MASTER THESIS

Cantilever biosensor for detecting attachment of proteins

-Toward the diagnosis of Alzheimer's disease-

タンパク質の結合能力を測るカンチレバーバイオセンサー

-アルツハイマー病診断を目指して-

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Abstract

As the population is ageing rapidly, age related dementia, e.g. Alzheimer's disease will be a crucial problem in the future. However, there is no cure of Alzheimer's disease; it worsens as progress and leads to death in the end. Nowadays, Alzheimer's disease is mainly diagnosed by brain images. However such diagnosis can detect the disease after highly progressed brain damage. Brain can't be recovered at that stage. Therefore, early diagnosis of Alzheimer's disease is necessary for slowing down a brain damage for better quality of life. From this observation, my research aims to develop sensors for early diagnosis of Alzheimer's disease.

The cause of Alzheimer's disease is not well understood. One hypotheses of Alzheimer's disease cause is related to tau proteins. Tau proteins attach to microtubules (MTs) and stabilize them. If the tau proteins are phosphorylated and tangled, the attachment of tau proteins and MTs is weakened. It can trigger the instability of MTs and collapse neuron's transport system for neural transmitters. In this research, I investigated the cantilever biosensor for detecting the attachment ability of tau proteins to MTs directly by mass change. The sensor was previously developed for the detection of insulin.

The cantilever is formed on the sidewall of the microfluidic device and is resonating at air-liquid surface for high mass sensitivity. The surface of the cantilever is sputtered with silicon dioxide for bio-affinity hydrophilic surface. After that, photothermal laser excites the cantilever and the velocity of the resonating cantilever is detected by laser doppler velocimetry. The frequency giving the maximum velocity is the resonant frequency. The resonant frequency of the cantilever decreases due to mass increase when the target molecules attach to the cantilever surface.

For detecting tau proteins, PLL coating and MTs immobilization are successfully done and confirmed by the resonant frequency change and fluorescence imaging. Tau attachment to MTs can't be detected, however, by the

resonant frequency change because of its small molecular weight. Therefore, anti-tau antibodies were flowed after tau proteins for bigger mass change. The mass change by antibodies was detected but there was a problem about non-specific bonding of anti-bodies.

Detecting the attachment of proteins related to cause of Alzheimer's disease is investigated. The proposed method of cantilever biosensor will be an excellent tool for early diagnosis of Alzheimer's disease.

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Contents

1 Introduction

In most of developed countries, population ageing is occurring because the fertility and the mortality are both decreased. The population of older people exceeds that of children now and will be double in 2050. It will make a lot of social problems in economics and healthcare systems.

1-1 Alzheimer's disease

Alzheimer's disease, mostly found in elder people ages more than 65, is predicted to affect 1 in 85 people globally in 2050 according to Brookmeyer, et al. [1] research. But Alzheimer's disease has no cure, worsens as it progress and leads to death in the end [2, 3]. Not only patients but also people around them are also suffering misery of Alzheimer's disease. Also at economic view, the total cost of care for individuals with Alzheimer's disease is expected to soar from 173 billion dollars in 2010 to more than 1 trillion dollars in 2050 in USA (figure 1-1-1).

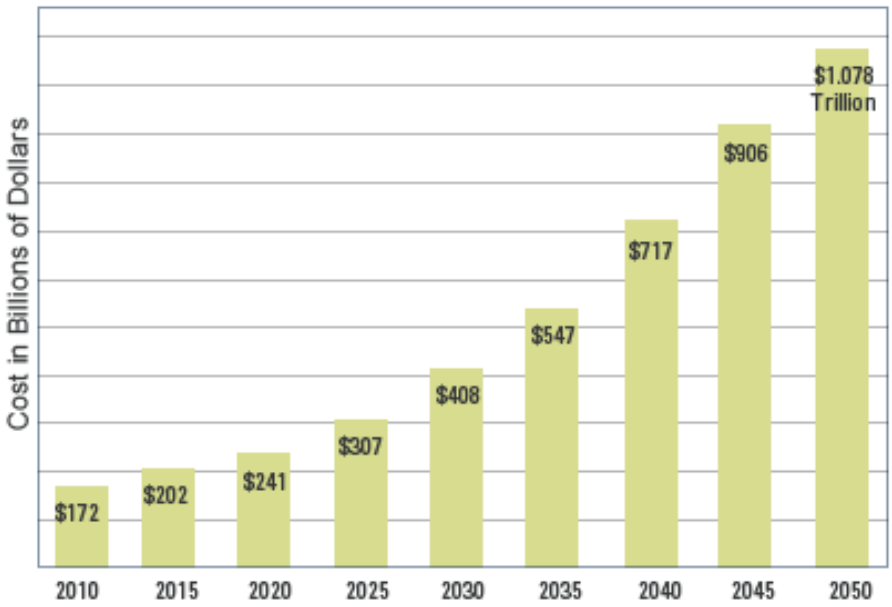

Figure 1-1-1. The cost for caring Alzheimer disease in the USA. From Changing the Trajectory of Alzheimer's Disease: A National Imperative, May 2010

(http://www.alz.org/documents_custom/trajectory.pdf).

1-2 Current diagnosis

Current diagnosis of Alzheimer's disease mainly depends on the record of mental decline and brain images. But they can only detect the decrease after severe brain damage occurred. For preventing brain damage and early diagnosis of Alzheimer's disease, we need accurate, reliable, low cost and easy-to-use test.

