修士論文

Real-time Measurement of DNA Degradation by Silicon Nanotweezers Coupled with Microfluidic Cavity

~Effect of Radiation Damage and Restriction Enzyme Digestion~

シリコンナノピンセットと Microfluidic デバイスによる DNA 損傷のリアルタイム測定

～放射線損傷と制限酵素切断の影響～

指導教員　藤田　博之　教授

東京大学　生産技術研究所

マイクロナノメカトロニクス国際研究センター

東京大学大学院

工学系研究科電気系工学専攻

37-126893

江　柏村

Po-Tsun Chiang

Abstract

Because cancer is one of the world's leading causes of death, taking 8 million lives per year, it is a big health concern to improve cancer treatment. [1, 2, 3] Radiation therapy and some chemotherapy depend on introducing DNA damage in cancer cells. In order to know the damaging process in detail, the purpose of this research is to measure/study the DNA degradation, including radiation damage on DNA and restriction enzymatic reaction on DNA, by using silicon nanotweezers. Silicon nanotweezers are the integrated electrical-mechanical tools which enable the manipulation of biological macromolecules and the measurement of their properties. [4] DNA manipulation by using silicon nanotweezers has been performed previously. [5, 6, 7]

For measuring DNA degradation well by silicon nanotweezers, the way of DNA trapping and stability of resonant frequency response of silicon nanotweezers are two of the most important points. We have already spent at least 2 years improving on these two points, but we are still struggling with the problem even now. I present some ways to improve them in this thesis. Basically by measuring the main mechanical resonance of the system, the rigidity of the molecules and the losses due to the molecules can be measured.

Before doing experiments by X-ray, I performed several experiments for measuring restriction enzyme digestion on DNA by using silicon nanotweezers. The results gave us the confidence that silicon nanotweezers can be used to measure DNA degradation in real-time and in solution.

Monitoring in real-time these properties, the effect of X-ray treatment can be studied under different conditions for a better understanding of such X-ray treatment for patients. We hope this research will paves the way for both fundamental and clinical studies of DNA degradation mechanisms under radiation beams for improving radiation therapy and other tumor treatment.

In Chapter 1, the introduction, background, objective, and originality of this research are described.

In Chapter 2, I describe the measuring tools and devices in the experiments of this research. Silicon nanotweezers are the measuring tools I used to measure the degradation of DNA in this research. I fabricate microfluidic cavity filled with solution, and insert DNA trapped by silicon nanotweezers into the chamber of microfluidic cavity. Coupled with microfluidic cavity, the DNA degradation in solution can be measured. I also touch upon the issue of the stability problem of the resonant frequency response.

In Chapter 3, I show how I improved the measuring system, including a parylene microfluidic cavity, insertion of silicon nanotweezers, and procedure of DNA trapping. The proof-of-concept experiments for stability of the resonant frequency response are also performed.

In Chapter 4 and 5, I present the experiments for the real-time measurement of DNA degradation. Chapter 4 describes the characterization of DNA molecular degradation by the restriction enzyme HindIII. The experiments in Chapter 4 verified that the DNA degradation in solution can be measured by silicon nanotweezers. Chapter 5 describes the measurement of radiation damage on DNA. I tried to find the biophysical mechanism of DNA damage due to radiation.

In Chapter 6, I discuss all results I obtained and tried to propose an ideal measurement system of DNA degradation by silicon nanotweezers.

In Chapter 7, I give the conclusions of this thesis, future works and prospects of this research.

Acknowledgement

I would like to first appreciate my adviser Professor Hiroyuki Fujita. He supports me and gives me many advices for this research. In addition, he also conducts me how to do the research and trains me for becoming an independent researcher. Moreover, not only for research, he also teaches me how to face my life in the future and encourages me by his personal experiences. Those experiences always support me when I face problems or feel discouraged. Also, I would like to thank Professor Dominique Collard. He gives me the chance to do this research, and teaches me the knowledge of silicon nanotweezers with patience. Although he is very busy as the director of LIMMS, he always shows his concern and discusses the experiment results with me.

I would like to express my gratitude to all my colleagues in silicon nanotweezers team. First, I really want to express my appreciation to Dr. Nicolas Lafitte. Thanks to his strict training with patience, I learned much knowledge of silicon nanotweezers. He also improved the LabVIEW software for monitoring measurement, and those works made experiments can be done more easily and efficiently. Second, I would like to thank Dr. Momoko Kumemura. She teaches me how to use the machine in cleaning room and bio room, helps me do experiments, and discusses experiment results for many times. Third, I want to express my appreciation to Dr. Laurent Jalabert. All the silicon nanotweezers we use in this research are fabricated by him. He even spends his private time fabricating nanotweezers. Next, I would like to thank my research partner Gregoire Perret. We always exchanged opinions, and his hard works and ideas helped us to improve the experiment system. Also, I would like to thank Dr. Cagatay Tahran. His elaborate way of thinking helps us to prevent some mistake and to reconsider the way of experiments. Besides, I would like to thank Professor Stanislav Karsten. His biology background helps us to consider experiment results in different ways. In addition, their human skills and their kindness which make everything easier, and they have been constantly giving me advices, ideas, knowledge, and help me to learn and grow up. It was a great honor for me to have the chance to work with them.

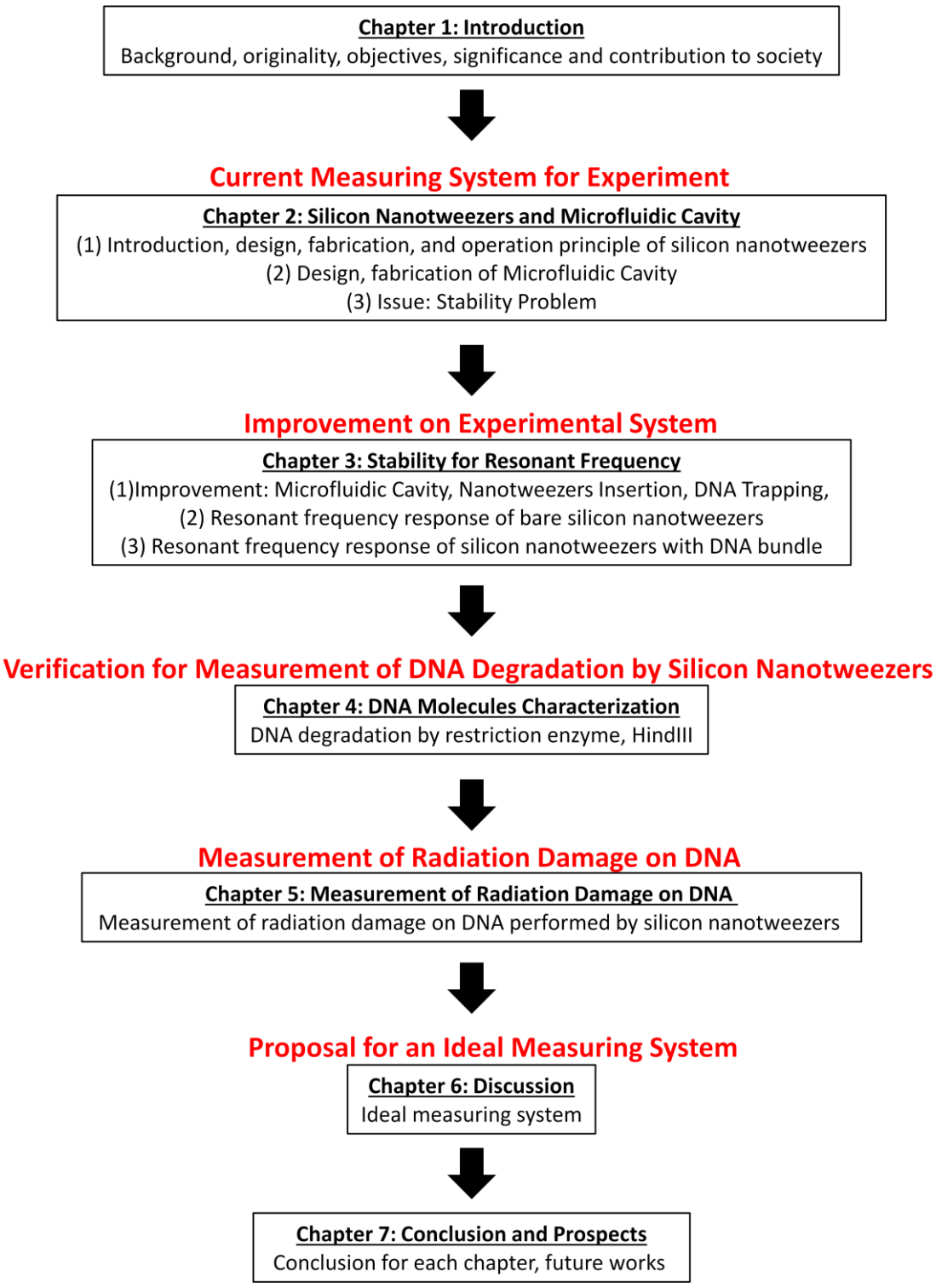
I would like to thank Professor Kiyoshi Miyagawa and Dr Atsushi Enomoto, Center for Disease Biology and Integrative Medicine in University of Tokyo. All X-ray experiments were done with their radiation machine, and they taught me the operation of radiation machine. Besides, their knowledge of X-ray and biology helped us to understand more about our results.

Also, I would like to thank members in Fujita Lab. Dr. Takaaki Sato, Dr. Atsushi Takei, Yuki Takayama, Shinsuke Nabeya, Hiroyuki Mitsuya, Jisu Lee, Quentin Delouvee, Godeffroy Valet, Shinsuke Yamada, Suzuki Akao, and the previous member Dr. Tadashi Ishida, Oya Koc, Islam Orazov, and Minoru Egawa. They treat me with kindness, teach me the life in Japan, and have good relationships with me. Besides, I would like to thank Dr. Manabu Ataka for his contribution for maintenance of clean room. Also, I would like to thank secretaries in Fujita Lab, Kazuko Okudaira, Akemi Yamada, Mayu Ueda, and Minako Makino. Their contribution helps us to focus on research and live in Japan more easily.

Finally, I would like to express my grateful feelings to Professor Ching-Fa Yeh, who is my teacher in National Chiao Tung University in Taiwan and has already retired. He introduced me to Professor Hiroyuki Fujita in my first year in Japan, 2010, and encouraged me to study in Japan. Without his help, I would not have the chance to study in University of Tokyo. In addition, I would like to appreciate my parents, Mr. Frank Chiang and Mrs. Su-chu Chen, and my brother Po-Shin Chiang. Their warm hearts always support the life of studying in Japan. Especially my father affords all the cost for life in Japan by working hard. Also, I would like to thank all the friends I met in Japan. They always encourage me and treat me well. Those encourage me to move forward and finish my study.

Contents

Structure of Thesis



1. Introduction

1.1 Introduction and Background

1.1.1 Cancer and Treatment

Cancer, which is also known medically as malignant neoplasm, remains leading cause of death globally. According to the statistics from the World Health Organization, over 25% of deaths were due to cancer in the world in 2012. [8] In Japan, according to the statistic from Japanese Governments, 30% of deaths were due to cancer last year. [9] The World Health Organization also indicates that the global number of cancer deaths may increase by nearly 80% by 2030, with most occurring in low- and middle-income countries. [8]

The reasons to cause cancer are diverse, complex, and only understood partially. [10, 11] There are many things known to increase the risk of cancer, such as smoking, exposure to radiation, dietary factors, and environmental pollutant. [13] DNA damage is considered to be the primary reason to cause of cancer. [14] There are more than 60,000 new natural DNA damages happen per human cell in one day. These natural DNA damages are due to endogenous cellular processes. In most cases, the speed of cell division and the speed of cell apoptosis are controlled and well-balanced. Even so many DNA damages happen every day, the system of DNA repair helps most damaged DNA to recover, and most cells having over damaged DNA move to the procedure of apoptosis. Therefore, when the deficiency in DNA repair happens, it would cause more DNA damages to accumulate, and increase the risk for cancer. [15] The process that makes normal cells transform into cancer cells called Carcinogenesis. Carcinogenesis is caused by mutation of the genetic material in normal cells, and it breaks the normal balance between cell proliferation and cell death. When cell division becomes uncontrolled (which means damaged cells divide before being repaired), the uncontrolled and often rapid proliferation of damaged cells may lead to benign tumors, and some types of these benign tumors may turn into malignant tumors (cancer). Malignant tumors can invade other organs, spread to distant locations (metastasis) and become life-threatening. [16, 17]

The main purpose of the treatment for cancer is to avoid malignant tumors spread. The tumor in patient's body should be removed as soon as tumor is discovered. There are many ways to treat cancer, such as surgery, chemotherapy, radiation therapy, immunotherapy, and hormonal therapy…etc. Here I simply introduced 3 main treatments for cancer: surgery, chemotherapy, and radiation therapy. [18, 19, 20]

Surgery

Cancer surgery is an operation to repair or remove part of your body to diagnose or treat cancer. It is the most direct way to treat cancer by removing tumors. It may be the only treatment on one patient, or it may be supplemented with other treatments, such as radiation therapy, chemotherapy, and hormonal therapy.

Cancer surgery is usually used for several goals. For example, the doctor can diagnose the stage of cancer by seeing tumor directly. Also, if it is possible to remove the cancerous tumors completely, it may be very efficient treatment for cancer. Sometimes doctor can only remove tumor as much as possible, and combine with chemotherapy or radiation therapy to make treatment more effective.

However, there are also some risks of cancer surgery. For instance, patients may feel painful after surgery, or the wound caused by the cancer surgery can become infected. While cancer can affect people of all ages, the risk of developing cancer generally increases with age because natural DNA damage happens more frequently for old people than young people. Nevertheless, especially in case for old patients, they do not have good immune and recovery system as young people, so they are more vulnerable to get infection after the surgery. Therefore, we need to find some way to treat cancer effectively but without the risks of pain and infection.

Chemotherapy

Chemotherapy is the use of medicine (chemicals) to treat disease. It is usually combined with other cancer treatment such as radiation therapy or surgery. Cancer cells divide and grow very quickly, and the medicine used in

chemotherapy is to reduce the growth rate of cancer cells. Chemotherapy drugs work in various ways: impairing mitosis (prevent cancer cell division), targeting cancer cell's food source, enzymes and hormones they require in order to grow, stopping the growth of new blood vessels that supply a tumor, and triggering suicide of cancer cells. A course of chemotherapy may be just a one-day treatment, or can last for a few weeks - it will depend on the type and stage of the cancer (how advanced it is). When combining with surgery, chemotherapy can be used to decrease the size of tumor before surgery, or to reduce the risk of death (such as infection and pain) after surgery. Chemotherapy can also help doctor to find the precise place of tumor without surgery when combining with radiation therapy. [21]

However, as the way to use one or more cytotoxic drug to treat cancer cells, during the treatment the normal cells may also be damaged and this causes many uncomfortable side effects on patients. The typical side effects, such as nausea and vomiting, alopecia (hair loss), fatigue loss and sleepy, loss of appetite, dry and sore skin, and flaky nails, are happened on over half of all patients. Other more serious symptoms like hearing impairment (deafness), neutropenia (low white blood cells), anemia (low red blood-cell count), and thrombocytopenia (low blood platelet count) may also happen to some patients. It is very hard to stand for even for young people. Although during past 20 years chemotherapy has been improved, but still hard to prevent side effects on patients.

The other treatment is radiation therapy, which is related to this research. I introduce radiation therapy in Chapter 1.1.2.

1.1.2 Radiation Therapy

Radiation therapy is a cancer treatment which uses high-energy radiation to kill cancer cells by damaging their DNA. Radiation therapy can either damage DNA directly or create charged particles (free radicals) within the cells that can indirectly damage the DNA. Cancer cells whose DNA is damaged beyond repair stop cell dividing or die. When the damaged cells die, they are broken down and eliminated by the body's natural processes. The length of radiation therapy depends on many factors, including, the type, location, and size of cancer. Most treatment takes 2 to 10 weeks, once a day

for five days in the row, and 1 to 5 minutes irradiation for one time. The radiation used for cancer treatment may come from a machine outside the body (External-beam radiation therapy), or it may come from radioactive material placed in the body near tumor cells or injected into the bloodstream (Internal radiation therapy). Some patients receive radiation therapy alone, and some receive radiation therapy in combination with chemotherapy or surgery. For example, a patient may receive radiation therapy before, during, or after surgery, depending on the type of cancer being treated. Different from surgery and chemotherapy, radiation therapy is one kind of moderate treatment for cancer and without hurting patient's body. [19, 20]

However, radiation therapy can damage normal cells as well as cancer cells, and this brings side effect on patients. There are several side effects caused from radiation therapy: skin changes (dryness, itching, peeling and blistering), diarrhea, nausea, vomiting, swallowing trouble and sexual changes. Therefore, treatment must be carefully planned to minimize side effects. Nevertheless, it is not easy to decide the most suitable dose of radiation for each patient, and that is why side effect from radiation therapy cannot be avoided completely. Different patients have different conditions of DNA composition and DNA related biomolecules, which means the operation of radiation therapy for each patient should be personalized to reach the best treatment. For personalized radiation therapy, it is necessary to better understand the biophysical mechanism of X-ray damage on DNA. [22]

1.1.3 DNA Damage by Radiation

Inside the nucleus of each human cell there are 46 chromosomes organized into two sets of 23 chromosomes, and DNA is packaged inside these chromosomes. [23] The DNA within our cells is continually being exposed to DNA-damaging agents, such as ultraviolet light, natural and artificial mutagenic chemicals and reactive oxygen species generated by ionizing radiation. [24, 25] When cells are exposed to ionizing radiation, radiation damage can occur in direct way and indirect way. **(Figure 1-1)**

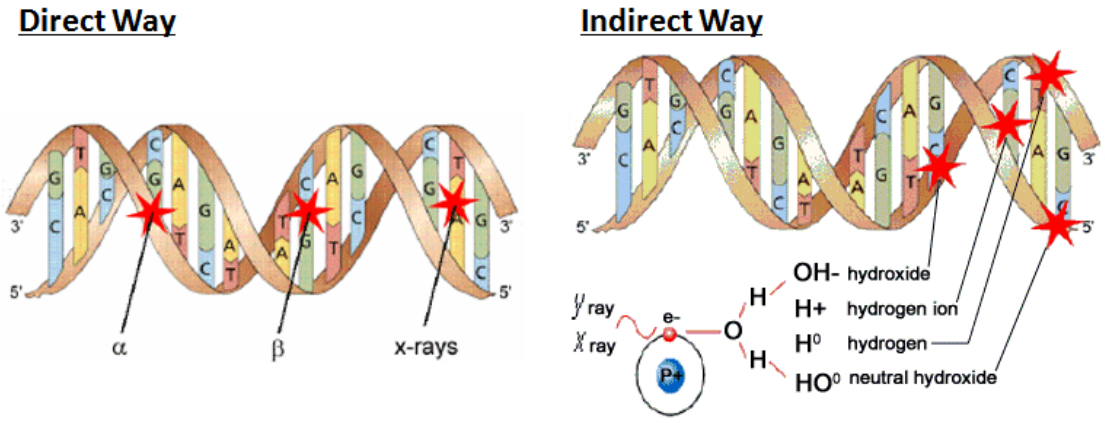


Figure 1-1 Direct way and indirect way of radiation damage on DNA [25]

Source: http://teachnuclear.ca/contents/cna_bio_effects_rad/direct_indirect/#notes

The direct way of radiation damage occurs when alpha particles (two protons and two neutrons), beta particles (an electron) or x-rays create ions which physically break one or both of the sugar phosphate backbones or break the base pairs (which are known as adenine, thymine guanine and cytosine) of the DNA. The bonding of these base pairs can also be affected by the direct way of ionizing radiation damage. However, heavy charged particles such as alpha particles have a greater probability of causing direct damage compared to low charged particles such as X-rays which causes most of its damage by indirect way. [26, 27]

Compare to the direct way, ionizing radiation can also impair or damage cells indirectly by creating free radicals. DNA damage due to indirect action occurs when radiation interacts with the water molecules, which are roughly 80% of a cells composition. The energy absorbed by the water molecule can result in the formation of free radicals, which are molecules that are highly reactive due to the presence of unpaired electrons (ions) on the molecule. Free radicals may form compounds, such as hydroxide or hydrogen peroxide, which is able to initiate harmful chemical reactions within the cells. As a result of these chemical changes, cells may undergo a variety of structural changes which lead to altered function or cell death. [23, 24, 26, 27]

There are many possibilities for the fate of DNA damaged by radiation. First, completely and perfectly repair themselves with the body's inherent repair mechanisms. Second, choose to die and make issue or organ functionally

impaired. This choice usually happens in high dose radiation. Third, repair imperfectly and replicate this imperfect structure, and with the progression of time, may be transformed by external agents (chemicals, radiation, lifestyle habits…) [28, 29]

The research related to radiation damage on DNA has been studied for several decades but still not enough. Many issues such as the threshold value of radiation dose for DNA chooses to repair or die are still not well-known. [30, 31] To improve radiation therapy, the well-known knowledge of radiation damage on DNA is necessary. Therefore, a good way for researching on bio-mechanism of radiation damage on DNA is required.

