Communicating Research to the General Public

At the March 5, 2010 UW-Madison Chemistry Department Colloquium, Prof. Bassam Z. Shakhashiri, the director of the Wisconsin Initiative for Science Literacy (WISL), encouraged all UW-Madison chemistry Ph.D. candidates to include a chapter in their Ph.D. thesis communicating their research to non-specialists. The goal is to explain the candidate's scholarly research and its significance to a wider audience that includes family members, friends, civic groups, newspaper reporters, program officers at appropriate funding agencies, state legislators, and members of the U.S. Congress.

Over 50 Ph.D. degree recipients have successfully completed their theses and included such a chapter.

WISL encourages the inclusion of such chapters in all Ph.D. theses everywhere through the cooperation of Ph.D. candidates and their mentors. WISL is now offering additional awards of $250 for UW-Madison chemistry Ph.D. candidates.

Wisconsin Initiative for Science Literacy

The dual mission of the Wisconsin Initiative for Science Literacy is to promote literacy in science, mathematics and technology among the general public and to attract future generations to careers in research, teaching and public service.

UW-Madison Department of Chemistry
1101 University Avenue
Madison, WI 53706-1396
Contact: Prof. Bassam Z. Shakhashiri
bassam@chem.wisc.edu
www.scifun.org

Single-Cell Detection of Antimicrobial Peptide's Attack on Live *E. coli* by Super-Resolution Fluorescence Microscopy

By

Yanyu Zhu

A dissertation submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

(Chemistry)

At the

UNIVERSITY OF WISCONSIN-MADISON

2020

Date of final oral examination : 09/03/2020

Final Oral Committee:

James C. Weisshaar, Professor, Chemistry

Samuel H. Gellman, Professor, Chemistry

Randall Goldsmith, Professor, Chemistry

Jue D. (Jade) Wang, Professor, Bacteriology

Chapter 6

Antimicrobial peptide LL-37 freezes bacteria

This chapter is written to communicate my research to the general public as a part of Wisconsin Initiative for Science Literacy (WISL) program

What is antimicrobial resistance?

Human health is always challenged by microbial invasion and infection. For example, we are experiencing a very difficult time now because of COVID-19, a disease caused by a highly infectious virus. The pandemic has had a substantial negative impact on the health and welfare of tens of millions globally. In addition to the virus, we are also faced with another threat: the spreading of infection by antibiotic-resistant bacteria. Pathogenic viruses and bacteria, though both "bad guys" for our health, are very different. Viruses do not have a cellular structure and cannot be killed by antibiotics. We rely on our own immune system to eradicate these invading pathogens. Bacteria, on the other hand, have a cellular structure. Many components of these cellular structures, such as the cellular membrane, which is the biological membrane that separates the interior of a cell from the outside environment, can be targeted specifically by some antibiotics.

Alexander Fleming discovered penicillin in the 1920s. The discovery and medical application of penicillin heralded the dawn of the antibiotic age. Antibiotics have saved millions of lives during the war between humans and pathogenic bacteria in the past century. However, bacteria can gain resistance to antibiotics through a process called "mutation." When bacteria multiply, one bacterial cell divides into two daughter cells. Before the bacterial division, the bacterium needs to duplicate its DNA to make two identical copies of DNA; one for each daughter cell. During the replication process, there is a chance that the replication machinery makes errors, so-called mutations (Fig. 6.1). As a result, the DNA sequence has changed and some cellular components would also change. For example, suppose there are 1,000 bacteria living on a growth plate and an antibiotic could kill the bacteria by targeting its cellular

membrane. In this example, 999 bacteria were killed by the antibiotic, but one bacterium survived the attack because its membrane components had changed due to mutation and antibiotics can no longer target it. This character can be passed down to the next generations, making all the daughter bacterial cells of this specific cell resistant to that specific antibiotic. The mutated strain can further change their cellular components to be resistant to other antibiotics. Gradually, this strain could possibly evolve into a notorious multidrug-resistant microorganism, or "superbug," which can be resistant to many known antibiotics. Therefore, as scientists, we need to design and develop new antimicrobial agents that could target these resistant pathogens.

