really difficult to make a flat road of the size of proteins. Besides, for a car to run on a road, it has to be in constant contact with the road, right? The tires can be in contact with the road thanks to gravity; but proteins are in an aquatic environment, where gravity has almost no effect, so you would need some other way to anchor the proteins to the road.

Daughter: Ohhhh, I get it. So, rotors aren't necessarily more convenient for living things. The itty-bitty world is interesting stuff. But how do they rotate? Do they run on electricity, like the motors we see around us?

Mother: Actually, ions flow into the cell from outside it. In the case of *E. coli*, they're hydrogen ions. The cells use the power of these ions to rotate, just like a hydroelectric turbine.

Daughter: Wow! Hydroelectricity! That sounds amazing.

Mother: Other bacteria use other kinds of ions. For instance, *Vibrio* bacteria use sodium ions.

Daughter: But…if ions keep flowing into the cell, won't it be full of ions? And how do the ions flow into the cell?

Mother: Good question. It would definitely be bad if ions kept flowing into the cell. Especially for hydrogen ions, since they affect pH.

Daughter: Oh yeah, I just learned in class the other day that pH is the concentration of hydrogen ions.

Mother: That's why there's a mechanism that works hard to pump the ions out. By doing that, the concentration of ions inside the cell is decreases, and the resulting difference in concentration is what lets the ions flow in. To use a technical term, it's called entropy.

Daughter: Entropy?

Mother: Well…basically, if you don't do anything with your room, things will keep piling up in it.

Daughter: ???

Mother: Also, since hydrogen ions and sodium ions are positively charged, if the charge inside the cell is negative, the ions will be drawn inside the cell. So, bacteria have come up with all sorts of mechanisms to rotate their motors.

Daughter: Wow, no kidding. So how do they rotate their motors to move around? Is there a propeller attached to the motor?

Mother: There's no propeller, but there's a reeeeeally long spiral-shaped hair attached to the motor. Bacteria move around by rotating the hair.

Daughter: Oh yeah, you said that bacteria move to favorable environments, right? They detect information with receptors, and they propagate the information inside the cell…okay, so they'd need a rudder then, too.

Mother: But it's not like that. Bacteria don't have a rudder like a ship does. Instead, they switch the rotation of their motor from clockwise to counterclockwise, and the effect of water causes the cell body to face a different direction at random. When that happens, the direction that the bacterium swims in changes at random. Bacteria change the probability of switching their motor rotation based on their environment so they can move in the direction they want. When they head toward an environment they like, they make a beeline for it and don't switch their motor rotation much. When they're heading toward an environment they hate, they switch their motor rotation a lot. It's like all types of cells face all kinds of directions.

Daughter: Oooh, that's really interesting. I've never seen a ship like that. In that case…if ions flowing into the cell cause the motor to rotate, does that mean ions flow out of the cell to make the motor rotate in the opposite direction?

Mother: That's not how it works. What we currently know is that ions always flow from outside to inside the cell. There seems to be something like a reverse gear somewhere in the motor that makes it rotate in the opposite direction, but we don't know much about this mechanism yet.

Daughter: Wow, so it's just like a gear shift in a car. A car can totally move backwards without changing the direction its engine rotates.

Mother: Information from the receptors, which I talked about earlier, is diffused in the cell and transmitted to the motor, but what's really interesting is that it seems gear changes happen just by the small number of molecules with information binding to the motor.

Daughter: Is that…the cooperativity that came up in what you were just talking about?

Mother: Right, right. The mechanism still isn't well understood, but lots of researchers are working on it.

In Conclusion

Daughter: Okay, I see…so I shouldn't underestimate bacteria. The size of a cell is on the order of micrometers, so the things that work in that environment are even smaller; it's the nanometer world, and all sorts of stuff happens there.

Mother: Yes, exactly! Bacteria may be lower life forms; but with just one cell, they act with a will of their own. In the nanoworld inside that cell, there's a whole world of things we don't yet understand. Makes you really want to peek inside, doesn't it?

Daughter: Yeah, totally! Hey, can I come to hang out at your lab sometime? I'm kinda psyched now. Plus, I don't really know what goes on at a university. I'll bet there are good restaurants there!

Reference

1. Zimmer, C. (2009). *Microcosm: E. coli and the new science of life*. New York: Vintage Books, Random House.

How Low Can You Go? The Numbers of Cells That Make Up Bodies: Large Numbers and Small Numbers

Kazuki Horikawa

This book deals with all sorts of numbers. Most of them have to do with the number of molecules in cells, with small numbers taking center stage. However, it may be difficult to understand how small these numbers are. To get a better sense of these numbers, we will try to think about the numbers of cells that make up the bodies of various creatures, great and small. Even if you know that the human body is made up of an incredibly large number of cells, you would have to be quite well informed to imagine what a large number it is. Even if you know that organisms much more primitive than humans are made up of very small numbers of cells, there is no way you would know the exact number. Here, through a conversation between parents and their child at the dinner table, we will think about how many or how few cells are in various organisms by comparing their numbers to grains of rice in a bowl.