1.1.4 Conventional Ways to Measure DNA Molecules Mechanical Property

Many biological reactions are too complex to be fully understood through the use of conventional ensemble techniques, where the individual behavior cannot be distinguished, and only average characteristics across billions of molecules can be measured. Since early 90s, the advances in instrumentation and techniques have enabled single molecule experiments. Since then, studies of biological processes at the molecular level have been undergoing in an explosive growth with especially remarkable discoveries on DNA properties.

Figure 1-2 shows the way to select a suitable single-molecule technique to study a given biological problem. [32] For measuring the mechanical properties of molecules, the most commonly known and used are quite obviously Optical Tweezers, Magnetic Tweezers and Atomic Force Microscopy (AFM). [33, 34, 35]

Now researchers have already understood the basic mechanisms and effectiveness of DNA damage by radiation, and the mechanical behavior of DNA. However, those experiments are usually performed by AFM or optical tweezers, which are difficult to be placed and operated under radiation beams; this prohibits in-situ monitoring of DNA damage in the practical situation. [34, 35] To improve radiation therapy, we need to understand radiation damage on DNA more precisely. Therefore, we need a measuring

system that can measure the radiation damage on DNA under in real-time. Also, because DNA acts in water in our bodies, it is better to measure radiation damage on DNA in aqueous solutions mimicking the cellular environment.

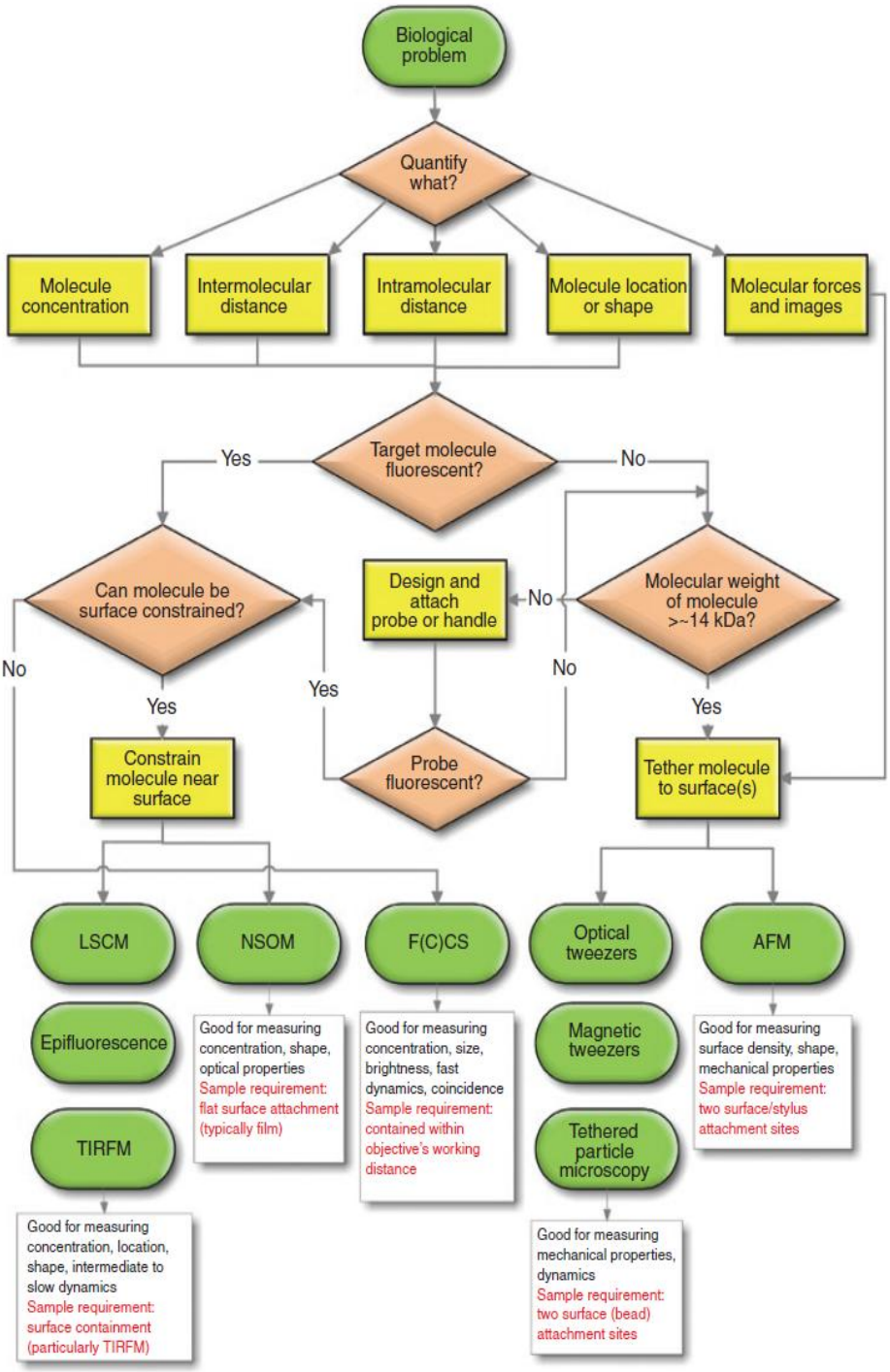


Figure 1-2

Flowchart to select a suitable single-molecule technique to study a given biological problem. [32]

1.2 Objectives and Significance

The research goal is to measure the degradation of DNA caused by radiation in real-time and in solution. As model cases, the influence of restriction enzyme reaction on DNA will be investigated. Besides, in this research, I try to measure the radiation damage on DNA with different doze and time of radiation, and try to understand the mechanism of the radiation damage on DNA with different condition. Also, I measured the digestion reaction on DNA by restriction enzyme in different concentration to understand the mechanism of restriction enzymatic reaction on DNA.

To measure the radiation damage on DNA in real-time and in solution, we need a device that is small enough and convenient to do the measurement. Our lab has been working on Micro Electro Mechanical Systems (MEMS) device for many years. MEMS is the technology to make very small mechanical devices by using semiconductor micromachining technique. By MEMS technology, we manufacture the Silicon Nanotweezers, which are one kind of MEMS devices for direct manipulation of biomolecules. Compared to AFM or optical tweezers, silicon nanotweezers are tiny and convenient to be placed under radiation beams, so we can measure the radiation damage on DNA in real-time. Besides, silicon nanotweezers characterization is based on electromechanical measurement and is not influenced by radiation beams. In addition, coupled with microfluidics, the response from the radiation damage on DNA is closer to the reality due to the measurement in solution.

To measure the DNA degradation by using silicon nanotweezers more precisely and efficiently, I improve the system for better stable resonant frequency response. These works, including the new way of DNA trapping, the sensing for insertion of silicon nanotweezers, and the microfluidic cavity coating with parylene, are presented in Chapter 3. As the model experiment, I did measurement of restriction enzymatic reaction on DNA. To avoid the influence from buffer solution or enzyme on silicon nanotweezers, I deposit gold on the surface of silicon nanotweezers' tip. Because gold is one material that is very to be influenced by chemical reaction, it protects nanotweezers from being influenced by buffer and enzyme solution. By the model experiments, we can make sure that silicon nanotweezers can be used to measure the DNA degradation in solution. After the model experiments, I

measured the DNA degradation by radiation.

In my Master research, I hope I can provide more information about the biophysical mechanism of the radiation damage on DNA and the restriction enzymatic reaction on DNA, and also establish a new way to detect the degradation of DNA. By developing this study, I believe that we could do some contribution in many medical and biotechnology field. For the contribution in medical field, this research could help improving radiation therapy and advancing the quality of cancer treatment. Also, the personalized radiation therapy and the development of a drug accelerating DNA treatment can be achieved in the future. For the contribution in biology and biotechnology field, we could provide one new way to analyze DNA degradation, and it may help us to understand the biomechanical properties of DNA.

2. Current Measuring Tools and Device

In this Chapter, I introduce silicon nanotweezers, which are the measuring tools in this research, and microfluidic cavity, which is the device I use to keep solution and in which tips of nanotweezers are inserted. These are built by previous workers in Fujita Lab.

2.1 Silicon Nanotweezers

Silicon nanotweezers are the tool we use to capture a DNA bundle and measure its mechanical characteristic. [4] Compared to AFM or optical tweezers, silicon nanotweezers are tiny and convenient to be placed under radiation beams; this allows us to measure the radiation damage on DNA in real-time. Besides, silicon nanotweezers characterization is based on electromechanical measurement and is not influenced by radiation beams. In addition, coupled with microfluidics, the response from the radiation damage on DNA is closer to the reality due to the measurement in solution.

2.1.1 Introduction to Silicon Nanotweezers

Silicon Nanotweezers are one kind of microelectromechanical system (MEMS) devices for direct manipulation of biomolecules. Silicon nanotweezers combine trapping capability, mechanical manipulation, and electrical/mechanical sensing at the molecular level. As the first step toward this idea, Hashiguchi et al. have proposed the concept of MEMS-based tweezers which are dedicated to the capture and manipulate DNA molecules. [36] Compare to other miniaturized systems developed for manipulating biological samples, this approach is oriented to the characterization of filamentary molecules.

The measurement and manipulation of DNA molecule by using silicon nanotweezers has already been performed by Yamahada, et al. [4]. In Chapter 2.1.2 to 2.1.6, I present the information details of silicon nanotweezers, including design, fabrication, measurement principle, and DNA trapping.

2.1.2 Design and Fabrication for Silicon Nanotweezers

Figure 2-1 shows a three-dimensional illustration of the Silicon Nanotweezers and the damped oscillator model of Silicon Nanotweezers. It consists of two sharp tips that act as electrodes for both DNA trapping by dielectrophoresis (DEP) and conductivity measurement of DNA molecules. One tip is fixed and the other one is driven by an electrostatic actuator. The motion (x-direction) of the movable arm can be measured by using two capacitances with gaps that vary in proportion to the electrode displacement.

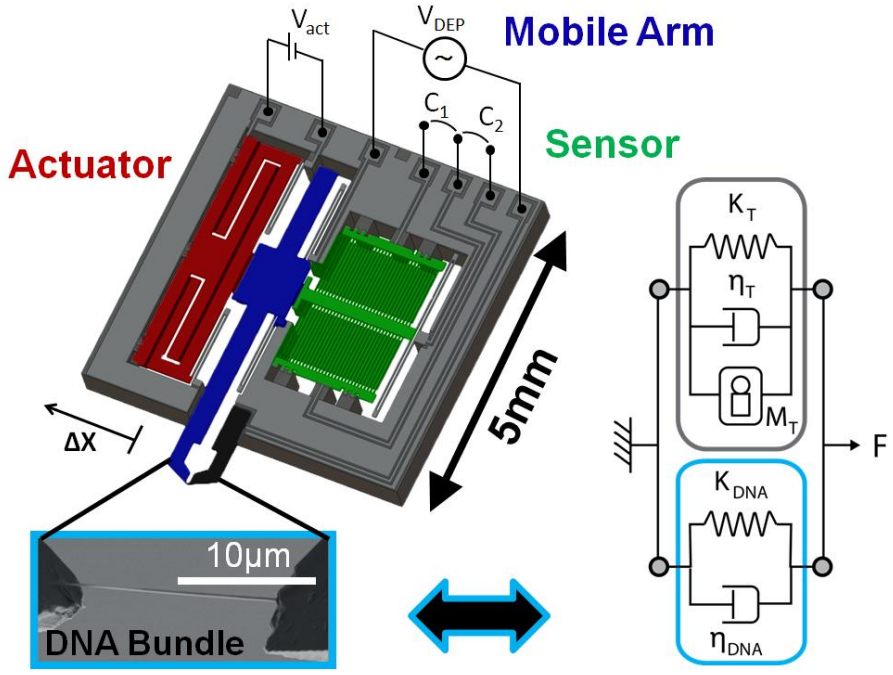


Figure 2-1 Structure of silicon nanotweezers and oscillator model

SNT consist of the following three parts:
(1) Two sharp tips (gap is 5μm~15μm)
(2) A series of comb-drive actuators
(3) A differential capacitive sensor for displacement

The fabrication of the silicon nanotweezers is based on reactive ion etching, local oxidation, and anisotropic etching of silicon. The starting material for our prototype was a SOI substrate having the following characteristics: (100)-oriented 25-μm-thick Si active layer/1.5-μm-thick buried oxide insulator/380-μm-thick Si handling substrate. The process flow is

summarized in **Figure 2-2**, and the different fabrication steps are enumerated as follows: [4].

(1) A thin Si_3N_4 layer is first deposited by low-pressure chemical vapor deposition and patterned to form rectangles aligned along the x-directions (mask #1).

(2) The Si_3N_4 and the Si over layer are etched by reactive ion etching (mask #2).

(3) Next, a local oxidation of silicon process is used to grow SiO_2 on the top and sidewalls of the structured Si.

(4) The Si_3N_4 layer is then removed.

(5) A KOH wet anisotropic etching of Si is performed to obtain {111} facets, which make sharp opposing tips.

(6) The buried oxide is removed by HF, and the handling Si is structured by deep reactive ion etching (using an Al mask on the backside: mask #3).

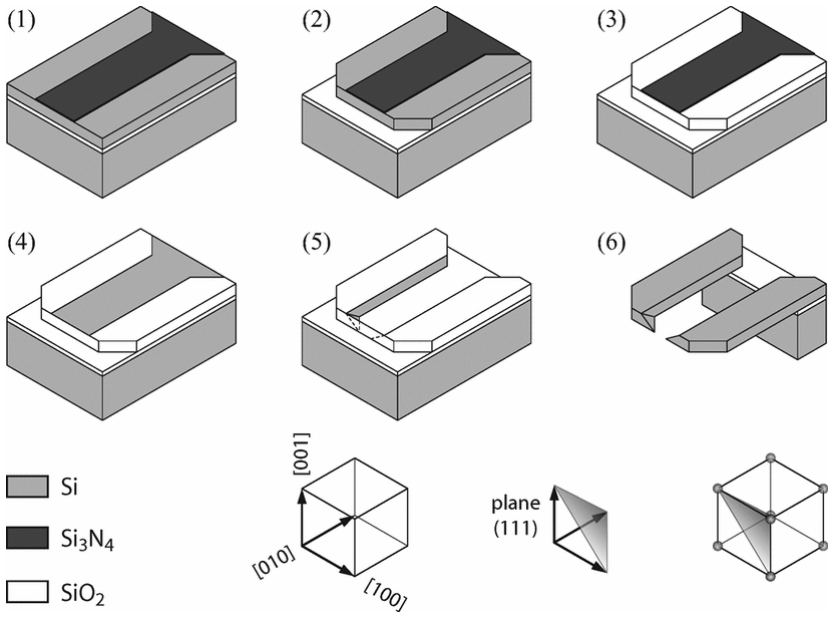


Figure 2-2 Fabrication process of the sharp silicon nanotweezers [4]

In the final step, a thin aluminum film is evaporated on the front side (not shown in Figure 2-3). Indeed, aluminum acts as an anchoring material for DNA molecules [37]. One should note that the process only requires three lithographic masks: one for defining the area of the sharp tips with silicon nitride (#1), one for microstructuring the silicon over layer (#2), and one for the backside etching of the handling substrate (#3).

2.1.3 Real-time Measurement Principle and Model

Figure 2-3 shows the steps of silicon nanotweezers measurement principle. For the measurement of the DNA bundle trapped by silicon nanotweezers, first, DNA is trapped in between two tips of silicon nanotweezers. Then, by changing the frequency of the driving voltages (1 V) added on actuators, we can measure the resonance characteristic by displacement sensing. This resonance characteristic reflects the mechanical characteristic of actuators and the DNA bundle trapped between two tips. By the oscillator model in **Figure 2-1**, we can extract the mechanical characteristic (stiffness) of the DNA bundle.

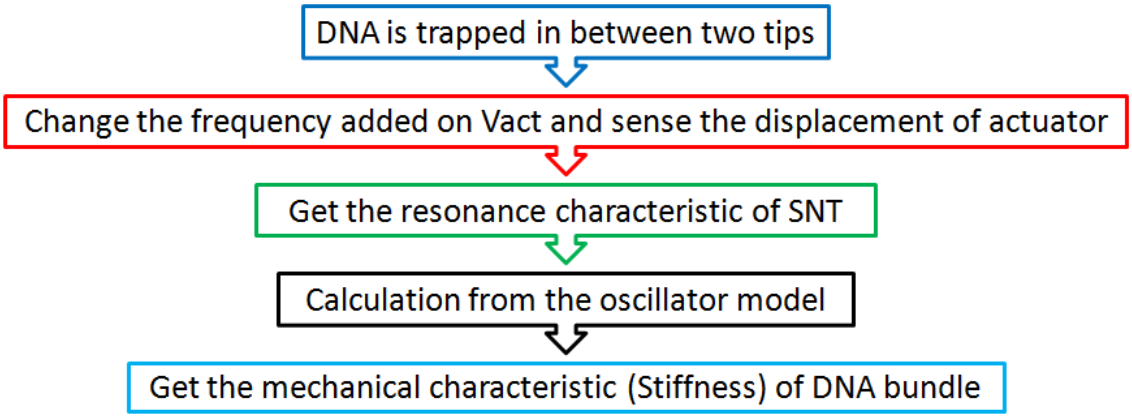


Figure 2-3 The measuring operation of silicon nanotweezers

Equation 2-1 shows the resonant frequency of bare silicon nanotweezers (F_0), while **Equation 2-2** shows the resonant frequency of silicon nanotweezers with the trapped DNA bundle (F_{DNA}). M is the mass of mobile part of silicon nanotweezers, 1.9×10^{-7} kg, k_{tw} is the stiffness of silicon nanotweezers, 50 N/m, and k_{DNA} is the stiffness of DNA bundle. By these equations, the stiffness of DNA bundle can be measured and analyzed. The characteristic of DNA bundle is presented in Chapter 2.1.6.

$$F_0 = \frac{1}{2\pi}\sqrt{\frac{k_{tw}}{M}} \qquad \textbf{(Equation 2-1)}$$

$$F_{DNA} = \frac{1}{2\pi}\sqrt{\frac{k_{tw} + k_{DNA}}{M}} \qquad \textbf{(Equation 2-2)}$$

Figure 2-4 shows the resonant frequency in different DNA bundle. By **Equation 2-2**, due to the difference of thickness, the resonant frequency (peak of curve) of SNT will be changed. The thinker DNA bundle is, the higher stiffness system is, the higher resonant frequency is.