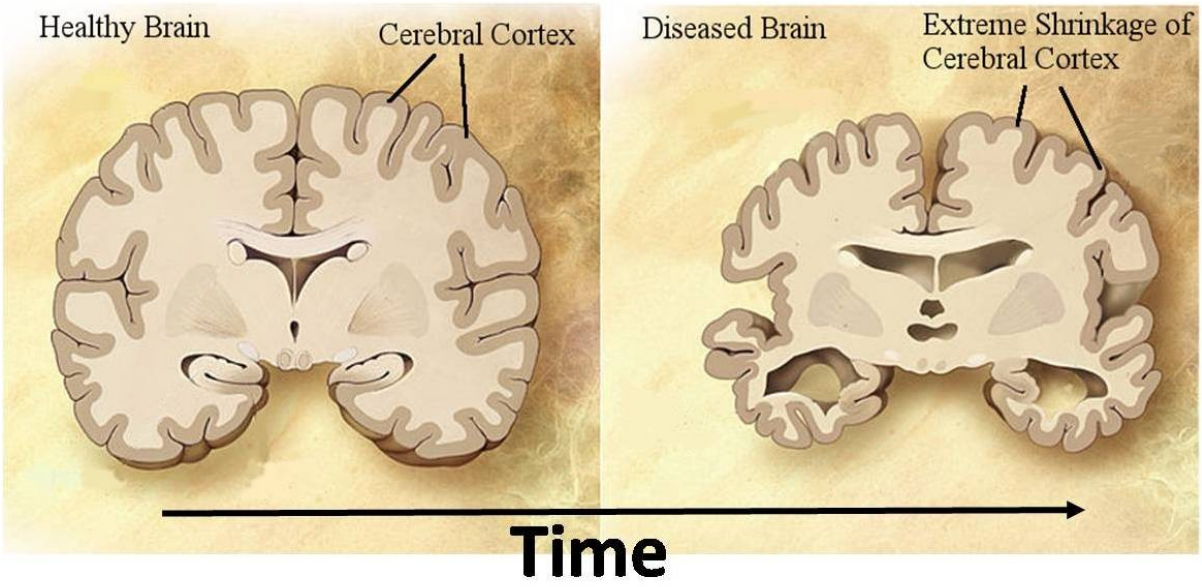

Figure 1-2-1. Schematic of brain image difference between healthy and diseased brain. (From http://neurosciencenews.com/single-traumatic-brain-injury-may-lead-alzheimers-disease/)

1-3 Microtubules and tau proteins

Tau proteins bind with MTs, which maintain the structure of cell and are platforms for intracellular transport, and stabilize them. But phosphorylated tau proteins form tangles and lose the function of binding with MT. Loss of binding function leads to the instability and collapse of MTs. In neuron systems, collapse

of MTs leads to deficiency of interneural transport of neuron transmitters and neurodegenerative diseases such as Alzheimer's disease (AD) in the end. (figure 1-3-1).

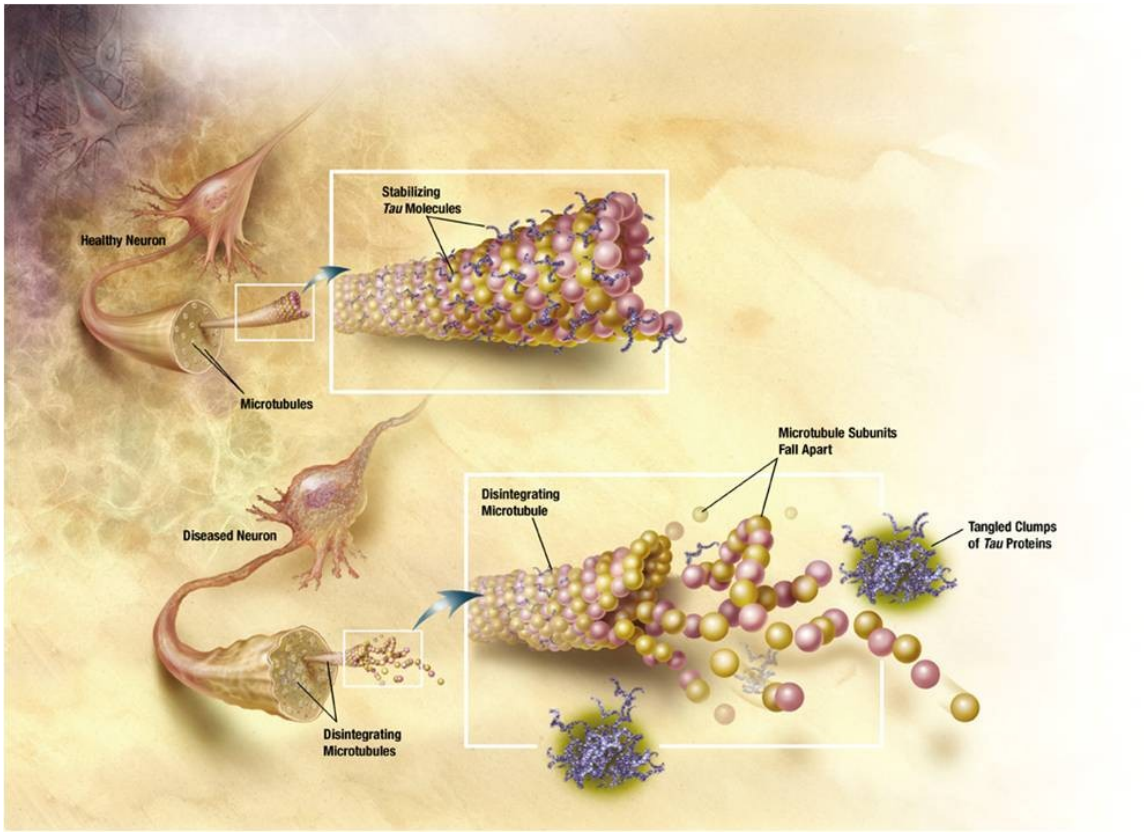

Figure 1-3-1. The effect of mutated tau to neuron systems. Illustration: National Institute on Aging/U.S. National Institutes of Health (From http://www.nia.nih.gov/alzheimers)

The state of tau proteins (healthy or tangled and mutant) and AD have strong relationship each other. Abnormally tangled tau proteins cause neuronal dysfunction [4-6]. Therefore, detecting tau proteins from the cerebrospinal fluid (CSF) samples for AD is great value to improve the diagnosis accuracy of the disease. The total tau concentration is in the range from 100 to 2000 pg/mL and increases with age. On the other hand, the concentration of phosphorylated-tau

(tau 181) is less than 50 pg/mL [7-9].