(At the dinner table)
Son: More rice please.
Mom: How much?
Son: A regular amount.
Mom: Okay, is this enough?
Son: Is that it? That's not much.
Mom: You don't look happy. If you're not happy, then tell me exactly how much you want!
Dad: Don't get into a fight over portions of rice. Everyone has different ideas of how much is a lot or a little.
Son: Okay, then how should I say it?
Dad: How about by weight?
Son: Come on, I have no idea how much a bowl of rice weighs.
Mom: And I am pretty sure I don't want to bother with a scale just to serve rice.

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Dad: Then how about saying how many grains of rice you want?
Mom/Son: That's an even bigger pain!
Dad: Hey now, numbers are really important. You know human bodies are made up of cells, right?
Son: Duh, everyone knows that. Muscles and nerves and stuff, it's all cells, isn't it?
Dad: Then, do you know how many cells make up the human body?
Son: I've heard the number before. It's 60 trillion, isn't it?
Dad: We thought for a long time that it was 60 trillion, but that number is out of date. A study done in 2013 showed it's only a little more than half that—about 37 trillion [1].
Son: Huh. A trillion is such a big number that I can't even imagine it, but when scientists actually sat down and figured it out, it turned out to be only about half of what they said before? They really messed up. But how did they count such a big number?
Dad: If you counted them one by one like grains of rice, at a rate of one per second, it would take 1.1 million years to count out. Even if you counted the cells at a pace of 580 per second, it would still take 2,000 years. That gives you an idea of what a ridiculously big number 37 trillion is.
Son: So, you're saying they didn't count 37 trillion cells one by one?
Dad: That's right. Basically, they figured out the densities of cells in the heart, the brain, and every other organ, and then did some multiplications to arrive at about that number. By the way, they got the former number of 60 trillion with a rough calculation in which they considered the standard weight of a cell as 1 ng and divided 60 kg, the standard weight of a person, by 1 ng. When they redid detailed calculations for each organ, they arrived at 37 trillion.
Son: So you're saying that 37 trillion is still vague, and that they might get a different number if they counted more accurately? There are lots of numbers in the world, and some of them may be arbitrary. I feel like I can't believe anything.
Dad: You're totally right. But if lots of different studies independently estimate the total number of cells in the human body in the tens of trillions, at least the order of magnitude shouldn't change. It's really hard to count anything accurately. It's a challenging issue for science. I'm interested to see what the estimate for the number of cells in a human will be in 10 years. By the way, speaking of numbers, do you know how many types of cells there are in the human body?
Son: By "types of cells", do you mean muscles and nerves and stuff?
Dad: Right, right. Even with broad classifications, the human body has about 300 different types of cells. That's a tiny number compared to 37 trillion, but you can probably only think of maybe 10 types of cells, such as nerve, muscle, and blood cells. So you realize that there are so very many different kinds of cells. If you look at the 37 trillion cells in the body by type, the most common type of cell is red blood cells. There are a huge number of them — 26 trillion.
Son: So 2/3 of the human body is red blood cells? What's the least common type of cell?

Dad: The least common are cells that are the precursors of eggs. In women past puberty, it's said there are about 200,000 egg cells.

Son: 200,000, huh? That's way smaller than a trillion, but it's still a pretty big number. It seems that lots of cells are needed to make up the human body, but what's the smallest number of cell types the body needs, and how many cells for each type?

Dad: It sounds like you're more interested in small numbers. In that case, a roundworm might be a good example.

Son: Roundworm? Is that some kind of bug?

Dad: It's not a bug. It's a type of animal called a nematode that lives in soil and eats bacteria. It's shaped like a thin tube. There's a species of nematode called *Caenorhabditis elegans* that's about 1 mm long and has a transparent body. It's been studied thoroughly, so we know exactly how many cells make up its body. A male has 1,031 cells and a hermaphrodite, 959 cells.

Son: Huh? You had been saying "about" until just now, but you're suddenly totally confident about these numbers.

Dad: Like humans and other animals, *C. elegans* starts as a single fertilized egg that divides and divides to form all the cells that make up its body, but *C. elegans* is surprisingly precise in when, where, and how many times its cells divide. Every individual organism has the exact same number of cells.

Son: It's more like a robot than a living thing.

Dad: Yeah, it really is like a robot the way its cells divide with almost no errors. All of its 1,000 or so cells have names are classified into 17 types. The most common type of cell is nerve cells, with 302 of them. It has 56 glial cells, which are related to nerve cells, and 95 muscle cells. On the other end of the spectrum, the least common type of cell is reproductive cells—it has only 2 of them. By the way, research on *C. elegans* led to the discovery of the mechanism by which its cells commit suicide. It was such a big discovery that it was awarded the Nobel Prize.

Son: I guess even organisms I've never heard of can be useful. So, if you were going to try to make a roundworm by collecting one of every cell type, you'd need at least 17 cells, then.

Dad: There's no way it could live with only one nerve cell and one muscle cell, but how many would you need? That's a good question. How many nerve cells do you need for intelligence to form? How far can you reduce the number of red blood cells without causing anemia? How many of which types of cells have to be assembled for the functions of an organism to emerge? They're all important questions.

Son: Then, just how many cells does an organism need to live?

Dad: The minimum is 1, of course. I'm sure you've heard of unicellular organisms. You know that *Escherichia coli* and yeast and similar organisms can live as a single cell, right?

Son: But bacteria and humans are completely different, aren't they?