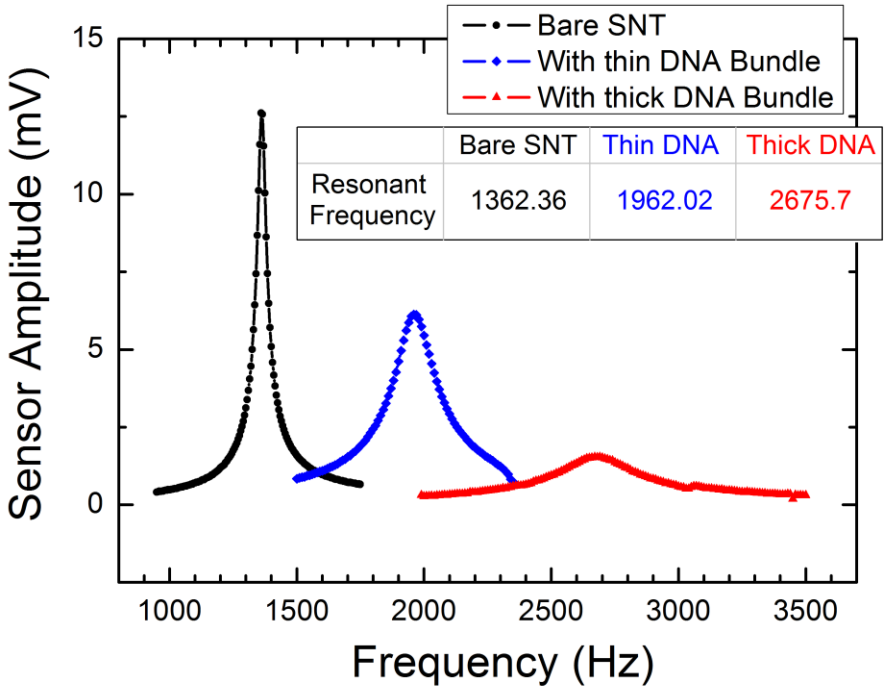


Figure 2-4 The resonant frequency (peak value) of different DNA bundles.

2.1.4 Trapping of DNA molecules

The first step for the characterization of DNA molecules is to trap molecules in between the two tips of silicon nanotweezers. Indeed, DNA molecules are routinely trapped in aqueous solution using dielectrophoresis forces. Washizu et al demonstrates the electrostatic orientation and dielectrophoresis (DEP) of DNA under a high-intensity field produced in microfabricated electrode system. [38]

(a) Principle of dielectrophoresis

DNA has many ionizable phosphate groups along its length. In solution, the DNA molecules become all charged and surrounded by a cloud of ions. The ions are electrostatically bonded to the molecule but are easy to move along. A DNA molecule is highly polarizable along its length.

Therefore the orientation of DNA is the same as that of nonspherical particles resulting from the interaction between the external field and the induced dipole. Washizu et al reported a study of the orientation of λ-phage DNA molecules with frequency dependence from 40 kHz to 2 MHz. The paper concludes that the optimal frequency for the orientation is experimentally 1 MHz. At this frequency, voltage up to 150 V_{pp} is applied to microfabricated electrodes with a gap of 60 to 150 μm, and the electric field strength is higher than 10^6 V/m.
Typically this method has been adopted for the precise positioning of DNA molecules trapping by Washizu et al. [39]. A single DNA molecule trapping was also performed by Kumemura et al. [40]

(b) DNA trapping

Based on these previous research and experimental works, the design of the silicon nanotweezers has been done in such a way to integrate conductive electrodes for DNA trapping by DEP. Therefore the trapping of a bundle of DNA molecules is achieved by applying an AC electric field on electrodes of tips.

For all the experiments including DNA trapping described in this thesis, we used a solution of double-stranded λ-DNA obtained from Takara bio Inc (http://www.takara-bio.com). λ-DNA is the DNA molecule of the bacteriophage λ. It is about 16.5-μm long and contains 48,502 nucleobase base pairs. The initial solution is concentrated in DNA molecules (0.35 μg/μL). The previous way is to dilute DNA solution for 2 to 10 times with DI water and then to trap DNA molecules by adding longer time (10 ~ 20 minutes) DEP voltage from the diluted solution. [42, 43] This way was to prevent from DNA molecules attach too much to other parts of silicon nanotweezers in addition to the tips of nanotweezers. However, practically this way is not easy to trap DNA bundle even adding the DEP voltage longer due to the low concentration of DNA solution. Even DNA trapping was

successful, during inserting silicon nanotweezers with DNA into other solution (DI water, buffer, or enzyme), the DNA bundle was easily to fall from the tips. The idea for improving DNA trapping is presented in Chapter 3.1.4.

2.1.5 Characterization of DNA molecules

Here I show **Equation 2-1** and **Equation 2-2** presented in Chapter 2.1.4 again.

$$F_0 = \frac{1}{2\pi}\sqrt{\frac{k_{tw}}{M}} \qquad \textbf{(Equation 2-1)}$$

$$F_{DNA} = \frac{1}{2\pi}\sqrt{\frac{k_{tw}+k_{DNA}}{M}} \qquad \textbf{(Equation 2-2)}$$

M is the mass of mobile part of silicon nanotweezers, 1.9×10^{-7} kg, and k_{tw} is the stiffness of silicon nanotweezers, 50 N/m. The mass of DNA bundle is neglected because it is much smaller than the mass of silicon nanotweezers. The difference between resonant frequency of bare silicon nanotweezers and the DNA bundle is shown as **Equation 2-3**, and we can drive **Equation 2-4**. By **Equation 2-4** we can get the stiffness of DNA bundle, k_{DNA}. From the previous work by Bustamante et al, the stiffness of a single λ-DNA molecule (16 μm) is approximately 3×10^{-5} N/m in solution [41]. We can use this reference value and to calculate equivalent number of DNA molecules trapped by tweezers in solution.

$$\Delta F = F_{DNA} - F_0 = \frac{1}{2\pi}\left(\sqrt{\frac{k_{tw}}{M}} - \sqrt{\frac{k_{tw}+k_{DNA}}{M}}\right) \qquad \textbf{(Equation 2-3)}$$

$$k_{DNA} = 4\pi\sqrt{MK_{tw}}(\Delta F) \qquad \textbf{(Equation 2-4)}$$

2.2 Microfluidic Device

In nature, DNA exists in the solution, so it is better to measure DNA degradation in solution to make the results closer to reality. In order to do experiments in solution, we fabricate a microfluidic cavity for the insertion of solution. We only need small volume of solution to do experiments, which means we can avoid unnecessary waste. Also since the solution filled in the microfluidic cavity is sustained longer against evaporation, we have enough time to measure and wait for reaction. Especially, for some DNA experiments such as DNA enzyme experiment, we need to keep using SNT for measuring DNA degradation for more than 40 minutes.

Figure 2-4 shows the fabrication of microfluidic cavity. It is made with one silicon chip (500 μm thick) and two slices of glass slits (36 mm x 24 mm, 150-μm-thick). The gap of the microfluidics device is 800 μm. we use this microfluidic cavity to fill solution, and insert silicon nanotweezers from the opening gap. The idea for this design is related to the evaporation of solution. We tested and found that the speed of evaporation from narrow opening is much slower with this design because the evaporation of solution starts from bigger opening in the back.

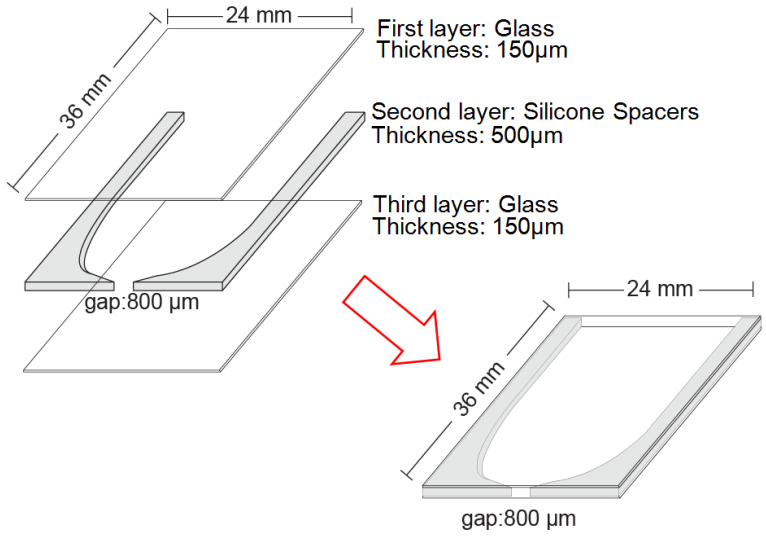


Figure 2-4 Fabrication of microfluidic cavity

2.3 Issue: Stability Problem of Resonant Frequency Response

Before trapping DNA and measuring the stiffness of DNA bundle, we have to make sure that the resonant frequency of bare silicon nanotweezers (without DNA) can be sustained at a stable value when bare SNT keeps being placed in the same environment. The stability of resonant frequency with bare SNT is very important because of two main reasons. First, we have to prove that the mechanical resonant characteristics of silicon nanotweezers are not influenced by DI water, buffer, and enzyme, so we can use silicon nanotweezers as a measuring tool in these different kinds of solution. Second, we can use the resonant frequency of bare silicon nanotweezers, as a standard value, to calculate the stiffness of DNA bundle by the oscillation model.

However, in many experiments we could only obtain unstable resonant frequency response. Sometimes even if we put silicon nanotweezers with DNA bundle into DI water, the resonant frequency keeps increasing while it should stay at a stable value since DI water does not influence on DNA bundle. Sometimes when we put trapped DNA bundle into buffer solution, the resonant frequency keeps decreasing although DI water and buffer do not influence on DNA molecules. Therefore, to find cause of the unstable resonant frequency response is the most important issue before we can apply silicon nanotweezers to measure DNA degradation.

Although the microfluidic cavity can decrease the speed of evaporation of solution, the meniscus surface of solution is concave. Due to the concave shape of solution surface, the tips of silicon nanotweezers need to be inserted deeper to touch the solution. (**Figure 2-6a**) Therefore, after inserting silicon nanotweezers into solution, too much solution attaches to the edges of nanotweezers and it changes the resonant frequency. (**Figure 2-6b**) In addition, the shape of meniscus surface obviously changes during several minutes after the insertion of nanotweezers and this also influences the resonant frequency. By these reasons it is very difficult to get stable resonant frequency response in liquid. I present some ideas and solutions to improve the stability problem of resonant frequency response in the next chapter.

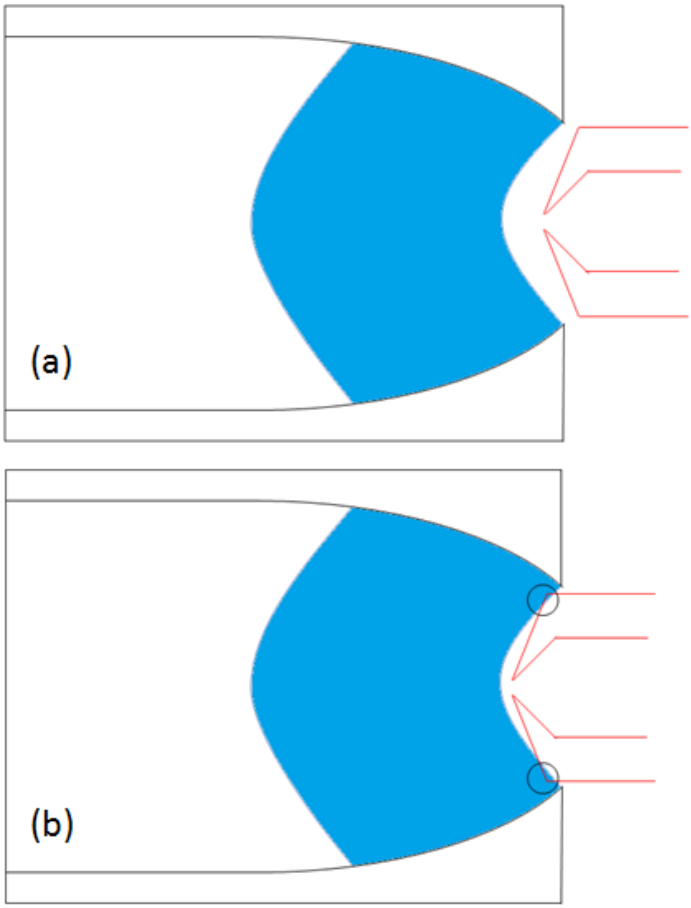


Figure 2-6

a. The concave meniscus surface of solution in microfluidic cavity

b. The edge of nanotweezers (black circles) touches solution before the tips

3. Improvement on Experimental System

Since the stability of resonant frequency response is the most important problem to solve before the measurement of DNA degradation, I considered many possible factors influence the stability of resonant frequency response, including attachment of DNA molecules to tips of silicon nanotweezers, the evaporation of solution and the meniscus surface change, and the insertion position of silicon nanotweezers into liquid. I tried to improve our system to reduce the influence factor to get stable resonant frequency response.

3.1 Improvement on Experiment System

To improve our measuring experiment system, I did many repeated experiments to test and tried to find the reason why resonant frequency response is difficult to be stabilized in solution.

3.1.1 Ideas for Improvement

At the beginning when we faced the stability problem, it was difficult to tell what caused this problem. After many experiments, I found that the meniscus surface of solution in microfluidic cavity changes by time and the volume of solution becomes less and less. This change of solution surface possibly influences the resonant frequency response.

Meanwhile, even if the solution in microfluidic cavity is inserted by the same volume in each time, the shape of meniscus surface is not the same in each experiment. Therefore, the insertion depth and insertion contraposition of silicon nanotweezers are not the same in each experiment, and this also influences the resonant frequency.

In addition, even if DNA bundle is trapped by nanotweezers, some of DNA molecules may fall down during immersion in solution because of the surface tension. And also sometimes DNA bundle is not well-trapped (even it looks like being well-trapped), or very hard to trap.

To solve on these problems, I improved the experimental system as follows: For meniscus surface change, we tried to use hydrophobic cavity. For insertion of nanotweezers, I used a microrobot (i.e. a computer controlled X-Y-Z stage) to control insertion of silicon nanotweezers, and sense the position of insertion. For DNA trapping, I tried to optimize the way of DNA trapping.

3.1.2 Parylene Microfluidic Cavity

To solve the problem of meniscus surface change, I consider using a hydrophobic material to make the microfluidic cavity. The idea is to make the surface of the microfluidic cavity hydrophobic. The hydrophobic interaction is mostly an entropic effect originating from the disruption of highly dynamic hydrogen bonds between molecules of liquid water by the nonpolar solute. By staying together, nonpolar molecules reduce the surface area exposed to water and minimize their disruptive effect. [44] Thus, the two immiscible phases (hydrophilic vs. hydrophobic) will change so that their corresponding interfacial area will be minimal. The speed of evaporation is related to the surface area of liquid, and by hydrophobic effect the surface area of liquid is minimized. Therefore, the evaporation of liquid in hydrophobic surface is much slower than in hydrophilic surface. The experiments performed by Shin, Dong Hwan, et al. proved that the water in hydrophobic surface evaporates much slower than the water in hydrophilic glass. [45]

To make the surface of the microfluidic cavity hydrophobic, I coated a 100 nm-thick parylene film on the surface of the microfluidic cavity and made it hydrophobic. Parylene is hydrophobic, and has strong resistance to acids, caustic solutions, gases and water vapor. By coating parylene on microfluidic cavity, the surface of microfluidic cavity becomes hydrophobic, and the meniscus surface of solution changes from concave to convex due to the cohesive force of solution. Due to this convex meniscus surface, it makes the tips of silicon nanotweezers only attached with small volume of solution and decreases the influence from meniscus shape change of solution. Also, the evaporation of solution becomes very slow because the surface area of solution is minimized.

Figure 3-1 shows the comparison between general microfluidic cavity and

parylene microfluidic cavity. The shape of meniscus surface in previous microfluidic cavity is convex, and after 30 minutes the surface line moved back a lot. In contrast, the shape of meniscus surface of parylene microfluidic cavity is obviously flatter, and the surface line did not change even after 1 hour.

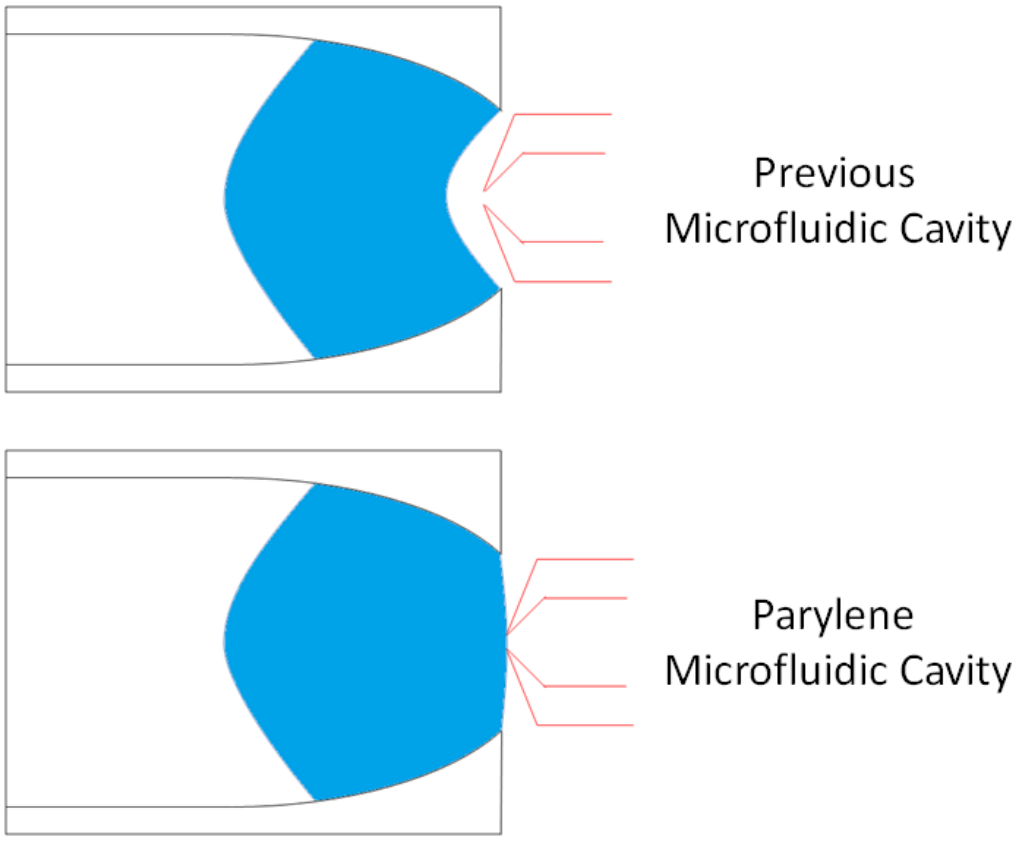


Figure 3-1

Previous microfluidic cavity and parylene microfluidic cavity

3.1.3 Insertion of Silicon Nanotweezers

The insertion depth and position of silicon nanotweezers has strong influence on the resonant frequency response. Although the insertion only influences less than 1 Hz, 1 Hz is not small if we compare to the typical resonant frequency change of 1 ~ 2 Hz due to DNA degradation. The insertion of Silicon nanotweezers is actually by moving the position of microfluidic cavity but not silicon nanotweezers. The tweezers are fixed at the same position, only the microfluidic device is moved by controlled motion of the microrobot. Not only the depth of insertion but also the speed of insertion and the position of insertion can be controlled. Usually, the middle of the microfluidic cavity (in x, y, z- direction) is the best position for inserting silicon nanotweezers. **(Figure 3-2)** This position is the most convex point of meniscus surface of solution. At this position, only the tips of the tweezers touch the solution, and it decreases the mass influence on silicon nanotweezers because solution does not attach to the edge of silicon nanotweezers much. To make sure the insertion of silicon nanotweezers is at the best position, the microscope is useful to observe where the middle of cavity in x-direction is. We cannot, however, determine the position of y-direction and z-direction. To determine the insertion position of silicon nanotweezers in 3-D, we sense the position by based on the change of sensor amplitude of silicon nanotweezers.