1- 4 ELISA

Enzyme-linked immunosorbent assay (ELISA) is one of the most commonly used methods to detect tau proteins in the CSF [10]. (figure 1-4-1). (a) Immobilizing antibodies on the substrate. (b) Flowing the solution including target moleculars with fluorescence markers. (c) Washing solution with buffer. Target moleculars attached to antibodies remain the substrate. It can be checked by fluorescence microscope. (d) If other moleculars flowed to the substrate, they don't attach to antibodies. (e) Moleculars washed away by buffer and can't be detected by fluorescence microscope.

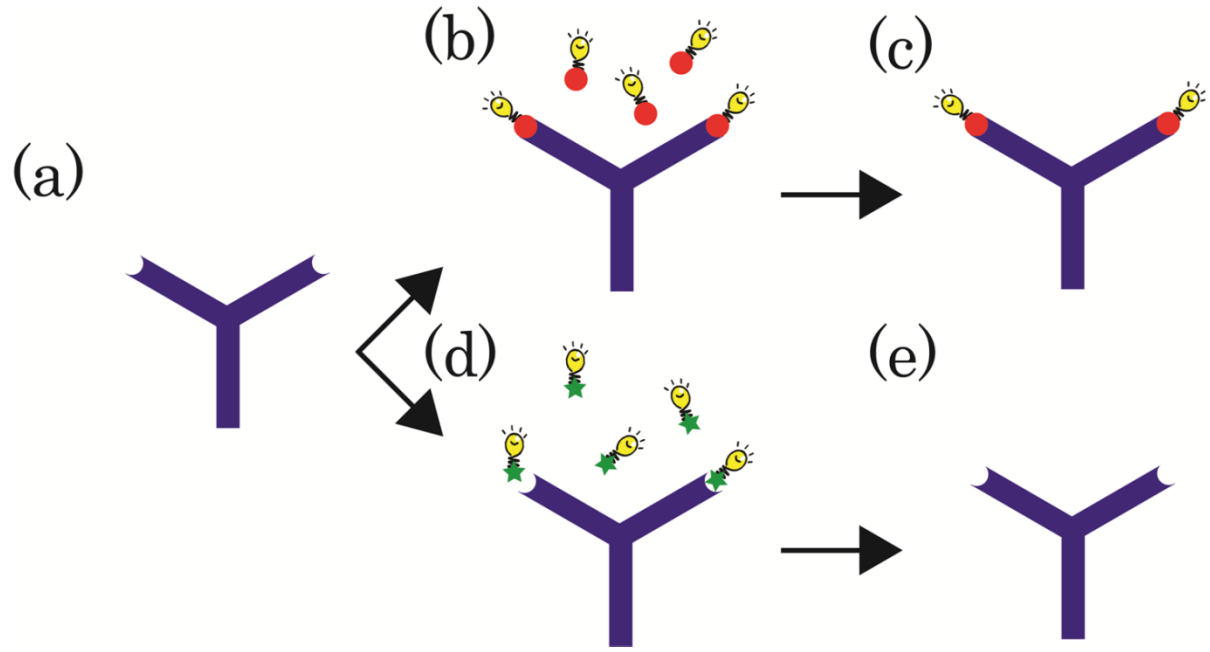

Figure 1-4-1. The principle of ELISA.

This method is composed of simple steps for detecting tau proteins. On the other hand, it can't detect the attachment of tau proteins to MTs and also needs expensive fluorescence materials.

1-5 Purpose

The purpose of this research is detecting the attachment of MTs and healthy/ tangled tau proteins. Toward this goal, the cantilever biosensor is used for detecting mass change by the attachment of tau proteins to MTs. As proteins attach to the surface of cantilever, resonant frequency decreases (the detail principle about cantilever biosensor is written in chapter 2). Healthy and tangled tau proteins can be distinguished by monitoring resonant frequency change. Healthy tau proteins have property to attach with MTs. From this attachment, resonant frequency will decrease by flowing tau proteins to cantilever biosensor immobilized with MTs. Tangled tau proteins, on the other hand, lose the function of attachment with MTs. No change of resonant frequency is expected when tangled tau proteins are flowed to cantilever biosensor.

1-6 Significance

This is the first research to directly sense tau proteins ability of attachment to MTs. This research will help understanding the difference in the attachment ability to MTs between healthy and tangled tau proteins. Also this cantilever biosensor needs only small amount of samples. This sensor also provides high sensitivity, easy experiment protocols. Significant potentials of cantilever biosensor for early diagnosis of Alzheimer's disease is showed by this research.