Dad: Indeed they are. The opposite of unicellular is multicellular. Let's think about the multicellular organism with the fewest cells. For starters, a multicellular system involves multiple cells with different roles assembling and then working

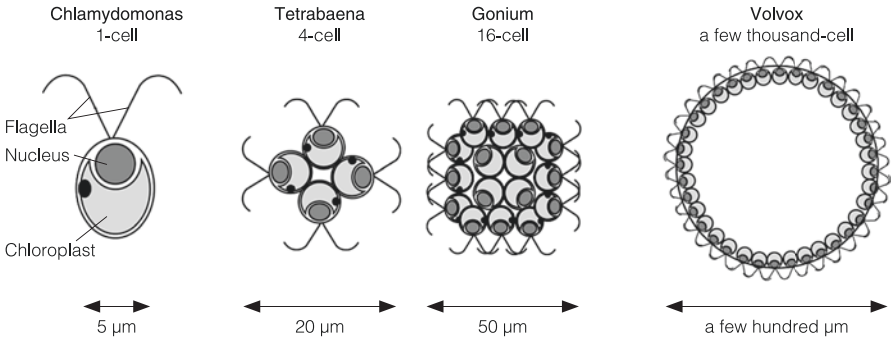

Fig. 1 The cell number variation of green algae

together to maintain life. In other words, a multicellular organism can't live if you break up its cells into individual units. Unlike a unicellular organism, you can't just assemble a bunch of the same type of cells and call it a multicellular organism. As for the organism with a multicellular system that's made of the smallest number of cells, have you ever heard of *Volvox*?

Son: I think it was in my high school biology textbook.

Dad: *Volvox* is a ball-shaped green algae that's about 1/10 of a millimeter in size, lives in places such as rice paddies, and is made up of thousands of small cells with two hairs called flagella (Fig. 1). Each individual cell closely resembles a unicellular organism called *Chlamydomonas*, but *Volvox* can't live if its cells are broken up, so it's classified as a multicellular organism.

Son: Thousands of cells? That's more than a roundworm has, so what's so interesting about that?

Dad: I'm just getting to the interesting part. There are relatives of *Volvox* that are made up of cells that resemble *Chlamydomonas*, just like *Volvox* is. There's *Pleodorina*, which is made of 128 or 64 cells; *Eudorina*, which has 32 cells; and *Gonium*, which has 16 cells (Fig. 1).

Son: The number of cells always doubles. It's like they're showing evolution in the way the number of cells changes.

Dad: Exactly. All of these relatives of *Volvox* are thought to have evolved from unicellular *Chlamydomonas*. If the number of cells that makes up the body doubles, then it seems that there should be a multicellular organism made up of as few as 2 cells, right? But no such organism has been discovered. *Tetrabaena socialis* is considered to be the multicellular organism made up of the smallest number of cells, with 4 cells (Fig. 1).

Son: Only 4 cells? That's a really small number. It can't live if the 4 cells are broken up, but it can if they work together. It really does seem like life.

Dad: If you're trying to understand what exactly life is like, focusing on the number of cells is a good place to start. Actually, lots of scientists have studied how many cells it takes for new functions to emerge.

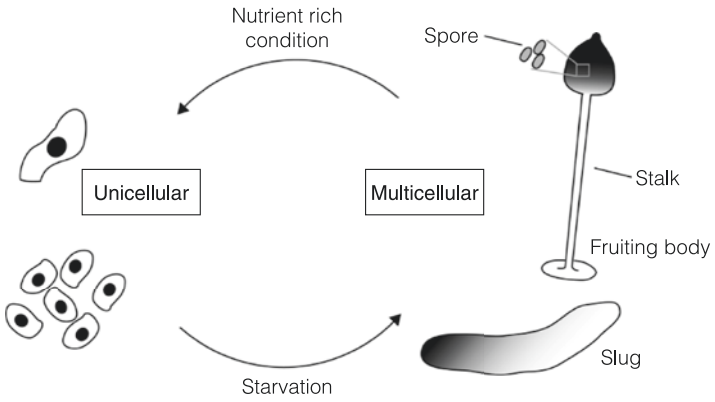

Fig. 2 Life cycle of *D. discoideum D. discoideum* cells switch the life style from unicellular to multicellular mode. Cells actively proliferate under the nutrient rich condition. Upon starvation, cells aggregate and develop into the multicellular slug and fruiting body

Son: So it's not just about counting cells, it's about finding out the minimum number of cells it takes for coordination to emerge, huh? Then if you could manipulate cell numbers at will, it seems that you could learn all sorts of things.

Dad: Unfortunately, for roundworms and almost all other animals, the number of cells that makes up the body is set in stone, so it's hard to manipulate cell numbers. But if you think in terms of an organism that can freely change its number of cells, you should look at a cellular slime mold, which can live as a unicellular and a multicellular organism. Cellular slime molds are very accessible organisms that can be found under piles of dead leaves. When they eat things like bacteria and grow, they act as unicellular organisms; but when they run out of sustenance, lots of cells congregate and form multicellular structures called fruiting bodies, which are shaped like mushrooms (Fig. 2).

Son: No kidding. It's like they evolve from unicellular to multicellular organisms as they live.