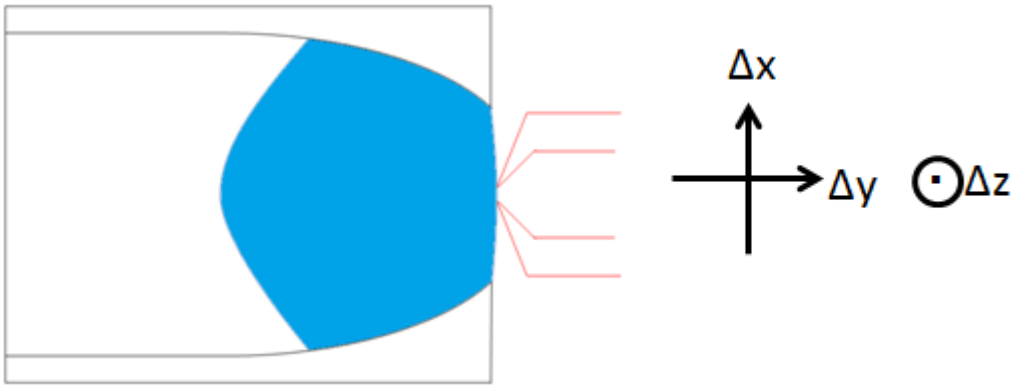


Figure 3-2

The x, y, z direction of insertion of silicon nanotweezers
The figure shows the view from the monitor of microscope

Z-direction

First I insert silicon nanotweezers into microfluidic cavity, and then move the cavity in z-direction slowly (1 μm per second). If the silicon nanotweezers touch the surface of microfluidic cavity, the amplitude of resonant frequency decreases suddenly. I monitor the amplitude change by phase locked loop measurement, and control the motion to stop automatically when tweezers touch the surface of the microfluidic cavity. After touching the upper surface of the microfluidic cavity, again I move the cavity in z-direction slowly to the lower surface of the microfluidic cavity, and again microrobot motion stops automatically when silicon nanotweezers touch the microfluidic cavity. By touching the upper and lower surfaces of the microfluidic cavity, we know the position of those surfaces, so we can determine the middle of cavity in z-direction.

Y-direction

The optimization of insertion position of y-direction helps to fix the insertion depth of silicon nanotweezers. As mentioned before, the insertion depth makes the resonant frequency change. If the insertion is deeper, the resonant frequency is lower. Therefore, it is necessary to know the insertion depth (the position of y-direction). The idea is also sensing the position of the meniscus based on the amplitude at the resonance. If the tips of silicon nanotweezers touch the meniscus surface of solution, the amplitude at the resonance decreases, and the microrobot moves microfluidic cavity 50 μm back (so that we know the silicon nanotweezers is 50 μm far from the meniscus surface of solution).

One thing has to be careful is that the position sensing cannot be performed when DNA bundle is trapped by silicon nanotweezers. Because the amplitude change is too small to detect, silicon nanotweezers is easy to be broken during the sensing.

By sensing amplitude change of resonant frequency we can determine the best position for insertion. This also helps us to fix the condition of each experiment and make our measurement more precise and repeatable.

3.1.4 DNA Trapping

The new way I suggest is to trap DNA with the originally high concentration of DNA solution. First I put silicon nanotweezers into a DNA droplet. The insertion depth into the DNA droplet should be as short as possible. Due to the high concentration of DNA solution, I only add DEP voltage for 2 minutes. The AC voltage depends on the gap of nanotweezers (typically from 5 to 8 μm), and it is usually from 5 ~ 8 V_{pp}. After 2 minutes we take silicon tweezers out from DNA droplet by the side of the droplet but not the frontage of the droplet. **(Figure 3-3)** By taking tweezers out sideways, we can prevent the attached DNA from being pulled out on the surface of the DNA droplet. In some cases, there are also some badly-attached DNA molecules still on the tips. After taking out from the DNA droplet, I insert the silicon nanotweezers into the other DI water droplet to wash the tips 2 ~ 3 times, making the badly-attached DNA molecules fall down and only well-attached DNA molecules stay on the tips. By this new way we can trap DNA molecules with high successful rate and achieve the stable resonant frequency response of nanotweezers.

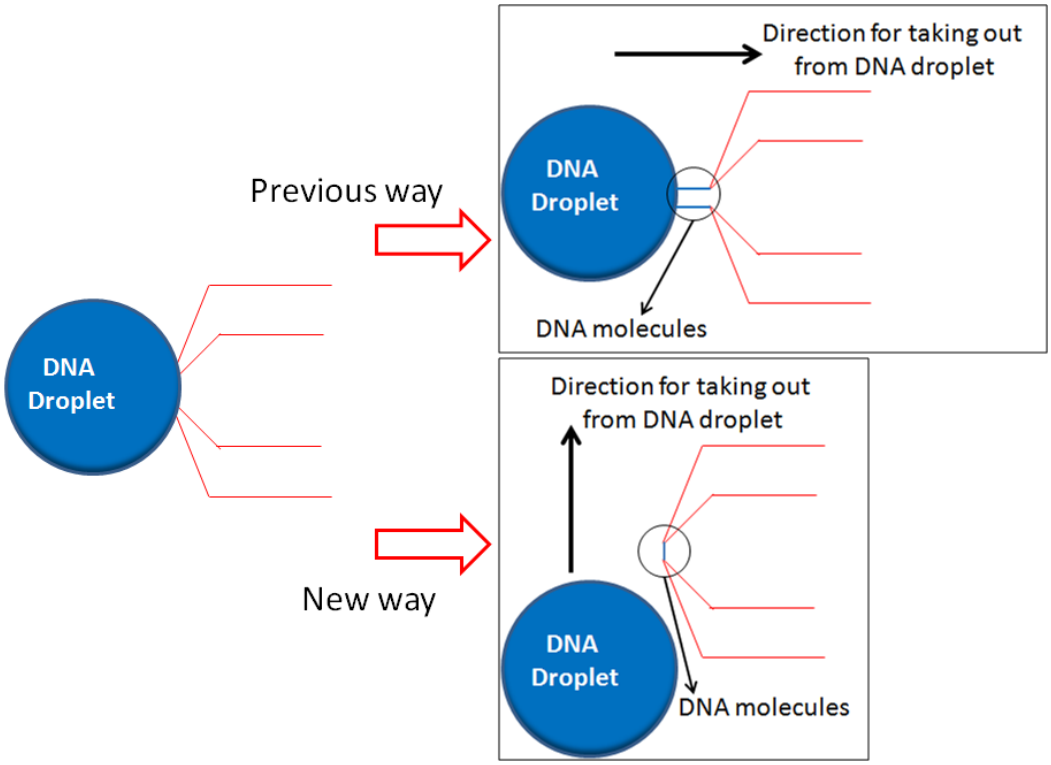


Figure 3-3

The new way for taking out from DNA droplet in DNA trapping is more efficient and decreases badly attached DNA molecules

To trap DNA by silicon nanotweezers easily and well, the other important point is the “freshness” of nanotweezers. The previous work (Yamamoto et al) shows that aluminum is a suitable material for trapping. [37] Usually a new silicon nanotweezers (which means it was not used for any experiment before) have a fresh aluminum deposited on the tips of tweezers. This fresh aluminum surface makes DNA easily attach to the tips. Therefore, after several experiments by the same tweezers, the tweezers should be deposited by aluminum again on the surface of tips. In addition, aluminum is an easily-to-oxidized material, and it is necessary to redeposit aluminum on the tweezers which are never used before but were fabricated (even had been deposited by aluminum) over 1 month ago.

In the previous work, Yamahata et al performed the DNA trapping by silicon nanotweezers with 15 ~ 20 μm gap between tips. [4] Now thanks to the well-fabricated silicon nanotweezers, the gap between tips can be even smaller than 4 μm with very sharp tips, the DNA trapping becomes easier due to this small gap. Instead of aluminum, gold is the other material that can be deposited on the surface of tips for DNA trapping I suggest. Unlike aluminum, gold has high resistance against oxidization and chemical reaction with other ions. Therefore, we can avoid the possible chemical reaction on the tip surface by using gold instead of aluminum especially when doing experiment in buffer or enzyme solution. The experiments performed by gold-deposited silicon nanotweezers are presented in the next chapter.

3.1.5 Procedure of DNA Trapping

As my experience of DNA trapping by using silicon nanotweezers, I wrote the procedure of DNA trapping for reader who wants to learn it.
The procedure for one DNA trapping experiment is shown as follows:

0. Before DNA trapping, check the resonant frequency of bare silicon nanotweezers and the gap between tips. Better to use the fresh nanotweezers to trap DNA.
1. Put the DNA droplet (usually 10 μL) on the edge of glass silt.
2. Insert the tips of silicon nanotweezers into the DNA droplet by very slow speed (1 μm per 1 moving step) and with the smallest possible insertion depth (under 10 μm) into the droplet.
3. Turn on the AC voltage (1 MHz, 8 Vpp for 8 μm gap tweezers) on DEP for 2 minutes.
4. Take tweezers out from the side of the droplet, moving with slow speed especially when the tips of tweezers almost leave from the DNA droplet.
5. Insert nanotweezers into another DI water droplet from the frontage of the droplet to wash tips and clean the badly-attached DNA molecules for 2 ~ 3 times. Keep turning on the AC voltage for DEP.
6. Turn off the AC voltage on DEP and measure the frequency response of nanotweezers with the DNA bundle.
7. After an experiment, put tweezers into the DI water droplet. After immersion in DI water for 1 minute, blow the droplet by an air blower to take all DNA molecules out from the tips.
8. See by microscope and measure the frequency response of nanotweezers to confirm the resonant frequency back to the frequency of bare silicon nanotweezers to make sure there is no DNA bundle between the tips.

3.2 Experiment for Confirmation of Stability

I have already presented several ways to improve the experiment system. Here I present the experiments for confirming the stability of resonant frequency response.

3.2.1 Bare Silicon Nanotweezers in Parylene Microfluidic Cavity

Here I presented some experiment results for stability of resonant frequency response. First I put bare silicon nanotweezers into the parylene microfluidic cavity filled with DI water. Because theoretically DI water and parylene do not influence silicon nanotweezers, we hope to see the resonant frequency stays at a stable value during the immersion in DI water.

In this experiment, I prepared the parylene microfluidic cavity filled with DI water (40 μL). Usually I waited 10 ~ 20 minutes for the stabilization of the meniscus surface of the solution filled in the cavity. Then, I sensed the best position of insertion, which is presented in Chapter 3.1.3, and inserted silicon nanotweezers (Al-A2, gap 8 μm) into the microfluidic cavity with very small insertion depth (shorter than 5 μm). After waiting for stable situation for 5 ~ 10 minutes, I measured the resonant frequency. **Figure 3-4** shows that the resonant frequency of bare silicon nanotweezers in the parylene microfluidic cavity filled with DI water. During 5000-second (1 hour and 23 minutes) measurement, the resonant frequency was kept at the stable value, around 1335.4 ± 0.05 Hz. By this result, we were sure that silicon nanotweezers can be used in DI water without any influence.

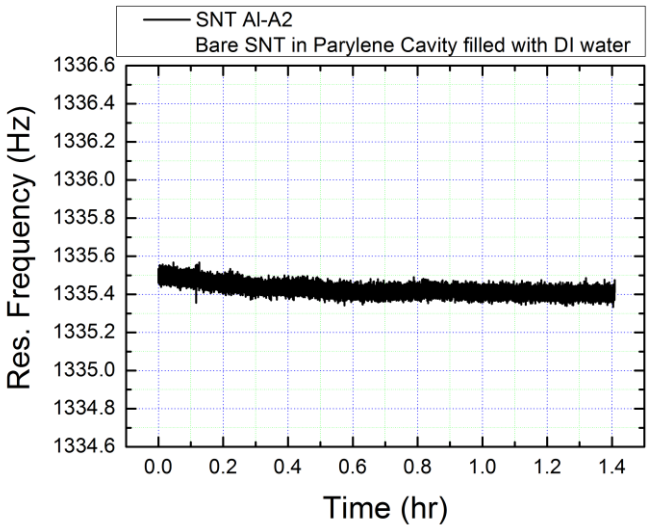


Figure 3-4 Bare silicon nanotweezers in parylene cavity filled with DI water

In addition to DI water, the case for buffer and enzyme solutions were also examined. The experiment procedure was the same as the experiment with DI water, which is presented in the last paragraph. I changed the solution to buffer and enzyme, and measured the resonant frequency of silicon nanotweezers. (Au E0, gap 8 μm) **Figure 3-5** shows the resonant frequency of bare silicon nanotweezers in buffer, which is stable enough. It also proves that silicon nanotweezers are not influenced by buffer. In addition, **Figure 3-6** shows that the resonant frequency of bare silicon nanotweezers (Au-D3, gap 11 μm) in HindIII enzyme solution is stable during 30-minute-long measurement. These results prove that buffer and enzyme do not influence silicon nanotweezers

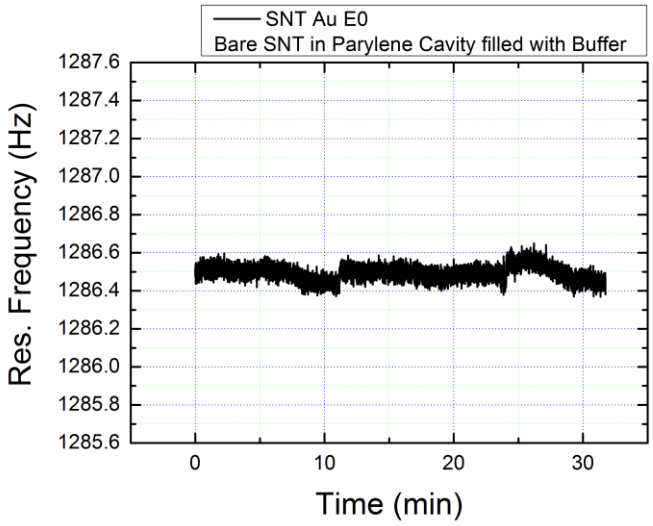


Figure 3-5 Bare silicon nanotweezers in parylene cavity filled with buffer

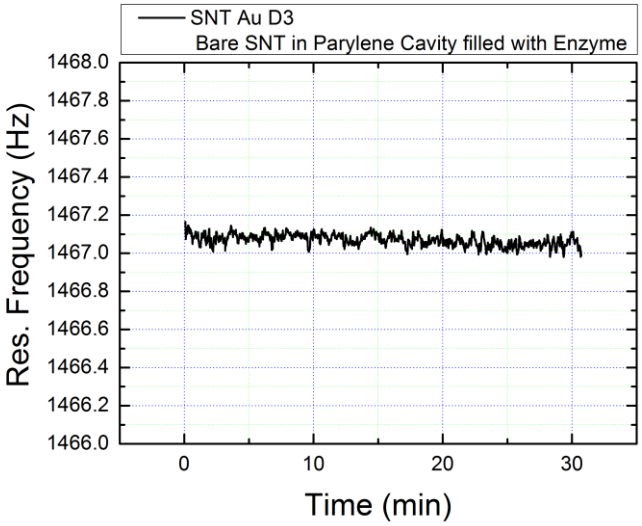


Figure 3-6 Bare silicon nanotweezers in parylene cavity filled with enzyme

3.2.2 DNA in Parylene Microfluidic Cavity

As was discussed before, it is not easy to get stable resonant frequency response of bare silicon nanotweezers in DI water and buffer. Therefore, it is definitely more difficult and complicated to get stable resonant frequency response of silicon nanotweezers with a DNA bundle on them. Based on my experience in experiments, several ways and some tips to trap the DNA bundle by silicon nanotweezers have been presented in Chapter 3.1.4 and 3.1.5. Here I present the resonant frequency characteristics of silicon nanotweezers with DNA in the parylene microfluidic cavity filled with DI water and buffer solution. We hope to see the resonant frequency can keep at a stable value because theoretically DI water and buffer do not influence on DNA.

Case in DI water

To make our experiments closer to the reality, it is necessary to do experiments in liquid because DNA exists naturally in solution. In this experiment, first I prepared the parylene microfluidic cavity filled with DI water (40 μL). It is necessary to wait 10 ~ 20 minutes for establishing the stable meniscus surface of the solution filled in the cavity. During the time waiting for the stable meniscus, I trapped DNA by silicon nanotweezers (Au-E2, gap 8 μm), following the procedure of DNA trapping as written in chapter 3.1.5, and put DNA into the parylene microfluidic cavity filled with DI water with very small insertion depth (lower than 5 μm). After waiting for stable situation for at least 10 minutes, I measured the resonant frequency, which is shown in **Figure 3-7**. At the beginning of insertion the resonant frequency is not so stable during the first 10 minutes, and it may be because of the unstable meniscus surface of DI water. After that, the resonant frequency started to stay at 1291.6 ± 0.05 Hz stably, which means the DNA bundle and the meniscus surface of DI water is in a stable condition.

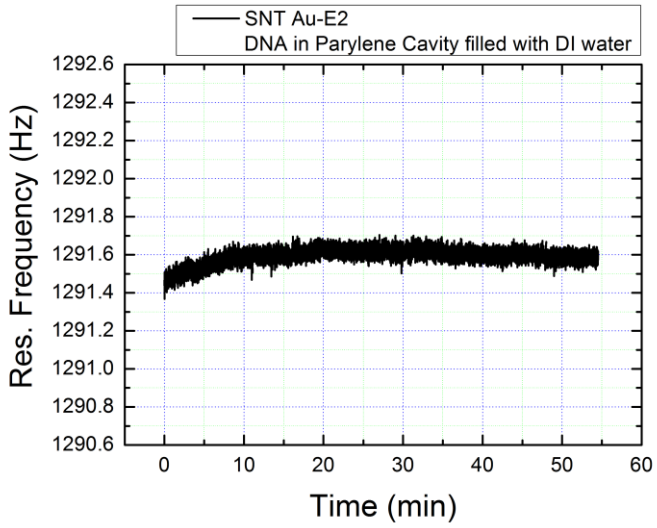


Figure 3-7 DNA in parylene cavity filled with DI water

As mentioned in Chapter 2.1.6, the stiffness of a single λ-DNA molecule is approximately 3×10^{-5} N/m in solution, and I used this reference value and the oscillator model of the silicon nanotweezers system to calculate the stiffness of the trapped DNA bundled and the equivalent number of DNA molecules trapped by tweezers in solution. Usually, the number of DNA molecules in the bundle is presumed, monitored and analyzed during experiments with the measurement in solution. The stiffness of the DNA bundle and the number of DNA molecules are calculated and shown in **Figure 3-8**. The average number of DNA molecules is 1900.

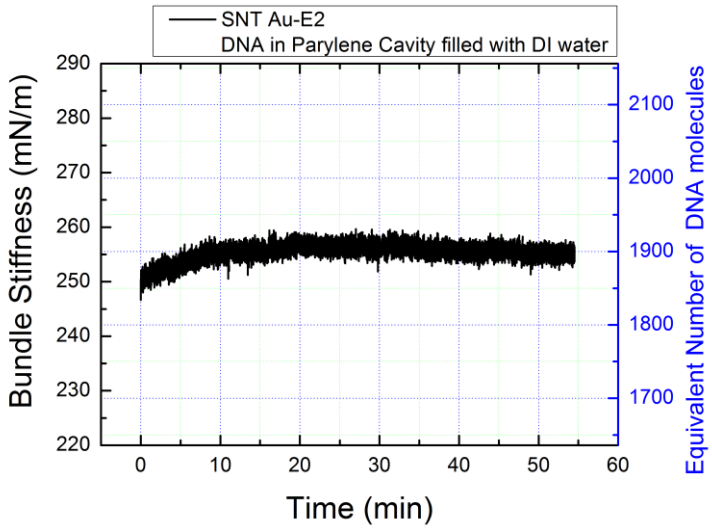


Figure 3-8 Bundle stiffness of DNA and number of DNA molecules in parylene cavity filled with DI water

Case in buffer

After the case in DI water, I started to try to use buffer, which is used to dilute enzyme, and used as the solution in the experiment. We want to make sure that only buffer (without any enzyme) does not cut or digest DNA bundle, as the control experiment for HindIII restriction enzyme reaction on DNA experiment. As I mentioned in chapter 3.1.4, I suggest using silicon nanotweezers deposited with gold instead of tweezers deposited with aluminum in the experiment with buffer and enzyme. In this control experiment, I use the gold-deposited silicon nanotweezers (Au-E2, gap 8 μm) to trap DNA bundle. Similar to the experiment procedure for the case in DI water, first I insert buffer (40 μL) into the parylene microfluidic cavity. During waiting for the meniscus surface of buffer becoming stable, I trapped DNA by adding AC voltage for 1 MHz, 8 V_{pp} for 2 minutes. After DNA trapping, I used a DI water droplet to wash the tips to remove the badly-attached DNA molecules, and then I insert the tips of nanotweezers with DNA bundle into the parylene cavity filled with buffer. **Figure 3-9** shows that during the 35-minute measurement, the resonant frequency kept at 1296.6 Hz stably, and which means that DNA did not react with the buffer.