1-7 Thesis structure

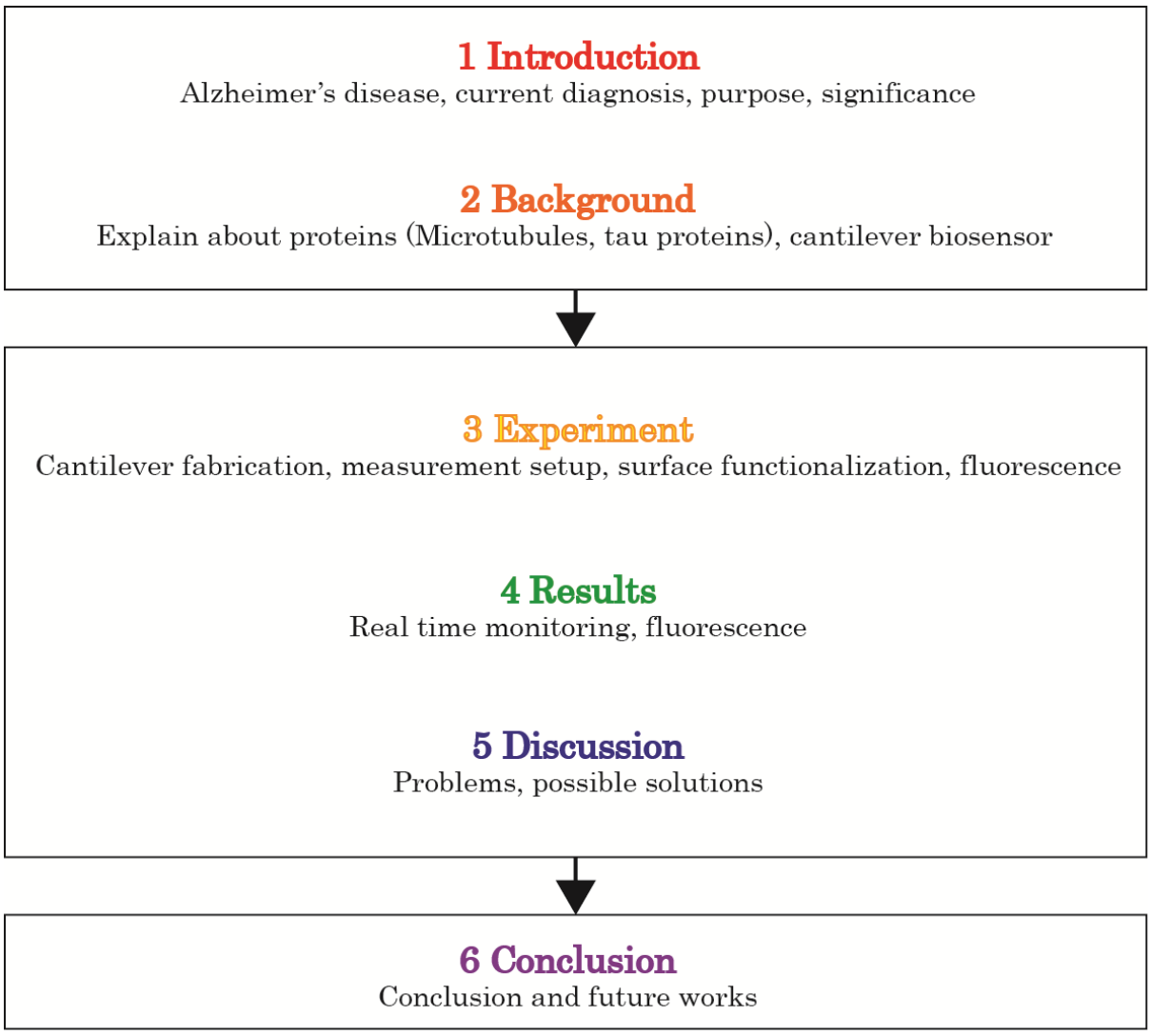

Figure 1-7-1 Flow chart of master thesis.

2 Background

The basic of bio materials and the principle of cantilever biosensor which are used in this research are explained in this chapter.

2-1 Microtubules

Microtubules are essential elements of cells. They make overall structure of cells by supporting extended morphologies [11-13]. They need to remain fairly straight to enable long-range transport since MTs network makes up the tracks for cargo-carrying motor proteins in the cells [14, 15]. MTs are also important at the formation of nervous systems. Tubulin's dynamics and tau proteins are finely controlled during the development of the brain's neuronal base [16].

Physically, MTs are hollow tubes composed of a lattice of α-β tubulins. They have 17 nm of interior space diameter and 25 nm of outer diameter. Tubulin heterodimers stack end-to-end to form protofilaments. These protofilaments bind laterally to form sheets that are rolled into a tube (figure 2-1-1)[17]. MTs have polarity, and can bind a multitude of associated proteins. These associated proteins can manipulate their rigidity and stability as well as crosslink and bundle microtubules.

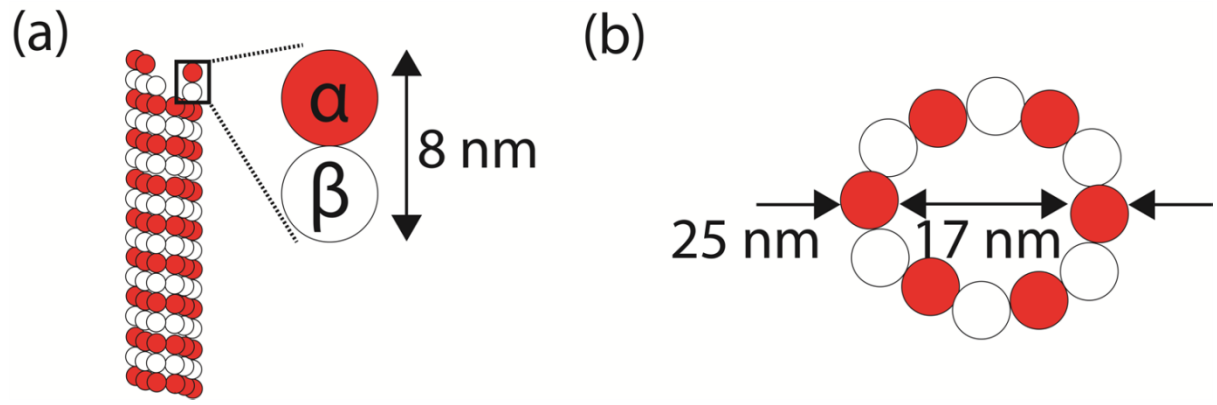

Figure 2-1-1. Structure of microtubules

Taxol is a chemical used to stabilize microtubules. Many researchers used taxol in vitro to stabilize microtubules against depolymerization after GTP-hydrolysis. Theoretical modeling implies that microtubules stabilized by taxol should be less stiff than unstabilized microtubules. But some cell biological researchers insist that MTs become stiffer [18-20].

2-2 Tau protein

Tau protein is protein which is abundant in neurons of the central nervous system[21]. Tau contains three domains, aminoterminal projection domain, a carboxyterminal domain of microtubule‐binding repeats, and a short tail sequence. In human body, there are six tau isoforms, and they have three or four microtubule-binding repeats [22, 23].

One of the well known functions of tau proteins is the attachment and stabilization of microtubules [24, 25]. On the other hand, Phosphorylation blocks the function of tau proteins, binding to microtubules. In patients with Alzheimer's disease, tau proteins become highly phosphorylated which makes tau proteins detach from microtubules [22]. This leads to collapse of MTs and neurons are dead in the end. Death of neurons triggers critical damage of neuron systems and it is most likely to lead to Alzheimer's disease.