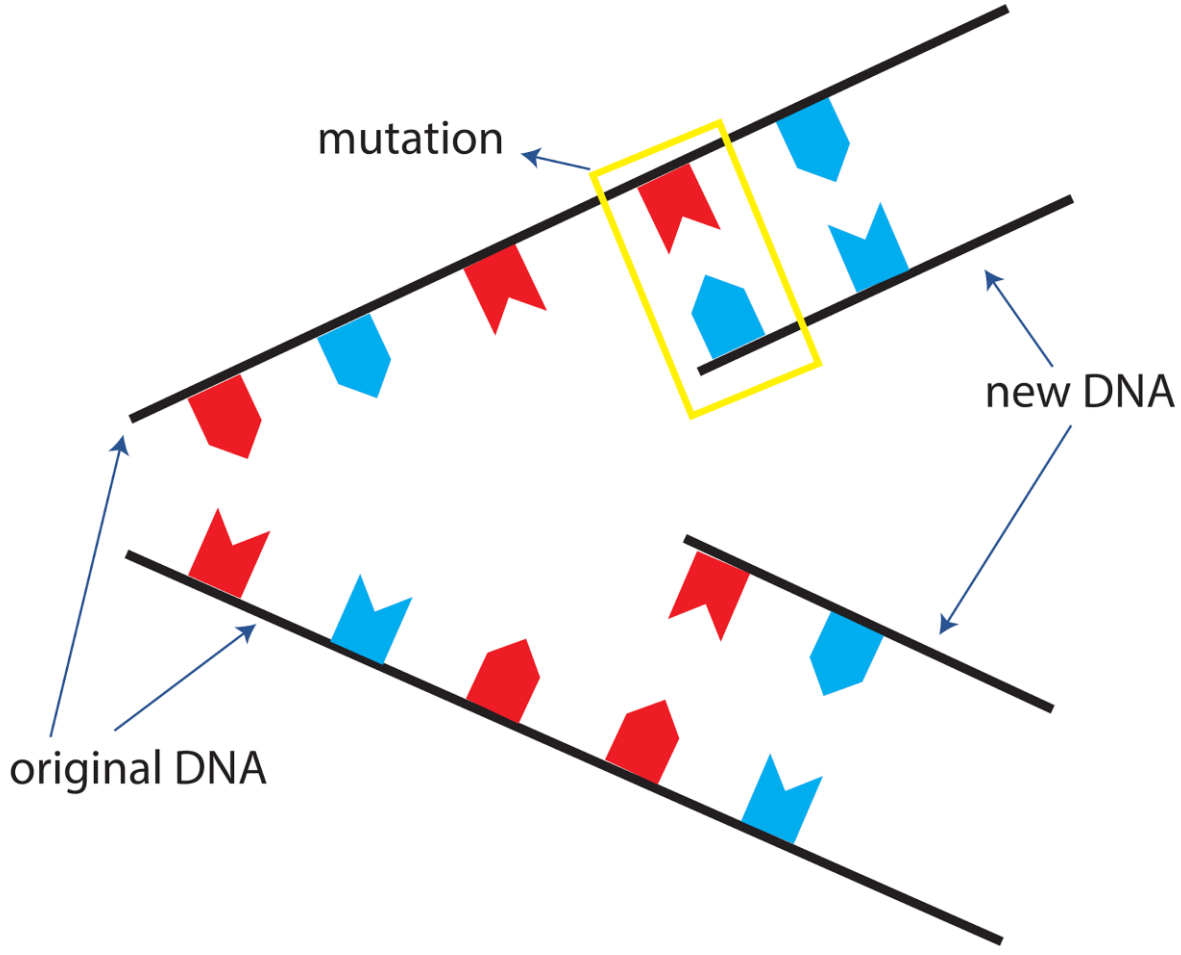

Fig 6.1 Schematic representation of mutation during DNA replication. Nucleotides are the basic structural unit of DNA. DNA is made up of four different types of nucleotides (different shapes

and colors in the figure). DNA has two strands (the two black lines in the figure) and each strand has a specific sequence of nucleotides. Under normal conditions, a nucleotide on one strand will match its corresponding nucleotide on the other strand. In the figure illustrated here, the correctly matching nucleotides should have the same color but different shapes. However, during replication, there is a chance that a wrong (non-matching) nucleotide is added by the replication machinery on the newly synthesized DNA strand. The sequence of this DNA strand is therefore changed.

What is antimicrobial peptide (AMP)?