Dad: Right. As a survival strategy, it joins with others of its kind to become multicellular, and it produces spores that can withstand desiccation and starvation. When it does, the stalk cells die and leave only a husk, lifting the spores off of the ground to help them be scattered in a new environment. Basically, even though a cellular slime mold is essentially a group of identical cells, some of the cells become spores in order to leave offspring, while the rest of the cells sacrifice themselves for the sake of the spores, their children. Because of this social nature, cellular slime molds are also called social amoebae.

Son: So, there's a spirit of cooperation even in cellular society.

Dad: By the way, these social amoebae have a unique trait: they can form multicellular structures of any size. But the size of a newborn animal is pretty much set. Human babies all weigh about 3,000 g when they're born; a baby is never born with 10 times the normal weight or height, right? But social amoebae can produce

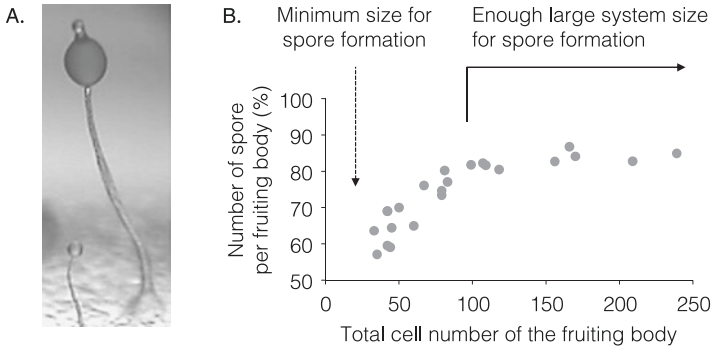

Fig. 3 Spore formation depends on the size of fruiting body. (**a**) Variation in the size of fruiting body. Large and small ones consist of ~10,000 and ~100 cells, respectively. (**b**) Spore forming ability of the fruiting body with different size. While 80% of cells differentiated into the spore in a large fruiting body (>100 cells), the percentage of spore cells decreased in the small fruiting body. Spore-less stalk consisting of as few as 18 cells suggested that 19 cells would be essential for the spore differentiation

spores from a massive size of 10,000 cells to a small size of hundreds of cells. And when they produce spores, the ratio of spores to stalks stays constant at 4:1. Science pretty much understands the mechanism behind accurately reproducing bodies of the same size but knows almost nothing about how cellular slime molds freely produce bodies 100 times their own size with the same pattern.

Son: It's normal for adults and children to be of different sizes, but I guess it's not normal for body sizes to be different at birth.

Dad: Social amoebae aren't born from eggs, but for now, it's okay to imagine they are. If you use these social amoebae, you can examine how few cells can make up a proper multicellular body. I'll tell you the answer first. If the social amoeba has at least 100 cells, it can produce spores with a 4:1 ratio of spores to stalks, but the fewer cells there are, the smaller the spore ratio gets; eventually, you would end up with an incredibly small fruiting body of 3 spores supported by 16 stalk cells. This means that a social amoeba needs to have at least 19 cells to divide into two types of cells (Fig. 3).

Son: What happens if it has fewer than 19 cells?

Dad: Then, it would end up forming just a stalk with no spores. Basically, all the cells would end up sacrificing themselves, and the social amoeba wouldn't be able to produce spores. So, in effect, the society would die out.

Son: So I guess that means that 19 cells is the borderline between self-interest and self-sacrifice.

Dad: Human society is like that too. There are plenty of instances in which something that would be hard for one person to accomplish alone can be done if lots of people get together. In the same way, we should be able to learn much more about the mechanism of cooperation among cells and the proteins that make them up by counting them accurately and manipulating their numbers at will.

Son: I'm really psyched about numbers now! I'm going to go count those grains of rice.

(The next morning)

Son: There were a total of 2,876 grains of rice in the bowl. It took me 3 hours to count them. It was hard, but now I feel like I have a more tangible grasp of numbers. But now, I don't want to look at rice again for a while, so I'll have bread for breakfast.

Dad: Okay, so do you know how many grains of wheat there are in a slice of bread?

Son: I get it! Numbers are important. Enough already… (sob)

Reference

1. Bianconi, E., Piovesan, A., et al. (2013). An estimation of the number of cells in the human body. *Annals of Human Biology, 40*(6), 463–471.

The Flow of Time Inside the Cell: The Time of Days Given by Molecules Driving the Circadian Clocks

Koji L. Ode and Hiroki R. Ueda

We All Live on an Earth That Rotates on a 24-h Cycle

Early one September morning, as a hint of fall was in the air, Professor Ruskin arrived at the university to work. When he looked out of the window at the sunrise-tinted sky, the scenery made him nostalgic for days gone by. Though he was now running a laboratory as a university professor, in his student days, he had at times greeted the morning after staying up all night working on experiments. The morning sun made him recall old times. His laboratory studied a phenomenon known as the circadian clock. All creatures on Earth live according to the rhythm of the planet environment, a 24-h cycle in which the sun rises in the morning and sets at night. Humans, as well as the sparrows that were chirping on the other side of the window at that moment, naturally wake up in the morning and go to sleep at night. Nocturnal mice have the inverse pattern and begin their activities at nightfall. All living things have their own daily rhythms, but they all sleep and rise every day according to a constant rhythm. This is so obvious that one may not be normally aware of it in daily life, but this bodily rhythm is generated by the molecular mechanism called circadian clocks.