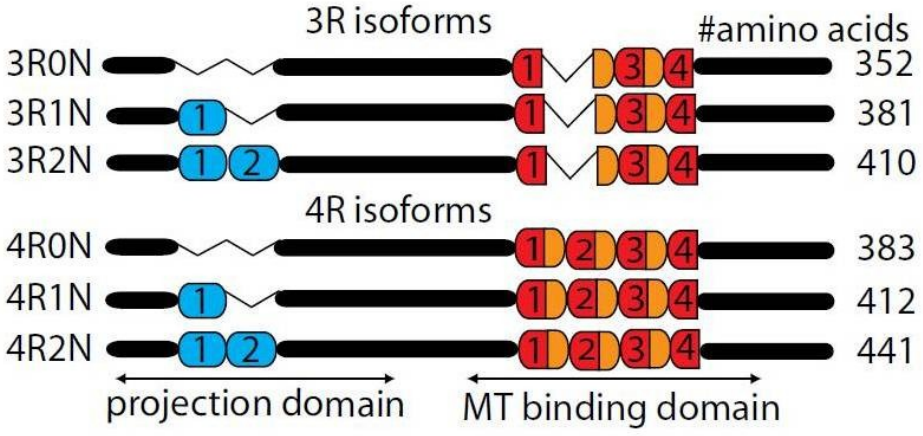

Figure 2-2-1. Schematic of tau isoforms[26] (From

http://www.pnas.org/content/105/21/7445.short).

2-3 Principle of cantilever biosensor

Micro sized cantilever biosensors, the surface of which is functionalized and utilized for specific biomolecular detection, have been widely used for its high sensitivity and simple structures. Two kinds of measurement principles are mainly adopted, static mode and dynamic mode. In the static mode, change in the surface stress induces the deflection and is measured by laser beam reflection onto the photodiodes array [27-29]. In dynamic mode, the mass of target biomolecules changes the cantilever resonant frequency. Most of researchers measured resonant frequency in the vacuum or air because resonant frequency is clearly monitored [30-33]. Measurement in the liquid, however, is highly desirable because of its prospective utility in biological applications. In the liquid, the vibration peak is much lower than in the vacuum and air [34, 35]. Recently, Park et al. developed a cantilever biosensor resonating at air-liquid interface [36, 37]. This biosensor depends on a meniscus membrane at a U-shaped micro slit for both keeping air-liquid interface and allowing resonating motion. It has 5.7 times larger single-to-noise-ratio (SNR) and 50% higher quality factor than a cantilever resonating in liquid [36, 37].

The change of mass loaded on the surface of cantilever translates into the resonant frequency. The relationship between the change of mass and the resonant frequency is described by following equations:

$$f_c + \Delta f = \frac{1}{2\pi}\sqrt{\frac{K}{M_c + \Delta M}}$$

$$= \frac{1}{2\pi}\sqrt{\frac{K}{M_c}}\left(1 + \frac{\Delta M}{M_C}\right)^{-\frac{1}{2}}$$

$$\approx \frac{1}{2\pi}\sqrt{\frac{K}{M_c}}\left(1-\frac{\Delta M}{2M_C}\right)$$

$$= f_c\left(1-\frac{\Delta M}{2M_C}\right)$$

$$\therefore\ \Delta M = -\frac{\Delta f}{f_c}2M_c$$

fc and Δf are the resonant frequency of the cantilever and its shift respectively. K is the spring constant of the cantilever. Mc and ΔM are the mass of cantilever and the additional mass attached on the cantilever surface respectively.

The Q factor of cantilever depends on the resonating environment (figure 2-3-1). Cantilever resonating in liquid, the damping by liquid lowers the Q factor of cantilever. For high resolution, cantilever resonating at the air/liquid interface or the air are desirable.

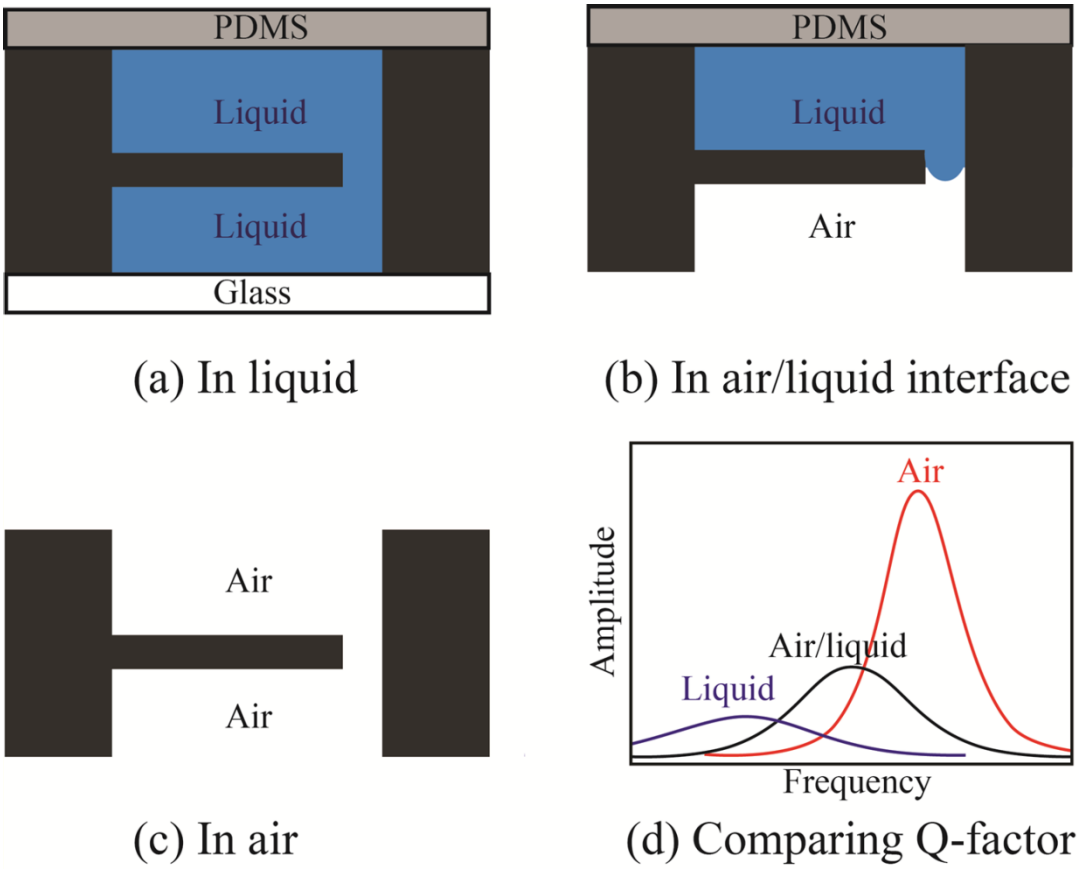

Figure 2-3-1. Comparison of Q-factor at different resonating conditions.