Most traditional antibiotics are small-molecule chemicals, which means that they have a low molecular weight. On the other hand, a peptide is a polymer. A polymer is a substance consisting of many similar connected units; each unit is called a monomer. A polymer can be very long and heavy since it contains many building units. If we regard a train as a polymer, then each train carriage is a monomer. Similarly, a peptide is a chain consisting of connected amino acids (Fig 6.2). Amino acids are the monomers and building blocks for peptides and proteins. Some amino acids carry a positive or negative charge due to their unique chemical structure. The charge of a peptide is the sum of the charge of all the amino acids that make it up. And if some peptides can kill bacteria, we call them antimicrobial peptides (AMPs, also called host-defense peptides). Natural antimicrobial peptides are short- and medium-sized peptides (typically <40 amino acids) that exhibit broad spectrum antibacterial activity (1, 2). They have been discovered in a variety of organisms, ranging from animals and plants to microbes (3, 4). For example, the AMP I discuss in this thesis, LL-37, is found in humans. Indolicidin originates from cows.

Melittin comes from bee venom. AMPs are evolutionarily conserved, which means that they are kept by the living species throughout many generations of evolutionary selection. They are the first-line natural defense to protect the host from invading pathogens. In addition to antibacterial ability, some AMPs also show antifungal and antiviral properties. They may serve as useful prototypes and templates for the design of new antibacterial agents. The peptide we studied intensively in this thesis is LL-37, which consists of 37 amino acids, including 11 positively charged amino acids and 5 negatively charged amino acids. Therefore, LL-37 has 6 positive net charge. This is important because both DNA and ribosomes are negatively charged molecules. I will explain later in this chapter how this positive charge on LL-37 gives it the ability to bind to and interact with DNA and ribosomes.

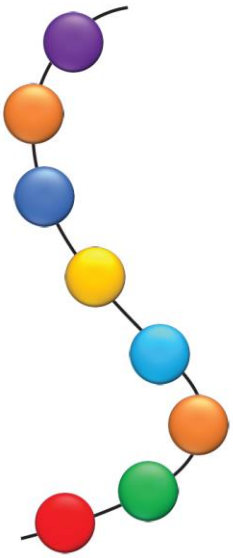

Fig 6.2 Schematic representation of a peptide chain and its amino acids. Each sphere represents an amino acid. Spheres of different colors represent different types of amino acids.

What technique do we use?

Scientists traditionally use bulk culture assays to study the attack of AMPs on bacteria. In these assays, scientists grow bacteria in a bulk culture and the properties of the cell culture are monitored by adding AMPs. This method provides the ensemble average information of the culture but fails to reveal cell-to-cell heterogeneity. For example, by using bulk culture assays we are able to detect that after adding AMPs to the cell culture, the overall cell culture, which might contain billions of bacteria, no longer grows. But have all the cells been attacked by AMPs? In fact, in our cell culture, some cells are dividing and are therefore more susceptible to AMPs. Some other cells could be more resistant to AMPs. Therefore, it would be better for researchers to study the attack of AMPs at a single-cell level.

Fluorescence microscopy is a powerful method for studying AMPs at a single-cell level. We can label the molecule of interest with some bioluminescent protein, for example, Green Fluorescent Protein (GFP). Then we can put the cell under a microscope and shine a laser on the sample. With this method, we can observe the fluorescence coming from the fluorescent protein inside the living cell. The location and motion of this fluorescent protein represent the location and motion of the molecule that we are interested in. This is like fixing a tag to a specific cow's ear. By detecting and tracking the location of the tag, we could spot the location and motion of that cow within a herd. Therefore, it allows us to detect, in real time and at a single-cell level, the dynamics and spatial distribution of proteins, protein-protein interaction, gene expression and cell division.

However, due to the physical nature of light, this technique suffers from a limitation - the resolution. The optical resolution of traditional microscopy methods is ~ 250 nanometer (nm), which means that they cannot tell apart two objects if they are within a distance of 250 nm,

approximately 1/400 of human hair diameter (1 nm = 0.000000001 meter). A typical *E. coli* cell is 1 micrometer (μm) thick and 4 μm long (1 μm = 1000 nm). Therefore, traditional optical microscopy can get the rough outline of an *E.coli* cell but fails to provide detailed subcellular structural information. Recently, a group of pioneering scientists have developed a novel technology called "super-resolution microscopy," which can achieve resolution around 30 nm (5-7).