"Good morning!"
All of a sudden, a chipper woman's voice broke the silence. It was first-year student Meg. She was using her summer break to help with experiments at the laboratory.

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Meg: You're here pretty early today, Professor.
Prof. Ruskin: I have some work I want to finish before noon. You're an early riser, aren't you?
Meg: I always wake up naturally around 7:00 AM. But I don't set an alarm on my phone, and my room is pitch-black because the curtains are closed. It's the work of the circadian clock, isn't it! I guess my body gets a hunch that morning is coming.
Prof. Ruskin: 'Hunch' is a nice perspective.

She can really get to the heart of the matter sometimes, Prof. Ruskin thought. She began to spend time in his laboratory when a bad grade on a test in his class prompted her to visit him to ask all sorts of questions in preparation for a makeup test. As she spoke to him, he realized that she was brimming with curiosity. She did not seem to be much good at memorization, but she was the type who pursued something with absolute gusto once she took an interest in it. Perhaps she would make a fine researcher.

Prof. Ruskin: I told you that the circadian clock, or the biological clock, as it's sometimes called, to put it in simple terms, is a function in our bodies that serves as a clock that tells the time of day. Meg, do you wear a watch?
Meg: Nope. I just look at the time on my phone. That reminds me, I read an article on the internet the other day that said wearing a watch is a point of etiquette for adults. Is that true?
Prof. Ruskin: I wonder. After all, etiquette and common sense change with the times. Still, even if you don't wear a watch, you can look at the time on a cell phone. For example, you might say to yourself 'the cafeteria is going to be crowded just after 12:00, so I'm going to get there early and wait in line.' The point is that you have a clock, so you can anticipate what's about to happen and act accordingly.

Once Prof. Ruskin had said this, he began drawing a figure on a whiteboard in a corner of the laboratory (Fig. 1).

The Body Has a Clock That Tells the Time of Day

Prof. Ruskin: Earlier, you said that you wake up even though you close the curtains and no alarm goes off, right? This is a bit of a stiff way to put it, but you could say that you're getting little time-related information from the environment.
Meg: You mean I don't have any clues to figure out what time it is?
Prof. Ruskin: Yes, exactly. But if you live a normal life, you hear the sounds of birds chirping and the newspaper being delivered, so it's not as if you have no clues at all. The light outside, meals, and all sorts of events occur cyclically every 24 hours, generally speaking. So, what would happen if you were to eliminate all time-related information from the environment? Imagine being shut up in your room, completely isolated from the outside world, no sound or light coming in

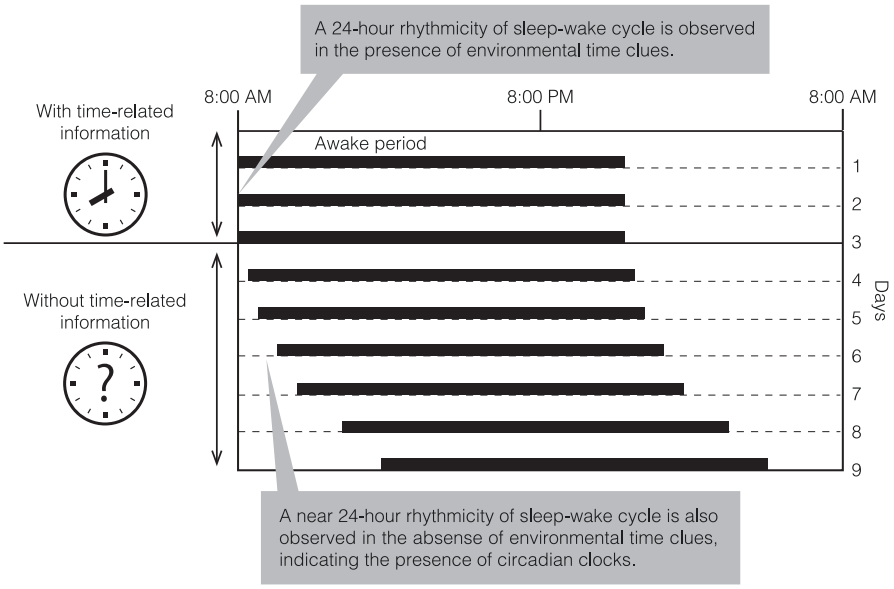

Fig. 1 Environmental time cues and the circadian clocks

from outside, eating meals at whatever times you please…do you know what would happen if you lived like that for 10 days or so? Of course, you wouldn't have a smartphone or a TV either.

Meg: Hmm,…well, first of all, you'd probably die of boredom.

Prof. Ruskin: It would be pretty boring.

As a university professor constantly having to deal with e-mails and meetings on a daily basis, being isolated from the outside world for 10 days might be pretty nice, Prof. Ruskin thought to himself.

Prof. Ruskin: When do you think you would end up getting up?

Meg: Let me see. In my case, since I can get up without an alarm and I'd eventually sleep after being up all day, I feel like I'd still go to sleep at night and get up in the morning.

Prof. Ruskin: That's right. Actually, there are experiments called isolation experiments where participants live without any time-related information from the environment [1].

The professor continued talking as he added to his figure on the whiteboard.

Prof. Ruskin: Even without light, sound, meals, TV, or any other clues to figure out the time, humans repeatedly sleep and rise on a nearly 24-hour cycle. You could say that your body has its own clock. This function, which measures the approximate length of one day, is called the circadian clock. When the time in your environment suddenly changes, such as when you go on a trip, your body's clock and the time in your environment will be out of sync, which causes jet lag.