3 Experiment

3-1 Cantilever design

The cantilever was fabricated on a silicon-on-insulator (SOI, 5 μm (Si)/ 1 μm (SiO_2)/ 450 μm (Si), Ultrasil) wafer for thickness uniformity. The size of a cantilever was 80 μm^L × 20 μm^W × 5 μm^T. It was surrounded by a micro-slit of 6μm in width. The cantilever was integrated in the microchannel (100 μm^W × 400 μm^H × 10 mm^L) by attaching a cover plate (figure 3-1-1). Meniscus membrane on the slit sustained the liquid pressure in the microchannel. It can be possible because the surface of microchannel is hydrophilic, and the surface contacting air is hydrophobic.

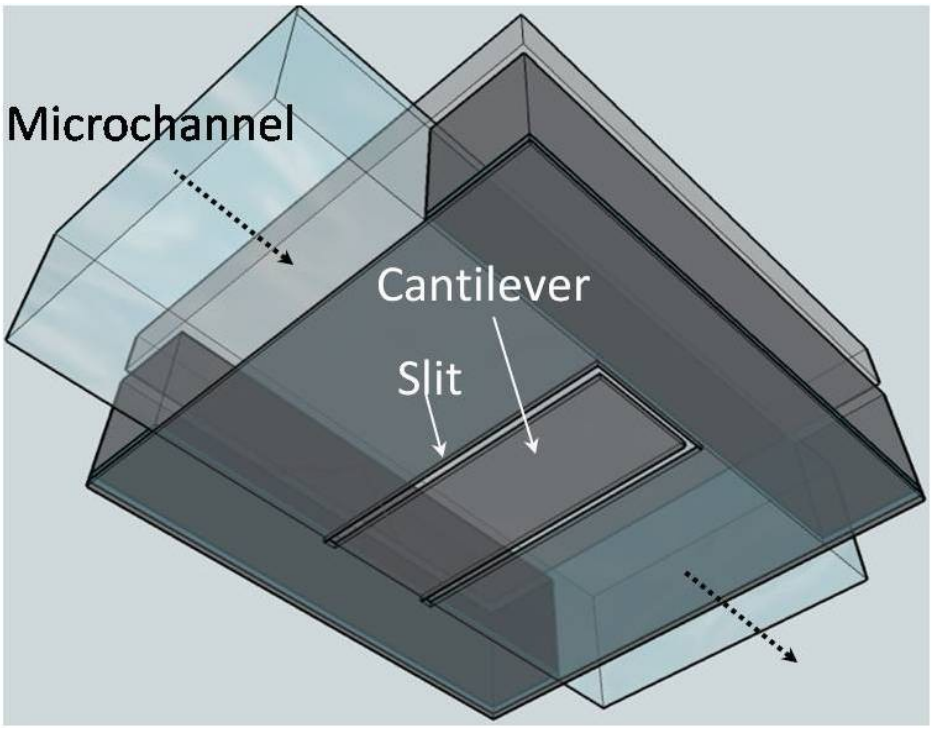

Figure 3-1-1. The schematic of cantilever biosensor resonating at air-liquid interface [37] (From http://pubs.rsc.org/EN/content/articlehtml/2011/lc/c1lc20608g).

3-2 Cantilever fabrication

Silicon on insulator (SOI) wafer was used to fabricate cantilever. (a) First, the SOI wafer was cleaned by a piranha solution (H_2O_2: H_2SO_4 = 1: 3) at 150°C on a hot plate. Then, a naturally formed silicon-oxide layer was removed by buffered hydrofluoric (BHF) acid for 30 seconds. (b) On the backside, the aluminum layer was deposited and patterned as a mask for etching a microchannel. (c) The cantilever was patterned on the 5-μm-thick silicon layer by a Deep Reactive Ion Etching (DRIE) process and protected by a photoresist (S1818). (d) The 450-μm-thick silicon layer was etched by DRIE process. (e) Then, the 1-μm-thick buried silicon dioxide layer was eliminated by BHF etching for 8 minutes. (f) Finally, SiO_2 was sputtered over the cantilever surface facing the microchannel side for obtaining bio-affinity hydrophilic surface. After SiO_2 sputtering, the cantilever was cleaned by piranha solution (H_2O_2: H_2SO_4 = 1: 3) for 20 minutes and rinsed by DI water. After the process, microchannel was formed by using a 2-mm-thick Polydimethylsiloxzne (PDMS, silicon-based organic polymer). The PDMS cover was cut out and attached to the device to form a microchannel. It has both the inlet and outlet for permitting the circulation of liquid through the microchannel by a syringe pump.

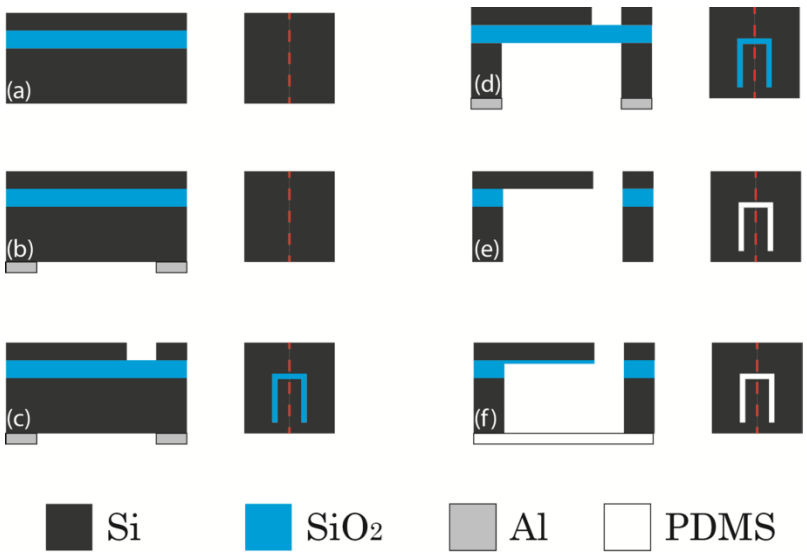

Figure 3-2-1. Fabrication process of cantilever biosensor.