The super-resolution microscopy that we applied is PALM (Photoactivated Localization Microscopy). A schematic of the PALM principle is shown in Fig 6.3. The molecule of interest is labeled with a photoactivatable or photoconvertible fluorescent protein. "Photoactivatable" or "photoconvertible" means that these fluorescent proteins can be switched between "on" and "off" states via laser, just as a light can be turned on and off by a light switch. By controlling the laser intensity, we can ensure that there are only one or two molecules in the "on" state per cell at any given time. Therefore, we can study each individual molecule. After some time, the fluorescent proteins will be bleached or photobleached, which means that they will enter a state that is unable to fluoresce anymore. For simplicity, we can think of this as each fluorescent protein having a specific "lifetime." When the fluorescent proteins are bleached, we can repeat the process by switching on and imaging another set of molecules. With this technique we are able to not only study the location of molecules of interest, but also track their motion at a very high resolution. Some scientists who developed the technique were awarded the Nobel Chemistry Prize in 2014.

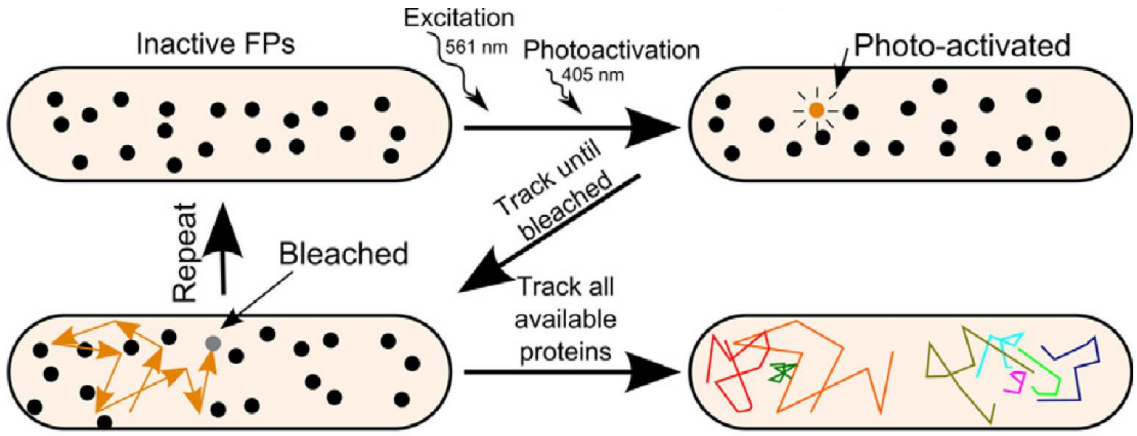

Fig 6.3 Schematic representation of the principle of PALM (Photoactivated Localization Microscopy). The protein of interest is labeled with a photoactivatable or photoconvertible fluorescent protein. We can locate and track each single molecule using this method. The trajectory we reconstruct can tell us the dynamic information of biomolecules. Figures are from ref (8).

What is my research about?

By using super-resolution microscopy, we studied the attack of AMP LL-37 on bacterial cells, in real time at high spatial resolution (9). We put the bacteria under a microscope and observed its behavior before and after we flowed LL-37 into the growth medium. The growth medium we use contains basic nutrients such as amino acids and vitamins that bacteria need for normal growth. The bacteria grown on such growth medium without LL-37 could provide us information about how bacteria behave when they grow normally.

Before they were assaulted by these peptides, the bacteria were growing happily and their DNA were moving around within the cell in a semi-random motion characteristic of life. Bacterial DNA is a long, circular polymer (a very long chain) confined in the cell. We focus on a segment of this long DNA chain, which we call DNA loci. Under normal conditions, the DNA

loci is not still. Instead, the DNA loci moves around because of the dynamic environment inside the living cell. We and others call this motion "jiggling." Other proteins, such as the ribosomes (the molecular machines that make protein), also move around to carry out their functions. The motion of these biomolecules is essential for the cell's viability. Several minutes after we flowed the peptide, the bacterial cellular membrane became permeabilized, which means that the cellular membrane was disrupted and some small biomolecules inside the membrane could leak into the environment. Seconds after membrane permeabilization, LL-37 entered the cell and the jiggling motion of DNA stopped.