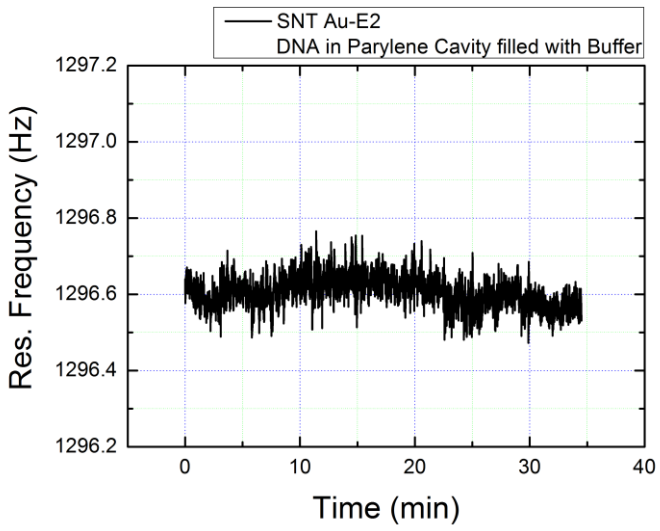


Figure 3-9 DNA in parylene cavity filled with Buffer

3.3 Discussion

I have presented how I improve the measuring system and some experiments to verify. I have improved the microfluidic cavity, and introduced the parylene microfluidic cavity. In addition, I could determine the best position for insertion in 3-D by sensing the amplitude of resonant frequency, and also established an optimized DNA trapping procedure. The experiment results show that we successfully kept the resonant frequency of bare silicon nanotweezers at a stable level in DI water, buffer and enzyme, and I proved that the frequency was stable over time, meaning there is no influence on bare silicon nanotweezers from DI water, buffer, and enzyme. Furthermore, I measured the resonant frequency of DNA bundle with silicon nanotweezers in DI water and buffer, and also proved that there is no influence on DNA bundle from DI water and buffer. Practically, it is still not easy enough to get the stable resonant frequency response of silicon nanotweezers with a DNA bundle. Fortunately, due to the improvement of measuring system, the stability problem has been reduced much. Thanks to the improvement of experiment system, we can start to measure the DNA degradation caused by restriction enzyme and radiation. These measuring experiments of DNA degradation are presented in Chapter 4 and 5.

4. SNT Monitoring Capability of DNA Degradation

By experimental confirmation of the stability of resonant frequency response, I proved the silicon nanotweezers (SNT) can be used in measurement in solution. The next step is to prove our SNT can measure DNA degradation in solutions in real time. In this Chapter, I describe the method for the kinetic characterization of bio-reactions on DNA with silicon nanotweezers. The experiments using HindIII restriction enzymes, which have the ability to cut DNA strands, are performed.

4.1 Experiment Procedure

Figure 4-1 shows the experimental process and idea of this research. Before measuring DNA degradation, we need to make sure that our silicon nanotweezers are not influenced by DI water, buffer, enzyme, (which are shown in Chapter 3.2) and X-ray (which is shown in Chapter 5.2). Before the x-ray experiment, we used HindIII enzyme, which is well understood and established for the function of cutting DNA strands, to do experiment (shown in Chapter 4). We want to prove that our system can measure DNA degradation in solution and in real-time by HindIII enzyme experiments.

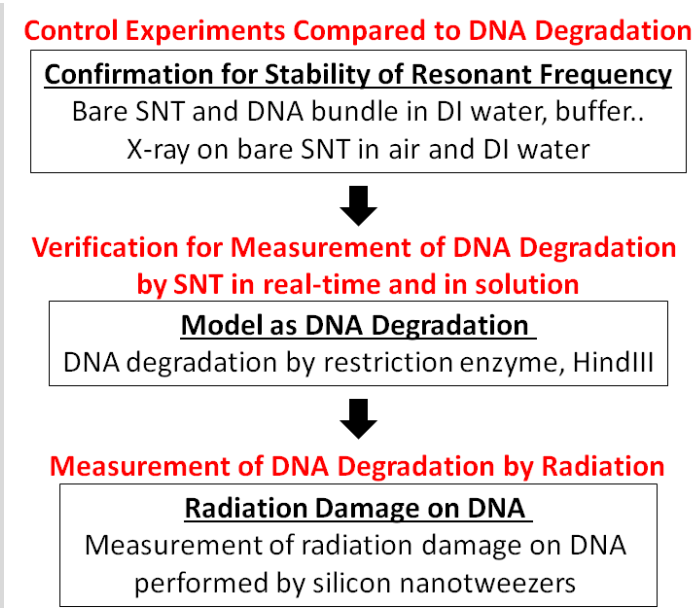


Figure 4-1 The experimental process and idea of this research

The experiment procedure is similar to the procedure of the experiments I presented in Chapter 3.2. To make it clearly, I present the experiment procedure as follows:

Control experiments

1. Bare silicon nanotweezers in buffer
   (To prove that buffer has no influence on bare silicon nanotweezers)
2. Bare silicon nanotweezers in enzyme
   (To prove that enzyme has no influence on bare silicon nanotweezers)
3. Trapped DNA with silicon nanotweezers in buffer
   (To prove that pure buffer has no influence on DNA)

The procedure of these three experiments has already been presented in Chapter 3.2.

HindIII Enzyme Experiment

The steps of the HindIII Enzyme experiment are as follows:

1. Insert bare silicon nanotweezers into the empty and clean parylene microfluidic cavity, and sense the best position for insertion (described in Chapter 3.1.3). Remember that you have to do position sensing without DNA bundle betweens the tips of tweezers.
2. Insert enzyme (40 μL) into parylene microfluidic cavity, and wait 10 minutes for the stable meniscus surface of enzyme solution in cavity.
3. During the waiting time, put a DNA droplet (10 μL) on the upper surface of microfluidic cavity, and trap DNA by the procedure presented in Chapter 3.1.5. After trapping, measure the resonant frequency of the silicon nanotweezers with a DNA bundle in air and take the picture of the DNA bundle.
4. After DNA is trapped by nanotweezers, insert nanotweezers into the microfluidic cavity, the insertion depth is 10 μm.
5. Monitor the change of resonant frequency, which shows the restriction enzyme HindIII reaction on DNA, for 30 minutes.
6. Take silicon nanotweezers out from enzyme, measure the resonant frequency of DNA bundle in air again, take a picture of residual DNA bundle, and then immerse nanotweezers into DI water droplet as quickly as possible. Otherwise the enzyme solution may be dry and some residue

may attach on the nanotweezers. The time for immersion of tweezers into DI water should be at least 1 minute.

7. Blow out DNA bundle by air blower, and measure the resonant frequency of silicon nanotweezers to make sure that DNA has been moved clearly.

4.2 HindIII Enzyme Cutting Reaction on DNA

Here I presented the measurement of restriction enzyme (HindIII) cutting reaction on DNA. HindIII is an enzyme used to cut DNA, and I try to prove that silicon nanotweezers can be used to measure the DNA degradation by these experiments.

4.2.1 Introduction to HindIII

HindIII enzyme is a type II site-specific deoxyribonuclease restriction enzyme that cleaves the double stranded DNA at a specific nucleotide sequence (AAGCTT). There are DNA restriction enzymes that play important roles in bacteria to protection it against viruses. They destroy viral DNA by cutting DNA in specific sites, and prevent insertion and transcription in bacterial DNA. They have been already been widely used by biologists and biochemists for several applications. [46, 47]

HindIII enzyme was purchased from New England BioLabs Inc (http://www.neb.com). HindIII was dissolved with appropriate buffer solution, and diluted with DI water. The HindIII restriction enzyme cuts (digests) λ-DNA in 7 restriction sites per molecule.

In this new development, I immersed a DNA bundle trapped by silicon nanotweezers in a solution containing HindIII enzyme. The goal is to characterize the kinetic of the interactions between the restriction enzymes, HindIII, and the DNA molecules. We hope we can do the real-time measurement of the digestion of the DNA bundle by monitoring the decrease of the resonant frequency of silicon nanotweezers, from which the mechanical parameters of the DNA bundle can be calculated

4.2.2 Control Experiment in Buffer and Enzyme

Before measuring the digestion of the DNA bundle by HindIII enzyme, we need to do some control experiments to get the stable resonant frequency response in two cases: "bare silicon nanotweezers in buffer" (to make sure that silicon nanotweezers are not influenced by buffer solution) and "DNA trapped by silicon nanotweezers in buffer" (to make sure that buffer does not react with DNA bundles without enzyme.) We hope the resonant frequency in these two cases can stay at a stable value for at least 30 minutes, which is also the measuring time for the enzyme experiment.

Bare silicon nanotweezers in buffer

First, I inserted bare silicon nanotweezers (Au-E2, gap 8 μm) into the buffer solution, and measured the resonant frequency of silicon nanotweezers. The insertion depth was 10 μm and the best position was sensed as presented in Chapter 3.1.3. The result is shown in **Figure 4-2.** During 35-minute experiment, the resonant frequency stayed at around 1293.7 ± 0.1 Hz stably; It means these silicon nanotweezers can stand for being immersed in the buffer solution.

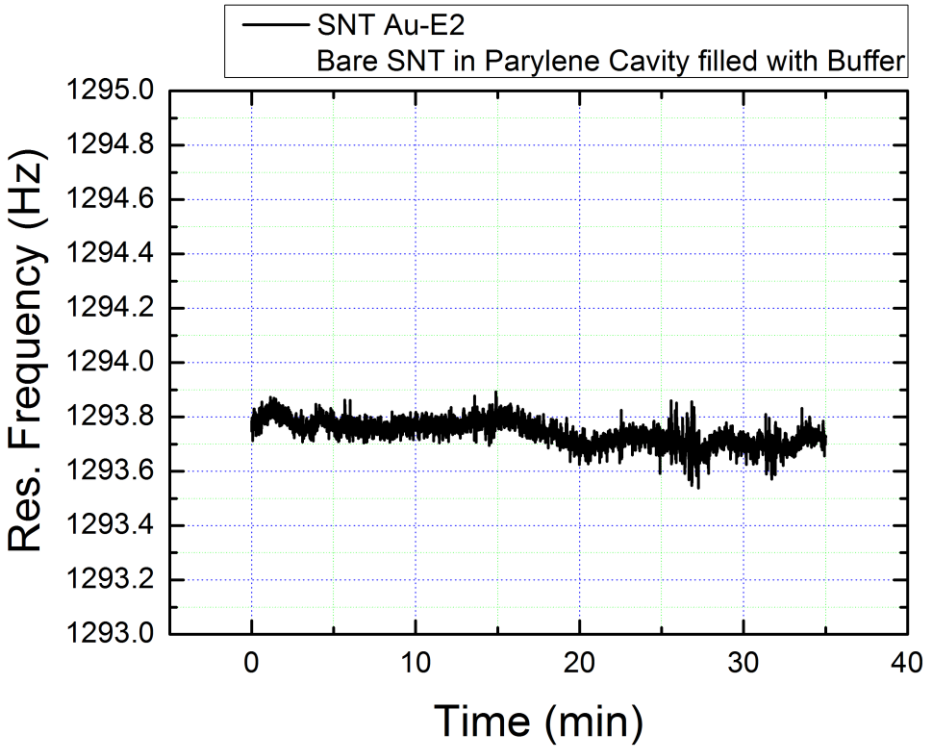


Figure 4-2 Bare silicon nanotweezers in parylene cavity filled with buffer

Bare silicon nanotweezers in enzyme

Second, I inserted bare silicon nanotweezers (Au-B2, 8.5 μm) into the enzyme solution. This is to prove that enzyme does not influence silicon nanotweezers. The insertion depth is 10 μm. **Figure 4-3** shows the results of this experiment. During 40-minute experiment, the resonant frequency stayed at around 1359.1 ± 0.03 Hz stably, which means there is no influence on silicon nanotweezers by enzyme.

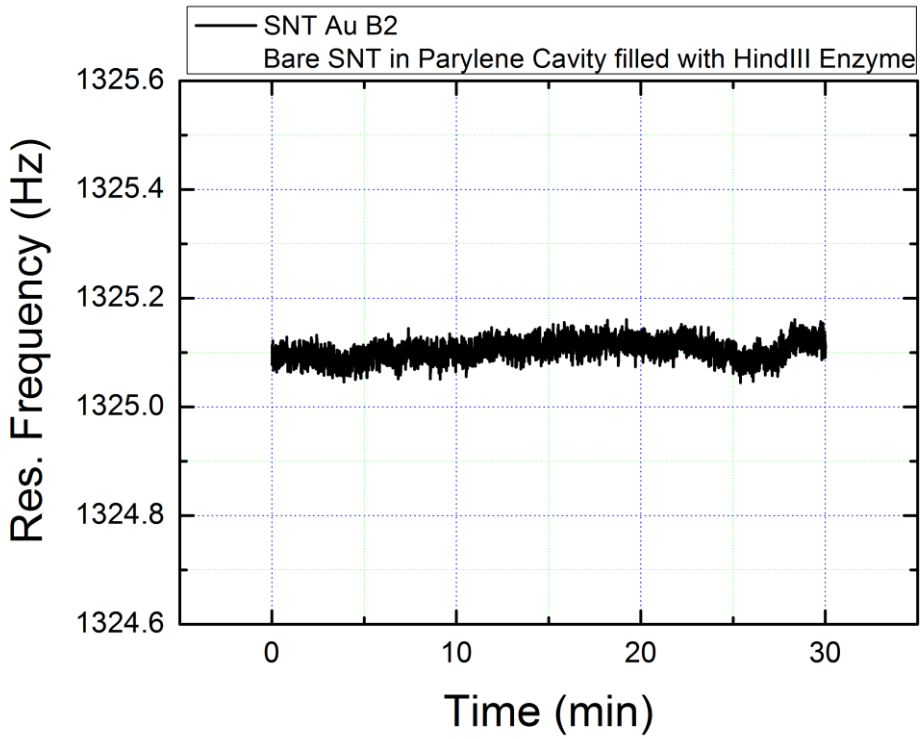


Figure 4-3 Bare silicon nanotweezers in parylene cavity filled with enzyme

Trapped DNA with silicon nanotweezers in buffer

Third, I inserted silicon nanotweezers with a DNA bundle into the buffer solution. The DNA bundle was trapped by dielectrophoresis, following the procedure as previously described in Chapter 3.1.5. The tips of nanotweezers are brought to the surface of the DNA solution (droplet) on the surface of microfluidic cavity and an AC voltage was applied between the tips (1 MHz, 8 V_{pp}) during 2 minutes.

Figure 4-4 shows the result of the experiment. During 35-minute experiment, the resonant frequency stayed at around 1296.6 ± 0,05 Hz stably, and is 2.9 Hz higher than the resonant frequency in the case of "bare silicon nanotweezers in buffer." This 2.9 Hz difference is due to the DNA bundle, and we could calculate the stiffness of this DNA bundle and the equivalent molecule number of DNA. The result shows the DNA bundle was not influenced in the solution that is only buffer and without enzyme.

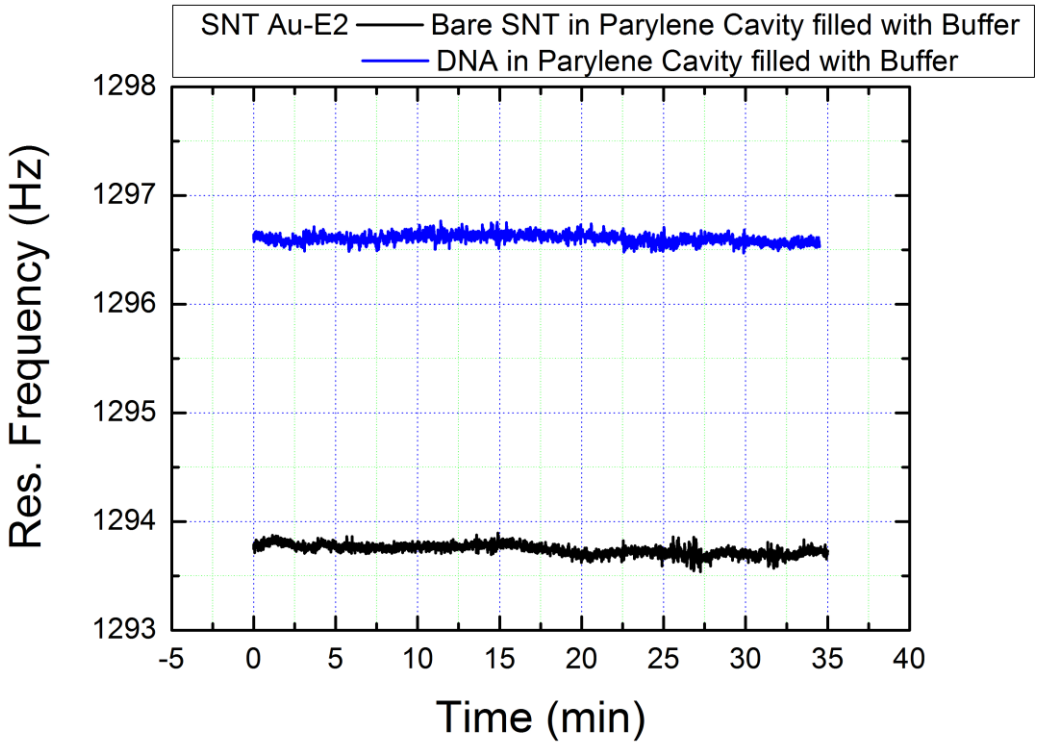


Figure 4-4 DNA in parylene cavity filled with buffer

4.2.3 HindIII Enzyme Experiment

By two control experiments, i.e. "bare silicon nanotweezers in buffer" and "DNA trapped by silicon nanotweezers in buffer," we have already proved that silicon nanotweezers and DNA are not influenced by DI water and buffer. Here I present the measurement result of HindIII enzyme digestion of a DNA bundle. The silicon nanotweezers I used in this experiment are deposited with gold and have the gap of 8.5 μm. I did the experiment with procedure presented in Chapter 4.1. In this experiment, the concentration of HindIII enzyme solution is 8 units (1 unit is defined as the amount of enzyme required to digest 1 μg of λ DNA in 1 hour at 37°C in a total reaction volume of 50 μL), and the volume of the enzyme solution in a parylene microfluidic cavity is 40 μL. By calculation I estimated that 1000 DNA molecules may be digested by 2 unit concentrated enzyme in 8 minutes at 37°C. The result is shown in **Figure 4-5.** During 60-minute experiment, the resonant frequency decreased from 1293.8 Hz to 1290.4 Hz, and this decrease was because of the HindIII enzyme cutting reaction on the DNA bundle.