3-3 Microfluidics

Syringe pumps are used for injecting biological sample to the surface of cantilever biosensor. The droplet of the sample is placed on the inlet of PDMS cover, and outlet is connected with syringe pump. Silicon rubber tubes are connected between the microchannel contacted with cantilever and syringe pump (Figure 3-3-1). Samples goes inside of cantilever channel by pulling syringe.

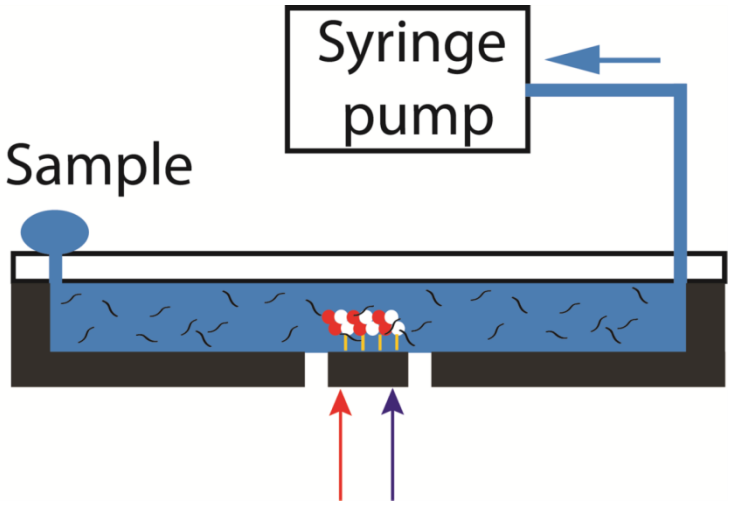

Figure 3-3-1. Microfluidics setup.

3-4 Laser setup for excitation and detection of vibration

The cantilever has to be vibrated for monitoring its resonant frequency. Some researchers used piezoelectricity for analyzing its resonant characteristics [38-42]. But devices have complex structures for actuation and detection. In this research, resonant characteristics of cantilever are monitored by using laser.

Laser setup for this research is composed of six parts, photothermal laser for actuating cantilever, laser Doppler velocimetry for detecting resonant frequency, close loop feedback control for tracking resonant frequency and real time monitoring, objective lens for focusing laser to cantilever, table, and CCD camera for adjusting position of cantilever.

The cantilever is vibrated by a pulse modulated photothermal laser (λ= 405 nm). The modulation frequency determine the actuation frequency. It excites a cantilever efficiently by transmitting a thermal energy to the cantilever [43, 44]. One of the advantages of this method is to excite any position on the surface of cantilever. The rated output power of the 405 nm laser diode for photothermal excitation is 30 mW. However, the average power is smaller than half of the value, because the power must be modulated in the rated range. In addition, the output power decreases with passing through the optical devices. The actual incident power at the cantilever surface is 2 mW.

The vibration of cantilever is detected by laser Doppler velocimetry (λ= 633 nm). The resonant frequency of cantilever is measured by the network analyzer (E5071C, Agilent technologies) at 100 Hz of resolution. This laser Doppler velocimetry detects the deflection velocity of vibrating cantilever. The signal detected by this method is not affected by averaged deflection changes of the cantilever.

The velocity signal detected by the laser doppler velocimetry is fed back to a lock-in-amplifier to compare the laser doppler velocimetry signal and the modulated photothermal laser signal. A proportional-integral (PI) controller keeps the phase difference at 90 degrees between excitation and vibration signals. The frequency data is converted to a voltage that represents the output of the sensor. The resonant frequency is saved in a data-logger programmed in LabVIEW.

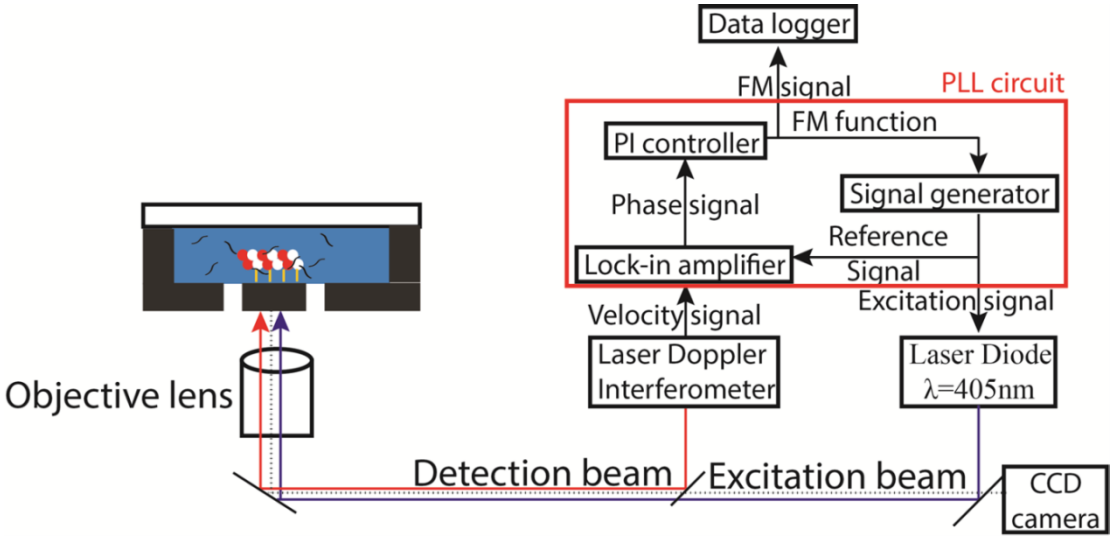

Figure 3-4-1. The laser setup for detecting resonant frequency of cantilever

The temperature is one critical factor of affecting cantilever's resonant frequency. Therefore, the liquid, thus the cantilever, was kept at the same temperature; this stabilizes the resonant frequency of the cantilever.