Four representative 1-hour DNA loci trajectories are shown in Fig 6.4, which shows that the DNA seemed to freeze after we flowed LL-37 through the growth medium. In addition to DNA, the motion of several essential proteins, such as ribosomes, also seemed to be reduced. Fig 6.5 shows 200 randomly selected ribosome trajectories in a normally growing cell and in cells after we exposed them to LL-37. The ribosome trajectories were more compact after we treated these cells with LL-37. The results indicate that the whole cytoplasm (the contents inside the cellular membrane) was rigidified by the LL-37. In other words, the cytoplasm turned from its dynamic, liquid-like state into a gel-like or glass-like rigidified state after LL-37. It is hard to imagine how a cell could survive in such harsh conditions.

We estimated that some 100 million LL-37 flooded the cell cytoplasm of each cell after membrane disruption. This means that the concentration of LL-37 inside the cytoplasm is 1000-fold more concentrated than the LL-37 concentration that we flowed into the growth medium (Fig 6.6). The cell behaves like a sponge to absorb huge amounts of LL-37 after membrane disruption. As stated above, each LL-37 carries 6 positive net charges. On the other hand, DNA, ribosomes and many small proteins inside the cytoplasm have large negative charges. These

negative charges are important for them to diffuse within the cytoplasm. The positively charged LL-37 can bind tightly to these negatively charged molecules, immobilizing and crosslinking them (forming connecting bridges between them). We speculate that the high concentration of positively charged LL-37 forms a dense network of linkages between these negatively charged biomolecules. This dense crosslink rigidifies the cytoplasm and causes permanent damage to the cell, turning it from a dynamic state into a gel-like or glass-like state which inhibits proper motion of its essential constituents. The cell got gummed up and could not be recovered. This is like throwing millions of wrenches into a delicately working machine. All of a sudden, everything stops and the function of the machine can no longer be repaired.

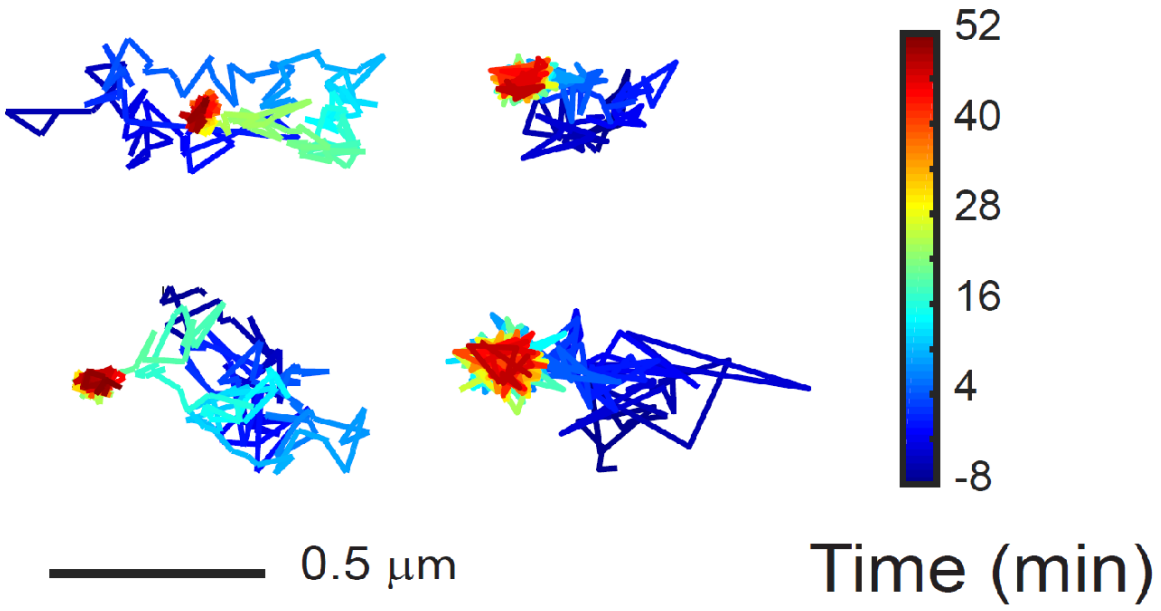

Figure 6.4 Examples of 1-hour time lapse trajectories of four DNA loci. Each trajectory represents the motion of a DNA segment. Time in minutes is color-coded as shown, with $t = 0$ time of injection of LL-37. The motion of DNA loci is largely reduced after flowing LL-37.