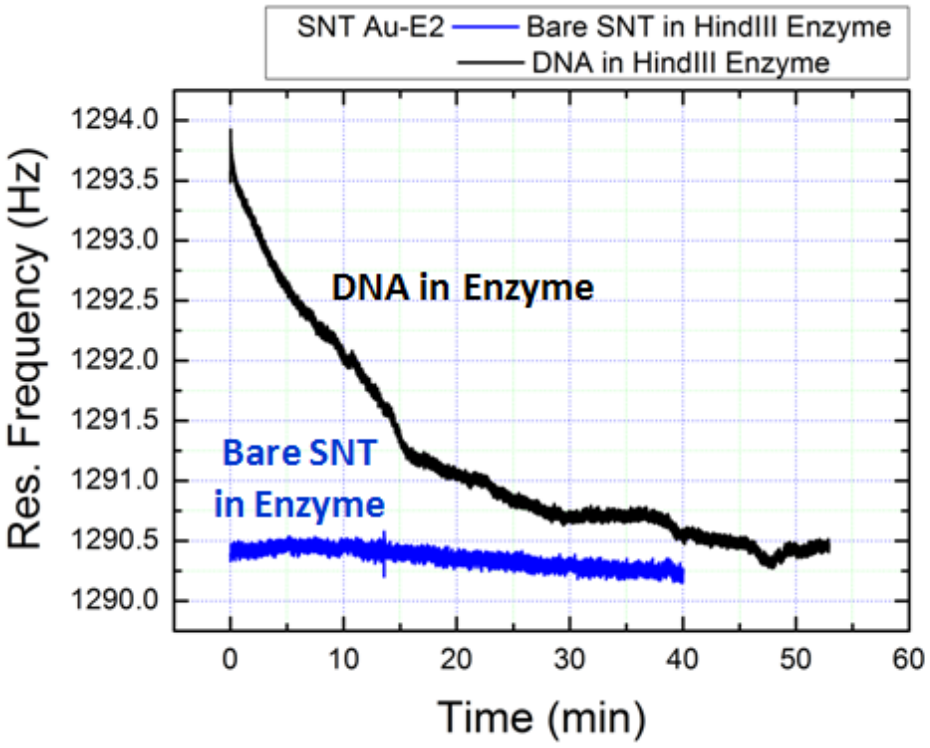


Figure 4-5

Blue curve: Bare silicon nanotweezers in parylene cavity filled with enzyme
Black curve: HindIII enzymatic reaction on DNA

By the calculation from resonant frequency and the oscillator model, the change of stiffness of DNA bundle and the change of the equivalent molecule number of DNA are shown in **Figure 4-6**. The number of DNA molecules decreased from 2200 to almost 0 in 50 minutes. The result shows most of DNA molecules were digested by HindIII Enzyme.

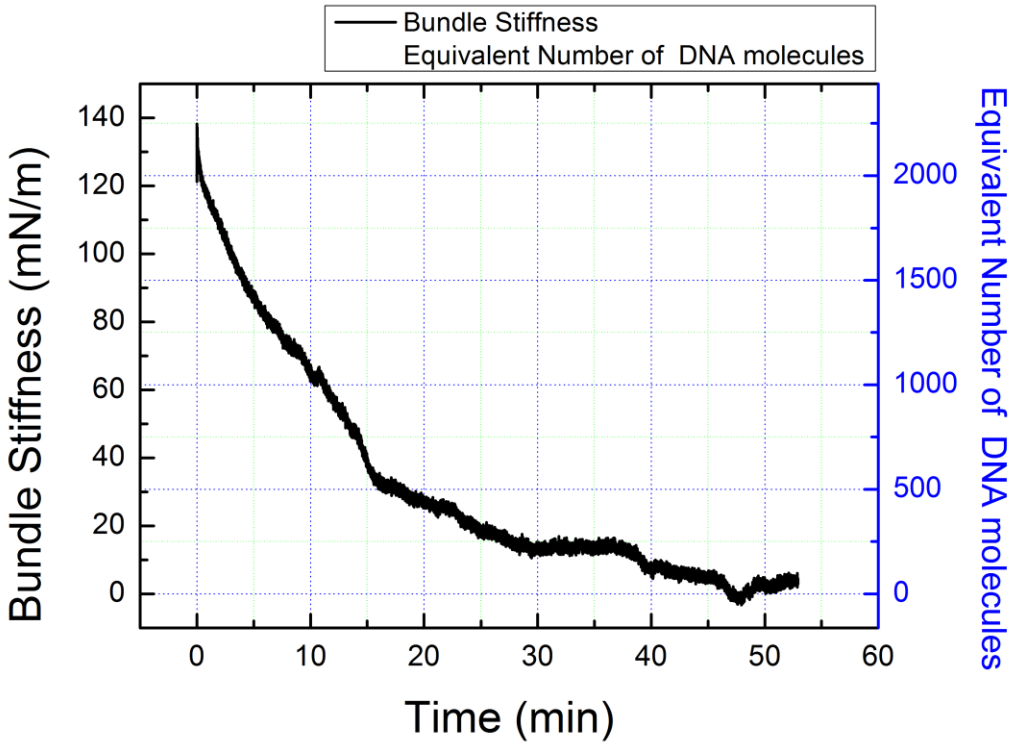


Figure 4-6

The decrease of the bundle stiffness of DNA bundle and the number of DNA molecules in HindIII enzyme

4.2.4 Discussion

The results show that HindIII restriction enzymatic reaction on DNA can be measured by silicon nanotweezers. Assume that the volume of HindIII enzyme for reaction is 10 μm cubed to 15μm cubed, the theoretical speed of digestion by 8-unit HindIII enzyme is 13 minutes to 43 minutes for 2200 DNA molecules, at 37°C.

Due to the information of HindIII enzyme, the reaction efficiency at 37 °C is 3 times faster than the reaction efficiency at 20 °C. Again, assume that the volume of HindIII enzyme for reaction is 10 μm cubed to 15μm cubed, the theoretical speed of digestion by 8-unit HindIII enzyme is 39 minutes to 129 minutes for 2200 DNA molecules, at 20 °C. In the experiment, the result is 50 minutes, at 20°C, which is in the range of theoretical value.

The HindIII restriction enzymatic reaction on DNA has already been widely-known and used in application. [44, 45] The initial purpose of this experiment is to prove that the silicon nanotweezers can be used for measurement of DNA degradation or other DNA bio-reaction experiments. We proved that it is possible to measure DNA degradation in real time and in solution by using silicon nanotweezers.

Based on these experiments and experience, I performed the X-ray damage on DNA experiments and present in Chapter 5.

5. Measurement of Radiation Damage on DNA

In this chapter, I present the real-time measurement of radiation damage on DNA by using silicon nanotweezers. We try to understand the bio-mechanical characteristic of DNA degradation due to radiation damage.

5.1 Experiment Procedure

The experiment procedure of radiation damage on DNA experiments is similar to the experiments I described in Chapter 3 and 4. The difference is that I shoot X-ray during the measurement. The radiation machine (Pantak HF350, 350 KV_p, 12 mA) is provided by Shimadzu Corporation (http://www.shimadzu.co.jp/). The dose of radiation is supplied 2.73 Gy per minute, and the distance from radiation source is 30 cm in our experiments.

The contents of experiments, including control experiments and radiation damage on DNA experiment, are as follows:

Control experiments

1. X-ray on Bare silicon nanotweezers in air
2. X-ray on Bare silicon nanotweezers in a parylene microfluidic cavity filled with DI water

Radiation damage on DNA experiment

1. X-ray on DNA in air
2. X-ray on DNA in parylene microfluidic cavity filled with DI water

Experiment procedure

As an example, the experiment procedure of X-ray on DNA in parylene microfluidic cavity filled with DI water is presented:

1. Insert bare silicon nanotweezers into the empty and clean parylene microfluidic cavity, and determine the best position for insertion (described in Chapter 3.1.3). Remember that you have to do position sensing without DNA bundle betweens the tips of tweezers.
2. Insert DI water into parylene microfluidic cavity, and wait 10 minutes for the stable meniscus surface of DI water in microfluidic cavity.
3. During the waiting time, put a DNA droplet (10 μL) on the upper surface of the microfluidic cavity, and trap DNA by the procedure presented in Chapter 3.1.5. After trapping, measure the resonant frequency of the silicon nanotweezers with DNA bundle in air.
4. After DNA is trapped by nanotweezers, insert nanotweezers into the microfluidic cavity, the insertion depth is 10 μm. (After touching the meniscus surface of solution, insert 10 μm deeper with very slow speed.)
5. Wait for the stable resonant frequency response (usually it takes 10 minutes ~ 20 minutes). If it is still no stable resonant frequency response, move tweezers 5 μm back then wait 10 minutes again. If it is still no stable resonant frequency response, move tweezers out from DI water, and repeat Step. 4.
6. Measurement of X-ray damage on DNA: After getting stable resonant frequency response, confirm the stable resonant frequency over 5 ~10 minutes, and then shoot X-ray for 5 minutes (total dose: 13.65 Gy), and then keep recording the resonant frequency for 5 ~ 10 minutes after irradiation to confirm the stability. Monitor the change of resonant frequency. The resonant frequency should decrease during the irradiation.
7. Take silicon nanotweezers out from DI water, measure the resonant frequency of the DNA bundle in air again, and then immerse nanotweezers into DI water droplet for cleaning. The time for immersion of tweezers into DI water should be at least 1 minute.
8. Blow out DNA bundle by an air blower, and measure the resonant frequency of silicon nanotweezers to make sure that DNA has been removed completely.

5.2 Control Experiment for Radiation on Silicon Nanotweezers

Before measuring the radiation damage on DNA, we have to make sure that our measuring tools, silicon nanotweezers, are not influenced by radiation.

X-ray on bare silicon nanotweezers in air

In this experiment, I shot radiation on bare silicon nanotweezers (Al E4, 8 μm) in air, and measure the resonant frequency by phase-locked loop measurement. The radiation time was 11 minutes and the total radiation dose is 30 Gy. **Figure 5-1** shows that before, during and after the irradiation, the resonant frequency stays at around 1364.2 ± 0.25Hz stably, which means that the radiation did not influence silicon nanotweezers in air.

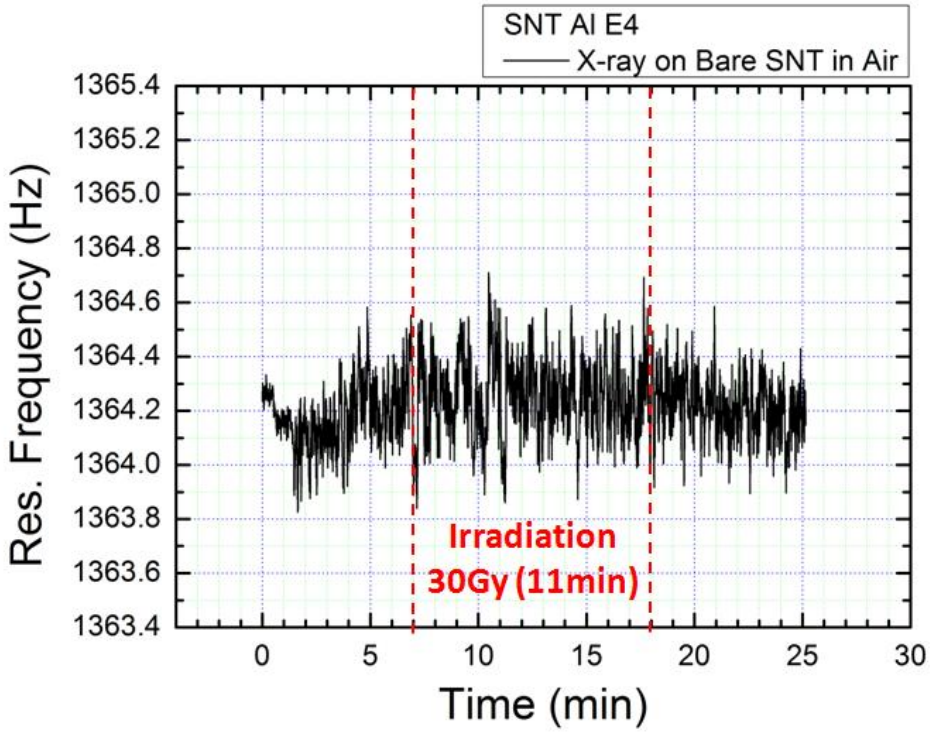
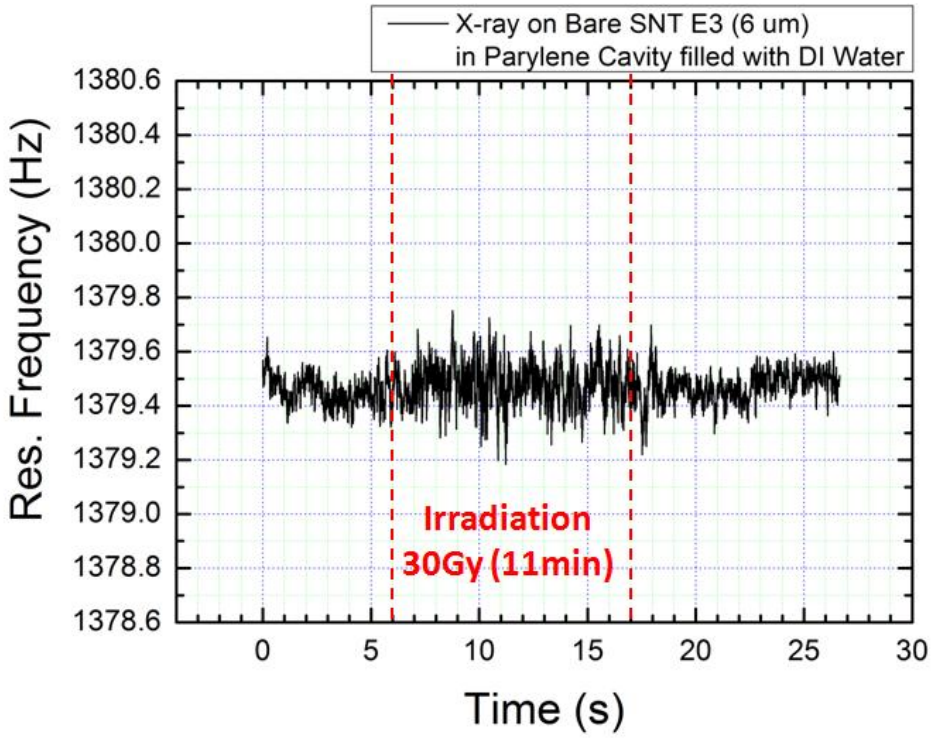


Figure 5-1 X-ray on bare silicon nanotweezers in air

X-ray on Bare silicon nanotweezers in parylene microfluidic cavity filled with DI water

X-ray excites water molecules and generates free radicals, and these radicals may influence silicon nanotweezers (Al-E3 gap 6 μm). To make sure that silicon nanotweezers can resist free radicals, we have to do the control experiment in DI water. In this experiment, I inserted silicon nanotweezers into a parylene microfluidic cavity filled with DI water, and then shot radiation on nanotweezers. The radiation time and dose are the same as before, which are 11 minutes and 30 Gy. **Figure 5-2** shows that the resonant frequency of bare silicon nanotweezers is kept stable during the irradiation even in DI water. The result shows that the resonant frequency was stable at around 1379.5 ± 0.15 Hz before, during, and after the irradiation. The results show that the silicon nanotweezers are not influenced by radiation, even in DI water.

Figure 5-2 X-ray on bare silicon nanotweezers in DI water



By these two results of control experiment, we prove that silicon nanotweezers are not influenced by radiation in air and in DI water.

5.3 Radiation Damage on DNA in Air

After two control experiment to prove silicon nanotweezers can stand for the irradiation, I started to do the "radiation damage on DNA" experiment. First I performed an initial experiment to make sure that our system is capable to measure the radiation damage on DNA, in air condition. Before irradiation, I trapped a DNA bundle by silicon nanotweezers. (Al-D0, gap 8μm). The DNA bundle was trapped by dielectrophoresis following the procedure presented in Chapter 3.1.5, and the AC voltage was applied between the tips (1 MHz, 8 V_{pp}) during 2 minutes. After trapping I waited 5 minutes, and then shot radiation on the DNA bundle with total dose of 30 Gy in 11minutes, and then waited 5 minutes. The result is shown in **Figure 5-3**. At the beginning, the resonant frequency was stable, and then the resonant frequency decreased from 1512 ± 0.5 Hz to 1509 ± 0.5 Hz during the irradiation. This means the DNA was damage by the radiation. However, after the irradiation, the resonant frequency increased. This increase may be caused by the drying effect of DNA bundle.

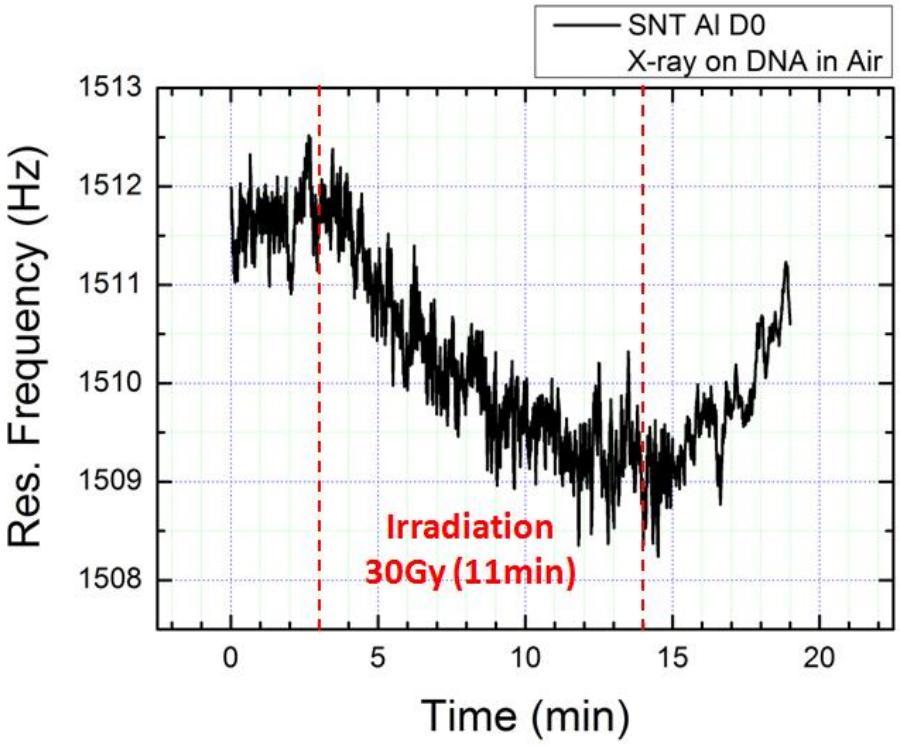


Figure 5-3 X-ray on DNA in air

5.4 Radiation Damage on DNA in DI Water

As mentioned before, DNA naturally exists in solution. To make our experiments much closed to the reality, it is better to measure the radiation damage on DNA in solution. Also, the radiation does not damage DNA directly but generate radicals in H_2O to hit and damage DNA strands. Therefore, radiation damages DNA in solution more easily than in air.

In Chapter 5.4, I present three experiments for the measurement of radiation damage on DNA in DI water by using silicon nanotweezers. Three of the experiments were all done with the same radiation dose (2.73 per minute, totally 13.65 Gy) and the same radiation time (5 minutes). The first experiment is done by using 8-μm-gap silicon nanotweezers with aluminum deposited, while the second and the third experiments were done by using 5 -μm-gap silicon nanotweezers.

First Experiment

To do this experiment, I trapped DNA by following the procedure presented in Chapter 3.1.5. Then, I put the DNA bundle into the parylene microfluidic cavity filled with DI water. The insertion depth of silicon nanotweezers (Al-E3, gap 6 μm) in DI water was 10 μm. After the insertion, I waited for the stable resonant frequency response for about 10 ~ 30 minutes. After I got stable resonant frequency response for several minutes, I started to shoot radiation on DNA. The result, shown in **Figure 5-4**, shows that the resonant frequency was stable at 1380.6 ± 0.03 Hz before irradiation, decreased from 1380.6 Hz to 1380.25 Hz during the irradiation, and was kept stable at 1380.25 ± 0.03 Hz again after irradiation. The resonant frequency of bare silicon nanotweezers is about 1379.8 ± 0.02 Hz in air, and 1379.5 ± 0.05 Hz in DI water. The comparison between real and control experiments is shown in **Figure 5-5**.