Meg: I see. Yeah, when you go to another country, even though you should be able to go to sleep when it gets dark there, your body has its own clock, and it's stuck on its own usual time.

Every Cell in the Body Is Equipped with a Circadian Clock

Meg: So, where is this clock?

Meg fired her question immediately.

Prof. Ruskin: It's in almost every cell in the body. You know that our bodies are made up of cells, cells have lots of genes made from DNA, and genes are retrieved as messenger RNA and proteins, right?

Meg: Yeah, that's that thing called gene expression, right? I learned that in your class!

Prof. Ruskin: Good. Lots of cells with different properties from various places in the body, like skin cells and liver cells, can be grown in a Petri dish…that is, they can be cultivated. When scientists observed gene expression while cultivating cells, they were shocked to find that for many genes, expression levels change according to a roughly 24-hour cycle. For instance, expression levels for some genes are high at 1:00 in the afternoon, while expression levels for other genes are high at 8:00 at night. Of course, cells don't have eyes or ears, so they don't take in time-related information from the environment, but they are still synchronized on a 24-hour cycle on their own.

Meg: Wow! No kidding!!

Meg replied with exaggerated surprise.

Prof. Ruskin: It was discovered about 20 years ago that each cell that makes up the body is equipped with a circadian clock in mammals [2], but researchers back then were all surprised.

How the Circadian Clock Works: Is It an Hourglass?

Prof. Ruskin continued to explain why there is a 24-hour cycle for gene expression in cells.

Prof. Ruskin: Genes aren't expressed haphazardly; they're turned on and off by proteins. For example, if a specific protein is present, expression of a specific gene will stop. When this happens, the protein may target the gene that produces it and whose expression it regulates. What's especially important in the function of the circadian clock is the gene suppressing its own expression.

Meg: Proteins that inhibit their own expression? That sounds kind of wasteful…

Prof. Ruskin: It's not wasteful at all. It's important for producing the oscillating phenomenon that repeats with a regular rhythm like a clock.

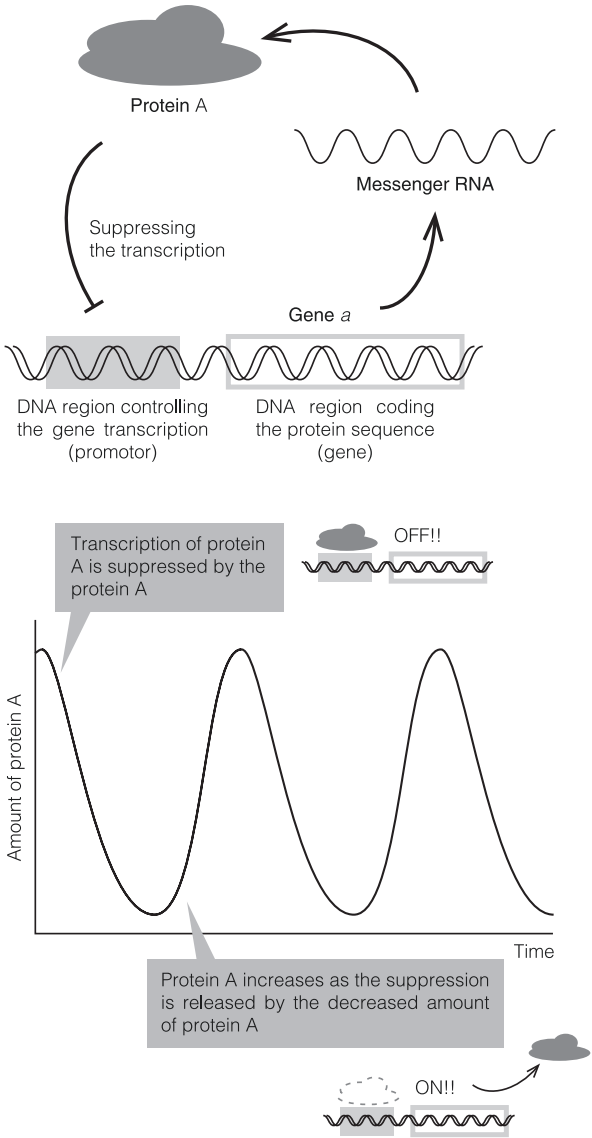

Fig. 2 Clock proteins drive the circadian clocks

Prof. Ruskin used the whiteboard to continue his explanation (Fig. 2). Here's how it works. Now, let's say that protein A is expressed from gene *a* (through mRNA). Protein A regulates the expression of several genes, one of which is gene *a*. When there is a large amount of protein A, the expression of gene *a* will be suppressed, meaning that the amount of protein A will decrease. When the amount of protein A decreases, the expression of gene *a* is no longer suppressed, and the amount of protein A increases again. Thus, the amount of protein A increases and decreases over and over.

Prof. Ruskin: These types of proteins that can suppress themselves are called clock proteins or clock genes. The rhythm of the circadian clock is thought to be produced by these clock proteins.

Meg: So cellular time is produced by increases and decreases in amounts of proteins. I guess it's like an hourglass, huh? And this hourglass can increase or decrease the amount of sand inside it on its own.