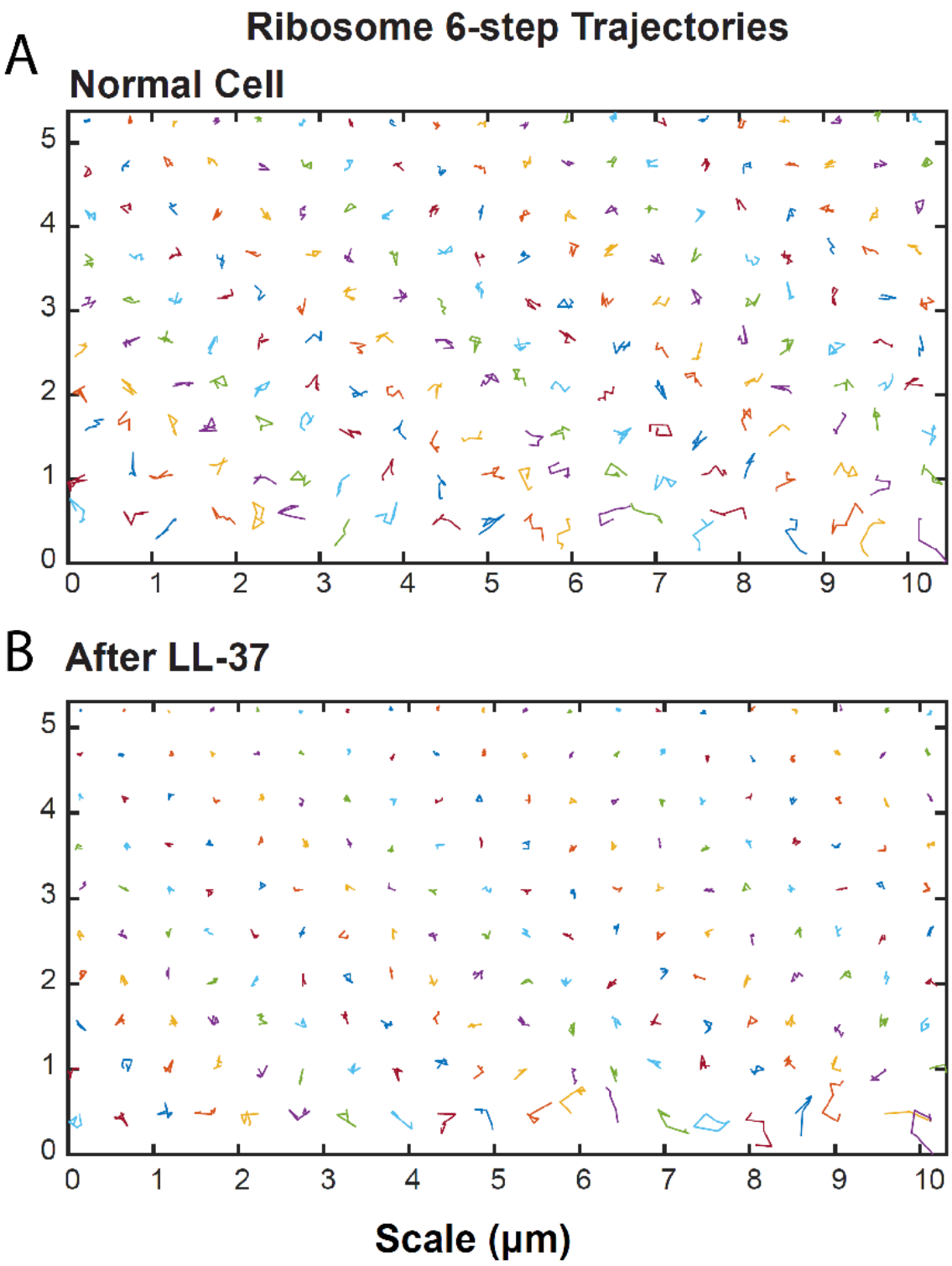

Figure 6.5 Galleries of 200 randomly selected 6-step trajectories of ribosomes taken at 30 millisecond (ms, 1 second = 1000 ms) per frame. (A) Cells in normal growth. (B) Cells 15

minutes after addition of LL-37. Scale is in μm. There are more compact trajectories after LL-37 treatment.