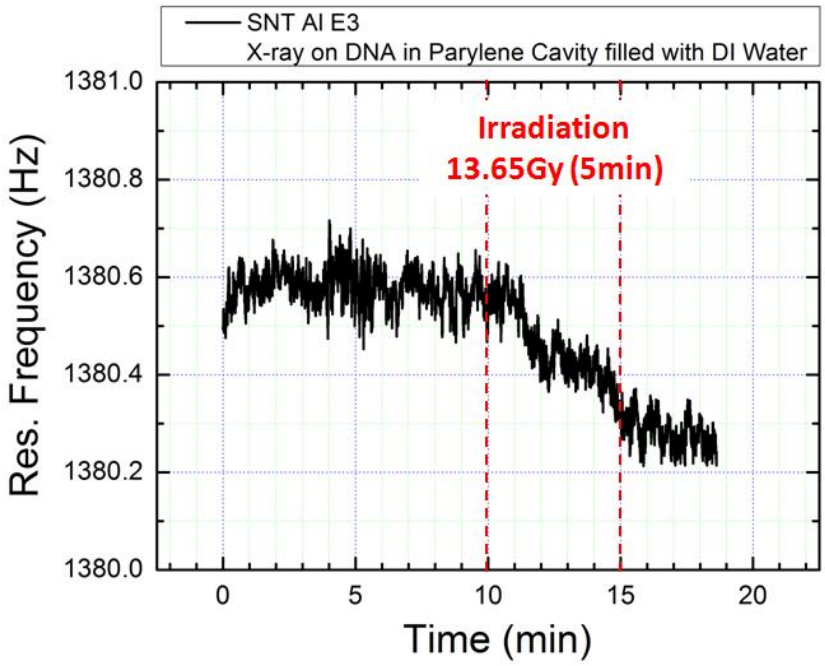


Figure 5-4 1st experiment for X-ray on DNA in DI water

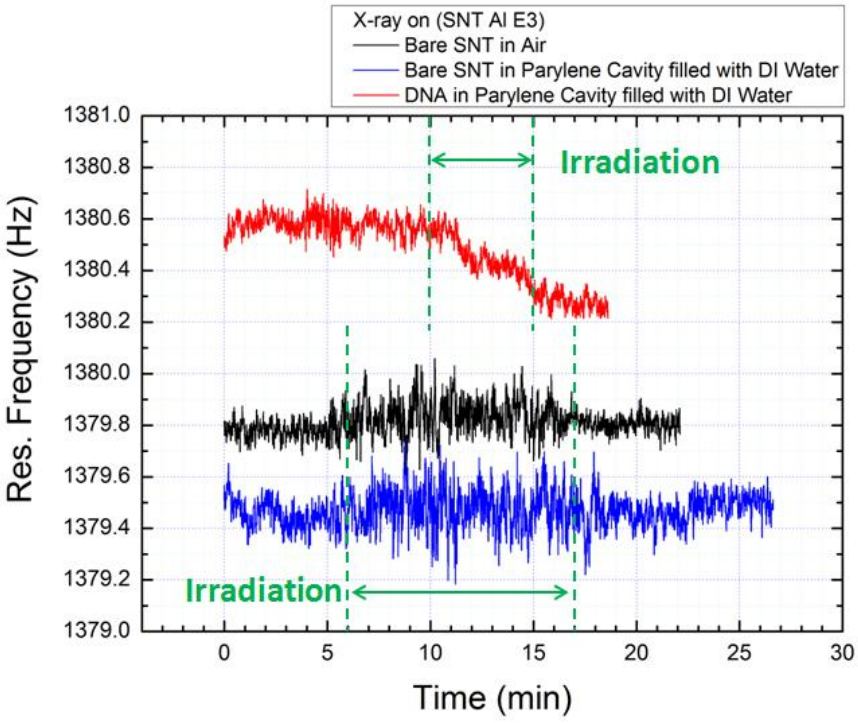


Figure 5-5 Comparison of real and control experiments for the 1st run
Black curve: X-ray on bare silicon nanotweezers in air
Blue curve: X-ray on bare silicon nanotweezers in DI water
Red curve: X-ray on DNA in DI water

We calculate DNA bundle stiffness and number of DNA molecules by the oscillator model. **Figure 5-6** shows the results that the number of DNA molecules decreased from 525 to 375, In other words, about 28.6% of DNA molecules (150 DNA molecules) were damaged by radiation.

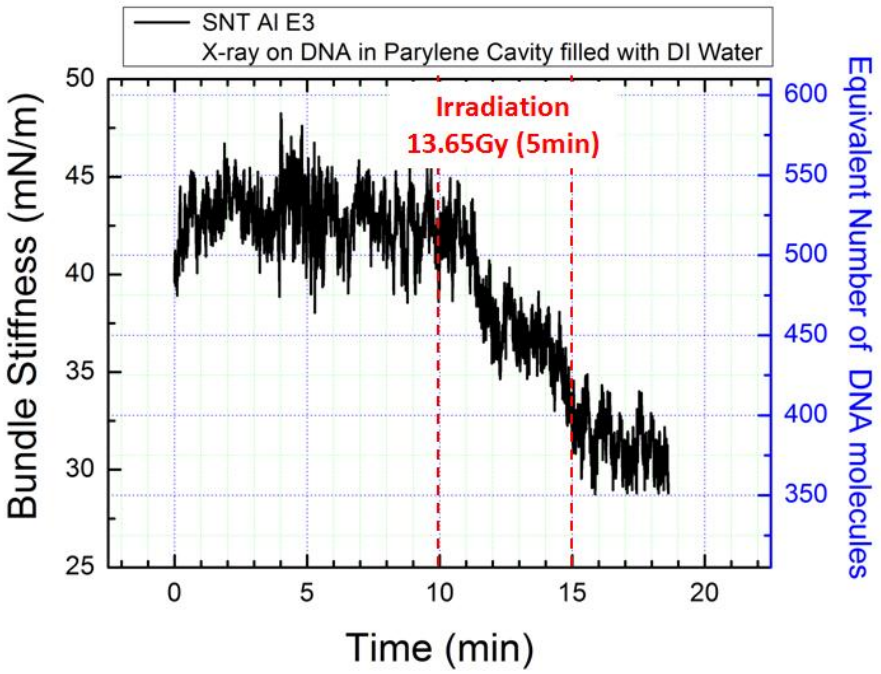


Figure 5-6

The decrease in the bundle stiffness of DNA and
the number of DNA molecules by irradiation in 1st experiment

Second Experiment

In this experiment, I followed the same experiment procedure as Chapter 5.1. Compare to the 1st experiment, the gap of silicon nanotweezers is smaller. (5 μm for 2nd experiment and 8 μm for 1st experiment), and the DNA bundle was much thicker than the bundle trapped in 1st experiment. I waited 6 minutes for the stable resonant frequency response, and then shot X-ray (totally 13.65 Gy, 5 minutes) on DNA in parylene microfluidic cavity filled with DI water. **Figure 5-7** shows the results of 2nd experiment. The resonant frequency was stable at 1409.85 Hz before irradiation, decreased from 1409.85 Hz to 1409.6 Hz during the irradiation, and was stable at 1409.6 Hz after irradiation.

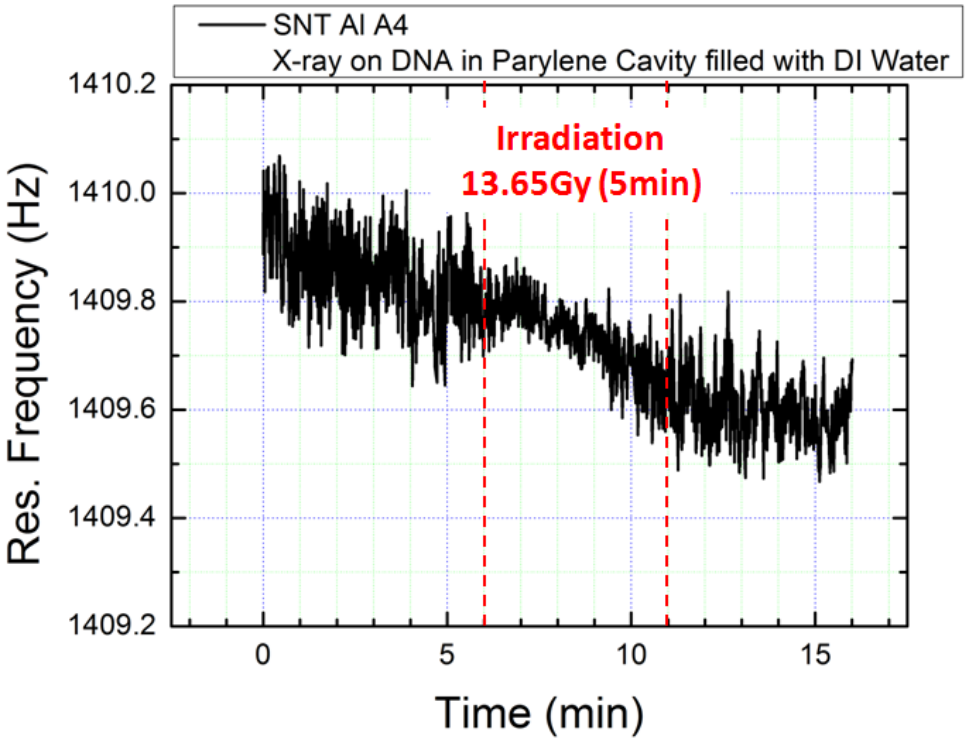


Figure 5-7 2nd experiment for X-ray on DNA in DI water

I calculate DNA bundle stiffness and number of DNA molecules by the oscillator model. **Figure 5-8** shows the results that the number of DNA molecules decreased from 8750 to 8625, In other words, about 1.4% of DNA molecules (125 DNA molecules) were damaged by radiation.

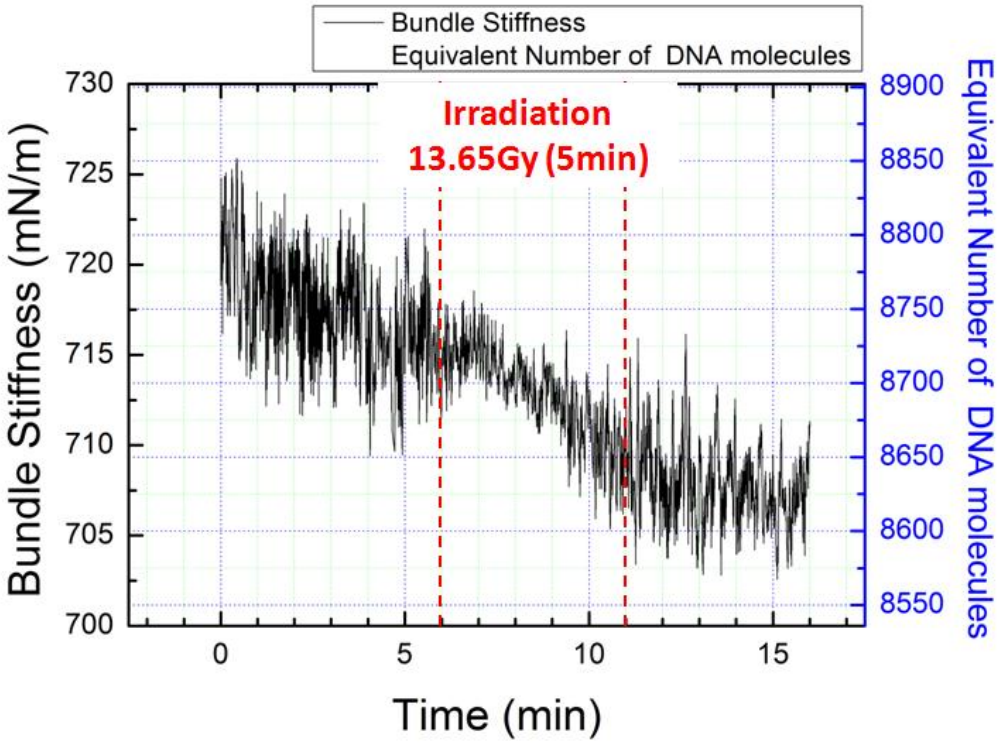


Figure 5-8

The decrease in the bundle stiffness of DNA and
the number of DNA molecules by irradiation in 2nd experiment

Third Experiment

I did the 3rd experiment with the same silicon nanotweezers and experiment procedure as the 2nd experiment. The difference between the 2nd experiment and the 3rd experiment is the DNA bundle in the 3rd experiment is much thinner than the DNA bundle in the 2nd experiment. I waited 5 minutes for the stable resonant frequency response, and then shot X-ray (totally 13.65 Gy, 5 minutes) on DNA in the parylene microfluidic cavity filled with DI water. **Figure 5-9** shows the results of the 3rd experiment. The resonant frequency was stable at 1389.55 ± 0.02 Hz before irradiation, decreased from 1389.55 Hz to 1389.45 Hz during the irradiation, and kept stable at 1389.45 ± 0.05 Hz after irradiation.

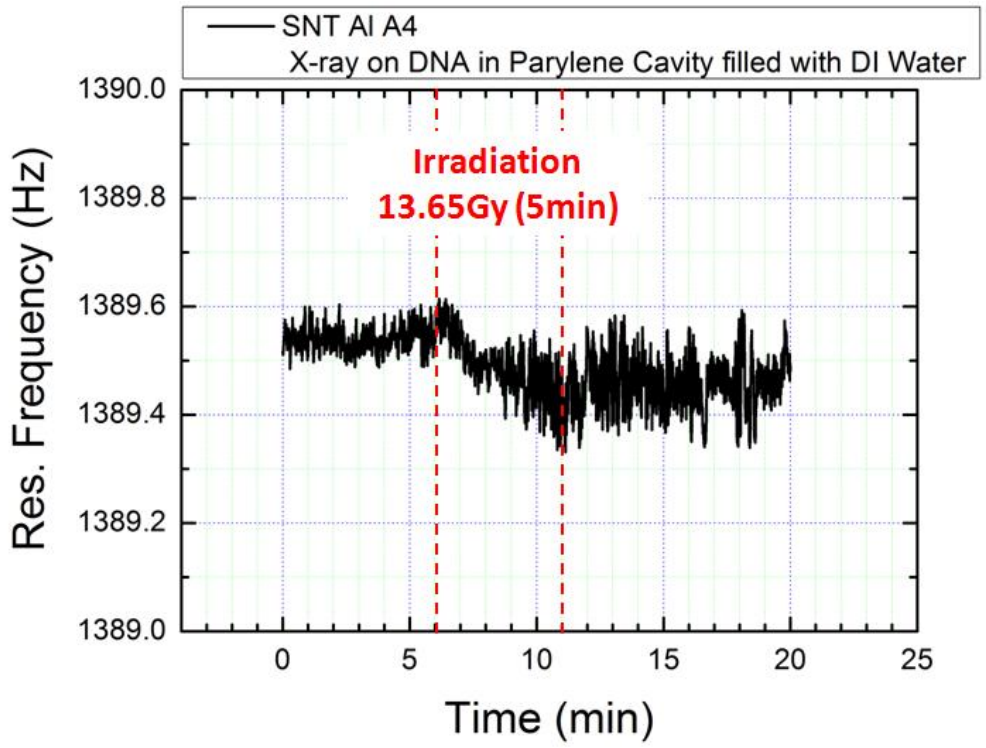


Figure 5-9 3rd experiment for X-ray on DNA in DI water

The DNA bundle stiffness and number of DNA molecules are shown in **Figure 5-10**. The results show that the number of DNA molecules decreased from 400 to 350, In other words, about 12.5% DNA molecules (50 DNA molecules) were damaged by radiation.

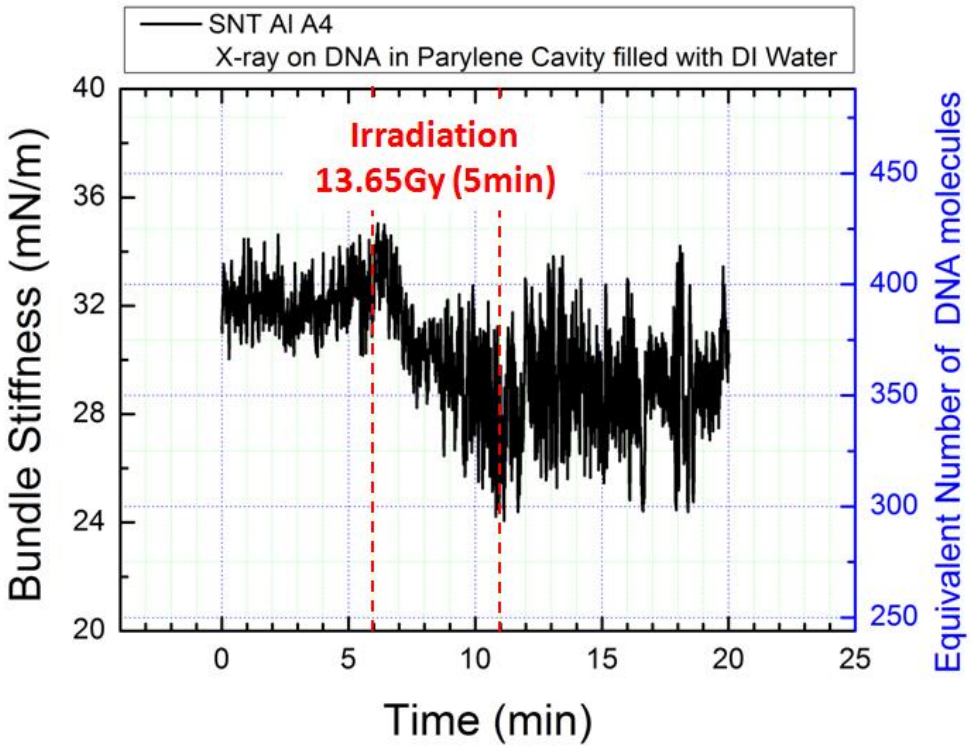


Figure 5-8

The decrease in the bundle stiffness of DNA and
the number of DNA molecules by irradiation in 3rd experiment

5.5 Discussion

I tried to compare the three experiments of X-ray on DNA in DI water. **Table 5-1** shows the comparison between the results of experiments.

	1st experiment	2nd experiment	3rd experiment
Gap of SNT	8μm	6μm	6μm
RF of bare SNT	1378.539 Hz	1391.140 Hz	1391.172 Hz
RF of DNA in Water	1380.6 Hz	1409.85 Hz	1389.55 Hz
RF of DNA in Air	1520.737 Hz	2342.112 Hz	1471.912 Hz
Number of DNA	525	8750	400
Reduction of DNA	150	125	50
Reduction of Stiffness	11.5 mN/m	10 mN/m	4 mN/m
Reduction (%)	28.4 %	1.4 %	12.5 %
Radiation per 1 DNA molecule	0.026 Gy	0.00162 Gy	0.0341 Gy

Table 5-1 Compare between three experiments for X-ray on DNA in DI water

Due to our hypothesis, the reduction of number of DNA is related to the dose of radiation. The dose of radiation is all 13.65 Gy in three experiments, however, only the reduction of number of DNA in 1st experiment and 2nd experiments are close (150 and 125) while the reduction in 3rd experiment is much smaller (50).

Undoubtedly, it is necessary to do more experiments with different condition. Actually, I tried several difference conditions. For example, change the dose of radiation to 27.30 Gy (twice of the radiation dose in previous experiment) with 5-minute radiation time. For another example, change the radiation time to 10 minutes (twice of the radiation dose in previous experiment) with 13.65 Gy. However, many experiments for radiation damage on DNA were not successful because the problem of stability of resonant frequency response still obstructed us. Therefore, I do not have enough data for radiation experiments. By only three experiment data, it is hard to see the reproducibility of experiments.

6. Discussions

I have already performed several experiments for measuring DNA degradation by using silicon nanotweezers. In fact, the number of experiments was hundreds. However, almost 70 percent to 80 percent of experiments were not good. The most difficult problem we faced is the stability of resonant frequency response. Now thanks to the improvement on silicon nanotweezers measuring system, it is much easier to get the stable resonant frequency of bare silicon nanotweezers in air and in parylene cavity filled with DI water. Nevertheless, it is still not easy to get stable resonant frequency response in case with DNA bundle. There are many factors can influence resonant frequency response of silicon nanotweezers, which are shown in the followings:

(1) DNA Trapping

Although I have already found the better way to trap DNA, which is presented in Chapter 3.1.4 and 3.1.5, it is still not good enough. It is still hard to trap the same stiffness of DNA bundle in each time, even by using the same silicon nanotweezers with the same voltage on DEP and the same time. Also, the condition of each silicon nanotweezers is slightly different (for example, the gap between tips, the tip radius, and the initial resonant frequency of bare SNT…). This difference makes it hard to trap the DNA bundle with the same stiffness in each DNA trapping.