From the Number of Circadian Clock Proteins, A New Question Arises

Prof. Ruskin: Right, an hourglass is a good analogy. But…

When Prof. Ruskin said this, he brought a small hourglass from a bookshelf.

Prof. Ruskin: Do you understand that the reason this hourglass can accurately measure time in its own way is because it has lots of sand in it? For instance, try focusing on a single grain of sand in the hourglass. The moment at which it falls from the top to the bottom is different from that of every other grain of sand. Some grains fall right away, and others don't fall so readily, but the time it takes for all of these different grains of sand to fall to the bottom is nearly constant.

Meg: I had never thought about that, but I guess it's true. When there's a school-wide assembly in the gym, it always takes about the same amount of time for all of the students to show up, but the time it takes for three friends to meet up in front of the train station is all over the place. Is that what you mean? It could happen that all three of them are late.

Prof. Ruskin: Right. The greater the number, the smaller the variation will be in the time it takes for something to happen. But when they actually measured the number of molecules in a cell, they found that the number of all-important circadian clock protein molecules per cell in one day is 20,000 at peak periods; and during off periods, the number is only 2,000 [3]. As I explained earlier, levels of expression rise and fall over and over in a 24-hour period.

Meg: *Only* from 2,000 to 20,000 protein molecules? …that still sounds like quite a lot to me, though.

Prof. Ruskin laughed, as if to say that he could understand why she'd think that.

Prof. Ruskin: I guess that was a bit of an overbearing expression. There was something about *Escherichia coli* in "Minorities and Small Numbers from Molecules to Organisms in Biology", the book you were reading yesterday, wasn't there?

Meg: Yeah, about numbers of flagella and stuff!

Prof. Ruskin: I bring it up because mammalian cells are bigger than the cells of microorganisms like *E. coli*. Volume-wise, mammalian cells are about 1,000 times bigger than *E. coli*. So, 2,000 molecules in a mammalian cell represent about the same concentration as 2 molecules in *E. coli*.

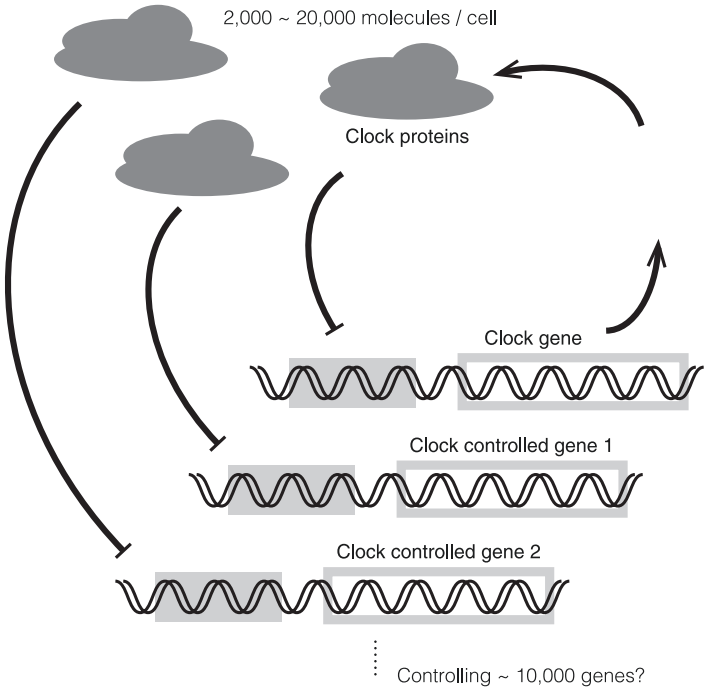

Fig. 3 The number of clock proteins and target genes

Prof. Ruskin explained that the question of whether 2,000 or 20,000 molecules should be considered large numbers or small numbers will be one of the themes of his research going forward (Fig. 3).

Prof. Ruskin: On top of that, it's now known that up to 20,000 or so clock protein molecules bind to DNA at approximately 10,000 sites to regulate gene expression [4].

Meg: So are you saying that the number of clock protein molecules is sometimes the same as or fewer than the number of sites they can bind to? It sounds like clock proteins are really busy and have major responsibility.

Prof. Ruskin: That's right. So, it may be that each of the 2,000 to 20,000 clock proteins produces a 24-hour cycle. Just like we all have our own clocks that we live by. When three friends are going to meet up at the station, if they all have clocks, they can meet up at a precise time. In the same way, each individual clock protein may have the ability to count out time (Fig. 4).

Meg: An individual clock protein can count out time! It's kind of unbelievable.

Prof. Ruskin: It really is something. However, this is getting away from mammals, but in bacteria called cyanobacteria, we now know that the shapes of proteins themselves change on a 24-hour cycle. On top of that, because the proteins produce these changes themselves, they continue to change shape according to a 24-hour cycle even if you take them out of the bacteria [5].