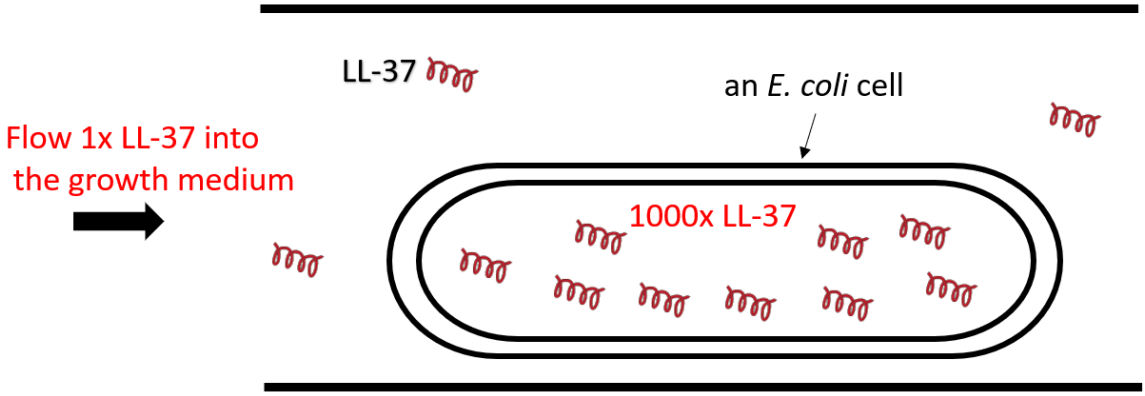

Figure 6.6 Illustration of the concentration of LL-37 inside and outside the *E. coli* cells after we exposed the cells to LL-37. *E.coli* cells absorb large amounts of LL-37. The concentration inside the *E. coli* cell is 1000-fold more concentrated than the concentration that we initially flowed into the growth medium.

How can my research benefit the public?

The era of multidrug-resistant bacterial infections necessitates the discovery of new antibacterial treatment and the elucidation of their antibacterial mechanism. Many traditional small-molecule antibiotics kill bacteria with a specific target. Some bacterial strains could survive the specific attack by mutation and then become resistant. This type of mutation poses a potential threat to our health. LL-37 is different from traditional small molecule antibiotics in that it can not only disrupt the cellular membrane but also rigidify the whole cytoplasm. The former mechanism causes the cell's content to leak out and the latter one inhibits the essential

motion of biomolecules. Therefore, LL-37 assaults the integrity of the whole cell, which cannot be simply counteracted by protein mutation.

Although not directly clinically related, our results help explain why resistance to antimicrobial peptides develops only very slowly. We revealed multiple effects of LL-37 on bacterial cells. A comprehensive understanding of all these effects and how they are interlinked with each other could be of medical significance. It could guide design criteria for synthesizing new antimicrobial agents that efficiently kill bacteria: the ability to disrupt the membrane and the ability to rigidify the cytoplasm.

References

1. Burton MF & Steel PG (2009) The chemistry and biology of LL-37. *Nat. Prod. Rep.* 26(12):1572-1584.
2. Scocchi M, Mardirossian M, Runti G, & Benincasa M (2016) Non-Membrane Permeabilizing Modes of Action of Antimicrobial Peptides on Bacteria. *Curr. Top. Med. Chem.* 16(1):76-88.
3. Zasloff M (2002) Antimicrobial peptides of multicellular organisms. *Nature* 415(6870):389-395.
4. Brogden KA (2005) Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nat. Rev. Microbiol.* 3(3):238-250.
5. Betzig E, *et al.* (2006) Imaging intracellular fluorescent proteins at nanometer resolution. *Science* 313(5793):1642-1645.
6. Rust MJ, Bates M, & Zhuang XW (2006) Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM). *Nat. Methods* 3(10):793-795.
7. Hess ST, Girirajan TPK, & Mason MD (2006) Ultra-high resolution imaging by fluorescence photoactivation localization microscopy. *Biophys. J.* 91(11):4258-4272.
8. Stracy M, Uphoff S, de Leon FG, & Kapanidis AN (2014) In vivo single-molecule imaging of bacterial DNA replication, transcription, and repair. *FEBS Lett.* 588(19):3585-3594.
9. Zhu YY, Mohapatra S, & Weisshaar JC (2019) Rigidification of the Escherichia coli cytoplasm by the human antimicrobial peptide LL-37 revealed by superresolution fluorescence microscopy. *Proc. Natl. Acad. Sci. U.S.A.* 116(3):1017-1026.