(2) Temperature

We also found that temperature can influence the resonant frequency response slightly. Therefore, the slight change in temperature of experiment environment may influence the resonant frequency. To do measurement more precisely, it is better to control temperature at stable value.

(3) Shape of Microfluidic Cavity

The fabrication of microfluidic cavity is cut by hand, so the shape of each microfluidic cavity is slightly different. This difference is possible to influence the shape of meniscus surface, and the resonant frequency response is possible to be influenced.

An ideal measurement of DNA degradation by using silicon nanotweezers should be performed under the condition with well-cleaned and fresh silicon nanotweezers and new and fresh microfluidic cavity for filling solution. By depositing aluminum or gold on tips of silicon nanotweezers can make it fresh and easy to trap DNA.

Here I present some tips to do experiments well by using silicon nanotweezers.

1. Before each experiment, check the resonant frequency of bare silicon nanotweezers and compare to the previous data. Check the gap of silicon nanotweezers because the tips may be abraded during the experiments.
2. It is better to avoid reusing the same microfluidic cavity used before. Especially for bio-experiment, clean experiment environment is necessary. That is also why it is better to use new silicon nanotweezers for each experiment.
3. It is better to use the DNA bundle with the similar stiffness in each time. Therefore the data is easier to compare.
4. Silicon nanotweezers are very sensitive, even change of light, temperature, or voice can influence the resonant frequency response.

The measuring system by using silicon nanotweezers is still not optimized. In our experiments, the stability problem still exists and easily influences the results. By the improvement presented in Chapter 3, the stability problem is eased off but still exists.

7. Conclusion and Prospects

7.1 Conclusion

7.1.1 Summary for each Chapter

Here I summarize each chapter of the thesis.

Chapter 1
I introduced cancer and its treatment, indicated that radiation therapy is the easiest treatment for cancer but still need to be improved, especially to reduce the side effect. I also introduced several conventional ways to measure DNA molecules, and indicated it is difficult to measure radiation damage on DNA in "real-time" by these ways. Then, I gave the objectives of this research, which is to measure DNA degradation in real-time and in solution.

Chapter 2
In this experiment, silicon nanotweezers are the measuring tools for DNA degradation. By measuring resonant frequency and Q-factor of silicon nanotweezers with a trapped DNA bundle, we can calculate the stiffness of the DNA bundle, the equivalent number of DNA molecules and the DNA degradation. For experiment in solution, we use a microfluidic cavity to keep the solution. However, the conventional measuring system has the stability problem of resonant frequency response.

Chapter 3
To improve the measuring system, I focused on interface effect and DNA trapping. By coating hydrophobic Parylene on the surface of microfluidic cavity, the meniscus surface of solution becomes convex and the speed of evaporation becomes slower. In addition, I sensed the change of amplitude when touching to the surface of microfluidic cavity, and made it possible to find the best position of insertion. Also, I presented an optimized procedure and some tips for DNA trapping. Besides, the experiments for confirmation of stability have been performed. The results show that the stability problem of resonant frequency response has been solved and prove that there is no

influence on bare silicon nanotweezers from DI water, buffer and enzyme.

Chapter 4

Three control experiments, bare silicon nanotweezers in buffer, bare silicon nanotweezers in enzyme, and DNA in buffer are been performed. The results show there is no influence on bare silicon nanotweezers in buffer and in enzyme, and also no influence on DNA in buffer solution without enzyme. After that, the HindIII enzyme experiment has been performed. The results show the resonant frequency decreased during the immersion in enzyme, and it verified that the silicon nanotweezers can measure the DNA degradation in real-time and in solution.

Chapter 5

Two control experiments, X-ray on bare silicon nanotweezers in air and X-ray on bare silicon nanotweezers in DI water, have been performed. The results show that X-ray does no influence on silicon nanotweezers in air and in DI water. After that, experiments of x-ray on DNA in air and x-ray on DNA in DI water have been tried. The results show that the resonant frequency decreased during the irradiation, which means we successfully performed the measurement of the X-ray damage on DNA. However, I could not find the good correlation between three experiments.

Chapter 6

Although I performed the measurement of DNA degradation by using silicon nanotweezers successfully, the percentage of successful measurement was too low (20 ~ 30 percent). The most difficult problem we faced is the stability of resonant frequency response. Some factors, such as DNA trapping, air-liquid-solid interface, or shape of microfluidic cavity, may influence the resonant frequency response of silicon nanotweezers. The silicon nanotweezers measuring system is still need to improve.

7.1.2 Conclusion

To improve radiation therapy, one of the ways is to understand biophysical mechanism of radiation damage on DNA. By using silicon nanotweezers, we can manipulate DNA molecules and measure the DNA degradation by sensing the resonant frequency of silicon nanotweezers.

Since the resonant frequency response of silicon nanotweezers are not stable enough in previous work, I improve the previous silicon nanotweezers measuring system, including the new way of DNA trapping, the new idea for sensing best position for insertion, and the new microfluidic cavity coating with parylene. The improvement makes the resonant frequency response of silicon nanotweezers much more stable than before.

After getting stable resonant frequency response in case of "bare silicon nanotweezers in DI water, buffer, and enzyme" and "DNA in DI water and buffer," I did experiments with HindIII restriction enzyme reaction on DNA. As model experiments, the results show that DNA degradation by HindIII enzyme can be measured.

After model experiments, I did the measurement of radiation damage on DNA by using silicon nanotweezers. The results show that silicon nanotweezers can resist radiation in air and in solution. Three measurement of radiation damage on DNA is successfully performed. The results show that DNA degrades during irradiation. However, I could not find the clear relationship between these three results.

Although three measurement of radiation damage on DNA has been performed successfully, the number of experiments is not enough for finding the model of radiation damage on DNA. The most difficult problem I faced is the stability of resonant frequency response. To solve this problem and improve the measuring system, I present the future work in Chapter 7.2.

7.2 Future Work

We have successfully measured the DNA degradation by silicon nanotweezers coupled with parylene microfluidic cavity. Since this achievement is just the initial work of DNA degradation measurement, there are many things need to be improved as follows:

(1) The stability of resonant frequency response

Because of the hydrophobic characteristic of parylene, the resonant frequency response is easier to be stable. However, in the case with DNA

bundle, we still have to improve the possibility of getting stable resonant frequency response. Sometimes we it is still hard to get stable resonant frequency response.

(2) Try different dose or time of radiation

Since we want to find the biophysical mechanism of DNA damage by radiation, we need to do measurement with different condition.

(3) Try different solution

Since the measurement of DNA degradation in DI water and in buffer has been performed, it is valuable to change the solution in experiment. For example, some buffer for DNA is easy for radiation to generate free radicals. By this kind of buffer, we can measure more obvious DNA degradation by radiation. For another example, we can also try to use some solution protect DNA from radiation.

(4) To amplify the resonant frequency of DNA bundle

Because the resonant frequency of silicon nanotweezers with DNA bundle is only slightly higher than the resonant frequency of bare tweezers in solution, sometimes the decrease of resonant frequency is too small to be analyzed. To make the decrease of resonant frequency more obvious, we can first amplify the resonant frequency of DNA bundle. By putting DNA bundle into zinc solution, the zinc ions attach to DNA bundle and make the stiffness of DNA bundle higher. [48] Therefore, the resonant frequency of DNA bundle is higher so the decrease of resonant frequency compared to bare silicon nanotweezers is more obvious.

(5) To control and measure the humidity and temperature in experiments

In fact, the resonant frequency response of silicon nanotweezers can be slightly influenced by temperature and humidity in experiment environment. We have already tried to make a close box for maintain the temperature and humidity in experiments, but it is still not completed. If we can measure radiation damage on DNA in specific temperature (for example, body temperature), the results of experiments can be closer to the reality.

7.3 Prospects

We focus on the measurement of the radiation damage on DNA in solution in this thesis. In fact, in addition to this research, there are many research can be tried by silicon nanotweezers measuring system.

While we want to improve radiation therapy by this research, in fact we are also able to help improving chemotherapy by silicon nanotweezers measuring system. For example, we can change the solution to chemical medicine solution, and measure the change of resonant frequency of DNA bundle during the immersion into chemical medicine solution. By analyzing the change of resonant frequency of DNA bundle due to different concentration of chemical medicine solution, it is possible to understand more about the reaction of medicine on DNA. Furthermore, it is also possible to use different medicine solution to do experiments.

In addition to DNA, one of the other ideas is to use silicon nanotweezers to trap other biomolecules, such as microtubules and cells. Some experiments have been done in our laboratory. [7, 49, 50] Many kinds of molecules are able to be trapped by silicon nanotweezers. In our laboratory, trapping metallopolymer by using silicon nanotweezers was succeeded. [50] Since so many applications can be provided by silicon nanotweezers, we can change the type of solution or the type of molecules to do different experiments.

We believe that the researches by using silicon nanotweezers measuring system have the potential to improve the treatment and medicine using for disease and the system for analyzing biophysical mechanism of biomolecules.

8. Reference

1. Parkin, D. M., Bray, F., Ferlay, J., & Pisani, P. “Global cancer statistics, 2002.” CA: a cancer journal for clinicians, 55(2), 74-108. (2005).
2. Siegel, R., DeSantis, C., Virgo, K., Stein, K., Mariotto, A., Smith, T. ... & Ward, E. “Cancer treatment and survivorship statistics, 2012.” CA: a cancer journal for clinicians, 62(4), 220-241. (2012).
3. Siegel, R., Naishadham, D., & Jemal, A. Cancer statistics, 2013. CA: a cancer journal for clinicians, 63(1), 11-30. (2013).
4. Yamahata, C., Collard, D., Legrand, B., Takekawa, T., Kumemura, M., Hashiguchi, G., & Fujita, H. “Silicon nanotweezers with subnanometer resolution for the micromanipulation of biomolecules." Microelectromechanical Systems, Journal of, 17(3), 623-631. (2008).
5. Kumemura, M., Collard, D., Sakaki, N., Yamahata, C., Hosogi, M., Hashiguchi, G., & Fujita, H. “Single-DNA-molecule trapping with silicon nanotweezers using pulsed dielectrophoresis.” Journal of Micromechanics and Microengineering, 21(5), 054020. (2011).
6. Kumemura, M., Collard, D., Tourvielle, R., Lafitte, N., Montagne, K., Yoshizawa, S. ... & Fujita, H. “Integrated MEMS platform with silicon nanotweezers and open microfluidic device for molecular and cellular biomechanical assays.” In Micro Electro Mechanical Systems (MEMS), 2011 IEEE 24th International Conference on (pp. 67-70). IEEE. (2011).
7. Tarhan, M. C., Yokokawa, R., Jalabert, L., Collard, D., & Fujita, H. “Biomotor-based nanotransport system constructed by pick-and-place assembly of individual molecules.” In Intelligent Robots and Systems (IROS), 2010 IEEE/RSJ International Conference on (pp. 5628-5633). IEEE. (2010, October).
8. The statistics from World Health Organization http://www.who.int/en/
9. The statistics from Ministry of Health, Labour and Welfare, Japan http://www.mhlw.go.jp/index.shtml
10. Jemal, A., Center, M. M., DeSantis, C., & Ward, E. M. “Global patterns of cancer incidence and mortality rates and trends.” Cancer Epidemiology Biomarkers & Prevention, 19(8), 1893-1907. (2010).
11. Smith, Robert A., et al. "Cancer screening in the United States, 2010: a review of current American Cancer Society guidelines and issues in cancer screening." CA: a cancer journal for clinicians 60.2 99-119. (2010).

12. Trédaniel, J., Boffetta, P., Buiatti, E., Saracci, R., & Hirsch, “A. Tobacco smoking and gastric cancer: Review and meta - analysis.” International Journal of Cancer, *72*(4), 565-573. (1997).
13. Jemal, A., Bray, F., Center, M. M., Ferlay, J., Ward, E., & Forman, D. “Global cancer statistics.” CA: a cancer journal for clinicians, 61(2), 69-90. (2011).
14. Hoeijmakers, Jan HJ. "DNA damage, aging, and cancer." New England Journal of Medicine 361.15: 1475-1485. (2009).
15. De Bont, Rinne, and Nik Van Larebeke. "Endogenous DNA damage in humans: a review of quantitative data." Mutagenesis 19.3: 169-185. (2004).
16. Tsugane, Shoichiro, and Shizuka Sasazuki. "Diet and the risk of gastric cancer: review of epidemiological evidence." Gastric Cancer 10.2: 75-83. (2007).
17. DeCensi, A., Puntoni, M., Goodwin, P., Cazzaniga, M., Gennari, A., Bonanni, B., & Gandini, S. “Metformin and cancer risk in diabetic patients: a systematic review and meta-analysis.” Cancer Prevention Research, 3(11), 1451-1461. (2010).
18. Miller, A. B., Hoogstraten, B., Staquet, M., & Winkler, A. “Reporting results of cancer treatment.” cancer, 47(1), 207-214. (1981).
19. Information from Cancer Treatment - National Cancer Institute http://www.cancer.gov/cancertopics/treatment
20. Types of Cancer Treatment | American Cancer Society http://www.cancer.org/treatment/treatmentsandsideeffects/treatmenttypes/treatment-types-landing
21. James, Nicholas D., et al. "Radiotherapy with or without chemotherapy in muscle-invasive bladder cancer." New England Journal of Medicine 366.16: 1477-1488. (2012).
22. Moding, Everett J., Michael B. Kastan, and David G. Kirsch. "Strategies for optimizing the response of cancer and normal tissues to radiation." Nature Reviews Drug Discovery 12.7, 526-542. (2013).
23. Genetic Science Learning Center, http://learn.genetics.utah.edu/
24. Direct and Indirect action of Ionizing Radiation on DNA, Teach Nuclear http://teachnuclear.ca/
25. Khanna, Kum Kum, and Stephen P. Jackson. "DNA double-strand breaks: signaling, repair and the cancer connection." Nature genetics 27.3, 247-254. (2001).

26. Jones, G. D. D., Boswell, T. V., Lee, J., Milligan, J. R., Ward, J. F., & Weinfeld, M. “A comparison of DNA damages produced under conditions of direct and indirect action of radiation.” International journal of radiation biology, 66(5), 441-445. (1994).
27. Ravanat, Jean-Luc, Thierry Douki, and Jean Cadet. "Direct and indirect effects of UV radiation on DNA and its components." Journal of Photochemistry and Photobiology B: Biology 63.1, 88-102. (2001).
28. Su, Tin Tin. "Cellular responses to DNA damage: one signal, multiple choices." Annu. Rev. Genet. 40, 187-208. (2006).
29. NDT Resource Center, Ionization and Cell Damage, http://www.ndt-ed.org/EducationResources/CommunityCollege/RadiationSafety/theory/ionization.htm
30. Baskar, R., Lee, K. A., Yeo, R., & Yeoh, K. W. “Cancer and radiation therapy: current advances and future directions.” International journal of medical sciences, 9(3), 193. (2012).
31. Mullenders, L., Atkinson, M., Paretzke, H., Sabatier, L., & Bouffler, S. “Assessing cancer risks of low-dose radiation.” Nature Reviews Cancer, 9(8), 596-604. (2009).
32. Walter, N. G., Huang, C. Y., Manzo, A. J., & Sobhy, M. A. “Do-it-yourself guide: how to use the modern single-molecule toolkit.” Nature methods, 5(6), 475-489. (2008).
33. Neuman, K. C., & Nagy, A. “Single-molecule force spectroscopy: optical tweezers, magnetic tweezers and atomic force microscopy.” Nature methods, 5(6), 491-505. (2008).
34. Fuller, D. N., Gemmen, G. J., Rickgauer, J. P., Dupont, A., Millin, R., Recouvreux, P., & Smith, D. E. “A general method for manipulating DNA sequences from any organism with optical tweezers.” Nucleic acids research, 34(2), e15-e15. (2006).
35. Regtmeier, J., Duong, T. T., Eichhorn, R., Anselmetti, D., & Ros, “A. Dielectrophoretic manipulation of DNA: Separation and polarizability.” Analytical chemistry, 79(10), 3925-3932. (2007).
36. Hashiguchi, G., Goda, T., Hosogi, M., Hirano, K., Kaji, N., Baba, Y. ... & Fujita, H. “DNA manipulation and retrieval from an aqueous solution with micromachined nanotweezers.” Analytical chemistry, 75(17), 4347-4350. (2003).
37. Yamamoto, T., Kurosawa, O., Kabata, H., Shimamoto, N., & Washizu, M. “Molecular surgery of DNA based on electrostatic micromanipulation.

Industry Applications," IEEE Transactions on, 36(4), 1010-1017. (2000).
38. Washizu, M., & Kurosawa, O. "Electrostatic manipulation of DNA in microfabricated structures." Industry Applications, IEEE Transactions on, 26(6), 1165-1172. (1990).
39. Washizu, M., Suzuki, S., Kurosawa, O., Nishizaka, T., & Shinohara, "T. "Molecular dielectrophoresis of biopolymers." Industry Applications, IEEE Transactions on, 30(4), 835-843. (1994).
40. Kumemura, M., Collard, D., Yamahata, C., Sakaki, N., Hashiguchi, G., & Fujita, H. "Single DNA molecule isolation and trapping in a microfluidic device." ChemPhysChem, 8(12), 1875-1880 (2007).
41. Bustamante, C., Bryant, Z., & Smith, S. B. "Ten years of tension: single-molecule DNA mechanics." Nature, 421(6921), 423-427. (2003).
42. Lafitte, N., Kumemura, M., Nagai, M., Jalabert, L., Collard, D., & Fujita, H. "An open microfluidic device with active valves for accurate trapping of DNA by silicon nanotweezers." In 14th International Conference on Miniaturized Systems for Chemistry and Life Sciences, MicroTAS2010 (pp. 1865-1867). (2010).
43. Lafitte, N., Kumemura, M., Jalabert, L., Collard, D., & Fujita, H. "Real-Time sensing of molecule binding on DNA with silicon nanotweezers." In 15th International Conference on Miniaturized Systems for Chemistry and Life Sciences, MicroTAS2011 (pp. 389-372). (2011).
44. TP Silverstein. "The Real Reason Why Oil and Water Don't Mix." Journal of chemical education, 75.1: 116. (1998).
45. Shin, D. H., Lee, S. H., Jung, J. Y., & Yoo, J. Y. "Evaporating characteristics of sessile droplet on hydrophobic and hydrophilic surfaces." Microelectronic Engineering, 86(4), 1350-1353. (2009).
46. Pingoud, A., & Jeltsch, A. "Structure and function of type II restriction endonucleases." Nucleic Acids Research, 29(18), 3705-3727. (2001).
47. Roberts, Richard J. "How restriction enzymes became the workhorses of molecular biology." Proceedings of the National Academy of Sciences 102.17: 5905-5908. (2005).
48. Lee, M. S., Gippert, G. P., Soman, K. V., Case, D. A., & Wright, P. E.. "Three-dimensional solution structure of a single zinc finger DNA-binding domain." Science, 245(4918), 635-637. (1989).
49. Guillou, H., Lafitte, N., Léal, R., Jalabert, L., Fujii, T., Fujita, H. ... & Collard, D. "Silicon Nanotweezers with double actuation and extended

stroke for single cell studies." In Solid-State Sensors, Actuators and Microsystems (TRANSDUCERS & EUROSENSORS XXVII), 2013 Transducers & Eurosensors XXVII: The 17th International Conference on (pp. 46-49). IEEE. (2013, June).

50. Lee, J., Yagi, K., Kumemura, M., Sato, T., Jalabert, L., Lafitte, N. ... & Fujita, H. "Characterization of Π-conjugated metallopolymer's mechanical stiffness by using silicon nanotweezers." In Solid-State Sensors, Actuators and Microsystems (TRANSDUCERS & EUROSENSORS XXVII), 2013 Transducers & Eurosensors XXVII: The 17th International Conference on (pp. 2684-2687). IEEE. (2013, June).