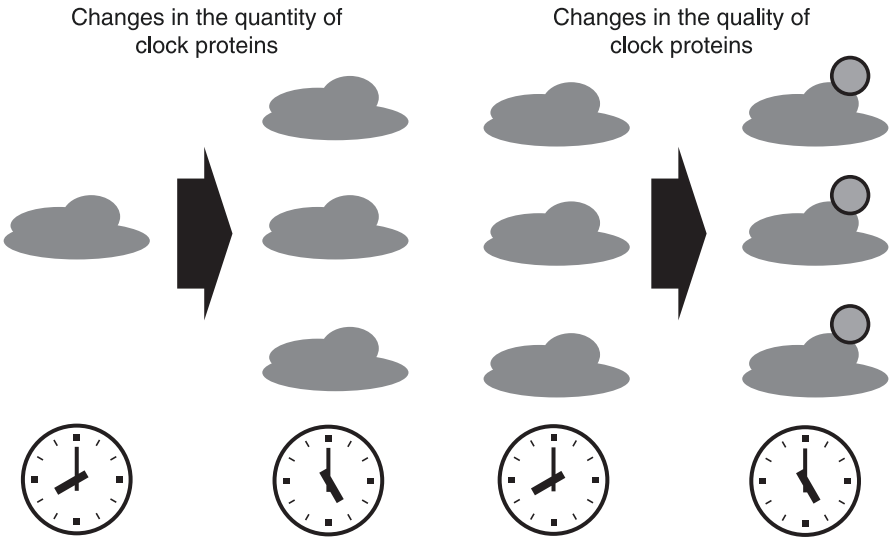

Fig. 4 Circadian timing system controlled by the changes in protein quantity and quality

Meg: So if you break open the hourglass and look at the sand, the sand has changed color over time. In that case, they can still count out time even if they don't have a large amount of sand. How can they do that?

Prof. Ruskin explained that in cyanobacteria, it's important that the states of proteins change due to a chemical modification called phosphorylation. However, in mammals like us humans, we have not yet discovered what sorts of chemical reactions are important for producing a 24-hour cycle.

Prof. Ruskin: We need to count out the number of molecules and thoroughly observe the aspects of each individual molecule we count out. If we can do that, we should be able to close in on what exactly is counting out the time of day. But we still have a lot of research to do to get there.

Meg: That sounds fascinating! Maybe I'll research that too. What should I study?

Prof. Ruskin: Let me see…first, you need to familiarize yourself with the circadian clock by reading some textbooks…But there's something you need to keep in mind. What's truly important and interesting is what is not yet known. In other words, things that aren't written in any textbook and don't show up on any test. Oh, your senior labmate should be here any minute. You'll be cultivating cells with him today.

Prof. Ruskin caught his breath with a "well then", and set about his work with a cup of coffee in one hand. First, he has to complete some manuscripts. He's been asked to write about the circadian clock in dialogue format in a way that's fascinating and easy to understand for high school and college students. "Well then, how am I going to do this?" he muttered as he faced his computer, fretting about how to convey the greatly fascinating circadian clock.

References

1. Aschoff, J. (1965). Circadian rhythms in man. *Science, 148*, 1427–1432.
2. Balsalobre, A., et al. (1998). A serum shock induces circadian gene expression in mammalian tissue culture cells. *Cell, 93*, 929–937.
3. Narumi, R., et al. (2016). Mass spectrometry-based absolute quantification reveals rhythmic variation of mouse circadian clock proteins. *Proceedings of the National Academy of Sciences of the United States of America, 113*, E3461–E3467.
4. Koike, N., et al. (2012). Transcriptional architecture and chromatin landscape of the core circadian clock in mammals. *Science, 338*, 349–354.
5. Nakajima, M. (2005). Reconstitution of circadian oscillation of cyanobacterial KaiC phosphorylation in vitro. *Science, 308*, 414–415.

Afterword

How did you enjoy *"Minorities and Small Numbers from Molecules to Organisms in Biology"*?

In addition to "minority", this book throws around the keywords "personality", "character", and "cooperativity". In fact, the studies themselves reported in this book were advanced thanks to cooperation among members with character (including some members with too much character, but I won't say who). Each one of us got into minority biology research in different ways and for different reasons. In my case, there was some incomprehensible data from a simulation more than a decade ago; back then, all I had was an armchair theory, but minority biology has become a field of research that lets me collaborate with experimentalists. I hope that you got a sense of the different focuses and modes of thought presented by the authors of this book, as well the connections among them.

If the conversations featured in this book were to be assembled in the form of a textbook, it would come across as some sort of field that is set in stone; however, our journey to the small numbers hidden in biological phenomena is an ongoing one. In particular, it remains to be determined whether there is a common theory of small numbers at the levels of molecules, cells, tissue, individuals, and societies; or whether there are different small numbers at work at each level. I think I would like this to be a principle that is common to all of the above levels, but the existence of different laws of interaction at every level is an attractive feature of biological systems, so I am not sure what I really want. We are still looking for companions on this journey.

We have received immeasurable assistance from Dr. Yasutaka Okazaki and Dr. Sue Lee at Springer Nature, and also from Mr. Jun Nagamoto at Nippon Hyoronsha. Much of the research introduced in this book has come along thanks to the support of a Grant-in-Aid for Scientific Research on Innovative Areas (KAKENHI) from the Ministry of Education, Culture, Sports, Science and Technology of Japan

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for “Spying Minority in Biological Phenomena”; and with the help of many co-investigators, research collaborators, and the engineering support team from 20 companies. As this book comes to a close, I would like to offer my deepest gratitude to everyone who lent their assistance.

Yuichi Togashi

Project “Spying Minority in Biological Phenomena”
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