3-5 Materials

BRB80 buffer is used in this research. It was made by 80 mM PIPES–NaOH pH 6.8, 1 mM MgCl2, 1 mM EGTA. Tubulin (Cytoskeleton, >99% purity) with concentration of 3 mg ml^{-1} was polymerized into MTs in BRB80 buffer containing 1 mM MgSO4 and 1 mM GTP, by incubating at 37 degree for 30 min. The MTs were stabilized and diluted in BRB80 (200-fold) containing 20 mM paclitaxel [45, 46]. The concentration of MTs is 0.02 mg/ml. Also, tau protein isoforms and mutants were purchased from Sigma (http://www.sigmaaldrich.com/catalog/product/sigma/t7675?lang=ja&region=JP).

3-6 Microtubule cutting

The average length of polymerized microtubules is about 18 μm. In this case, microtubules can make bridge over the 6-μm-gap of the cantilever slit. Cutting

microtubules shorter than 6 μm is necessary to avoid the bridging of MTs over the slit. Two methods were tried to cut microtubules, pipetting (100 times) and vortexing (10 min).

I used flow cell to check the length of cut MTs. It is composed of 2 sheets of coverslips and spacers between them (figure 3-6-1). The size of flow cell is 2 cm (length), 0.5 cm (width), 0.2 mm (height), total volumn is 20 μl.

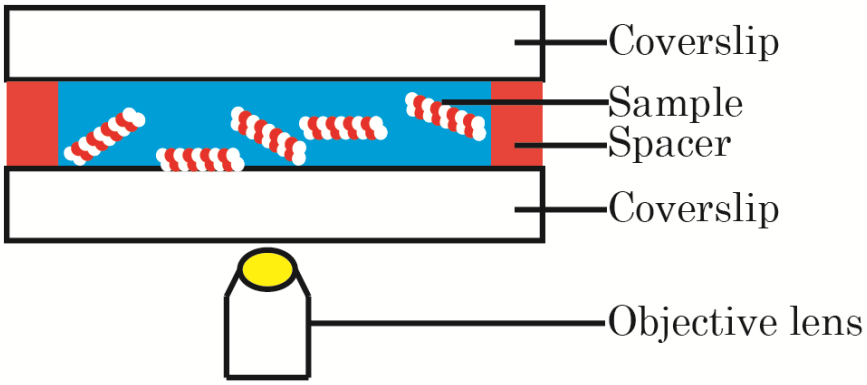

Figure 3-6-1. Schematic of flow cell to check cut MTs length.

First, Poly-L-lysine (PLL, concentration of 0.01 %)is filled in the flow cell for 2 minutes. Then the PLL solution was washed in the flow cell by buffer. After washing, fluorescence MTs are injected and kept for 2 minutes. Finally, after washing MTs solution, I checked MTs length by microscope. The results are shown in figure 3-6-2.

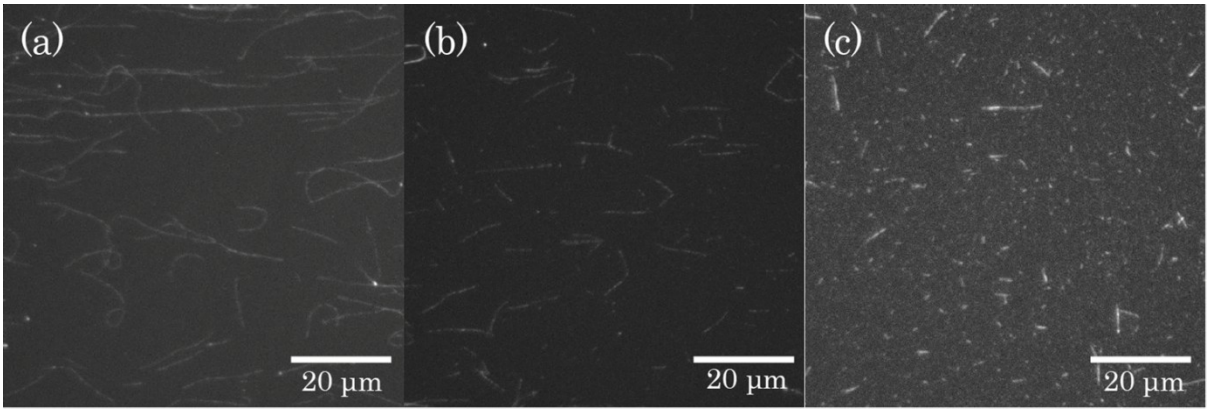

Figure 3-6-2. MTs length observation by different cutting methods. (a) Original length of MTs after polymerization. (b) Cutting MTs by pipetting. (c) Cutting MTs

by vortexing

The average length of MTs after polymerization is 18 μm. MTs cut to 6.4 μm in average-length by high sheer force of pipetting. After applying vibration of vortex for 10 min, MTs cut into 2.8 μm in average-length. From this experiment, MTs could be cut around 2.8 μm for cantilever immobilization by the vibration of vortex. I also realized that MTs can be cut by vibration from outside.

3-7 Surface functionalization

The surface of cantilever is SiO_2 after the sputtering to make it hydrophilic. The surface needs to be functionalized for detecting tau protein attachment to MTs (figure 3-7-1). (a) First, 50 μl of PLL (0.01 %) is flowed into the cantilever with the flow speed of 20 μl/min. (b) After that, washing the microchannel by BRB 80 until the resonant frequency is stabilized. (c) Then, the solution of MTs, which is cut into around 2.6 μm of length is injected into the microchannel. (d) After that, washing the microchannel by BRB 80 until the resonant frequency is stabilized.

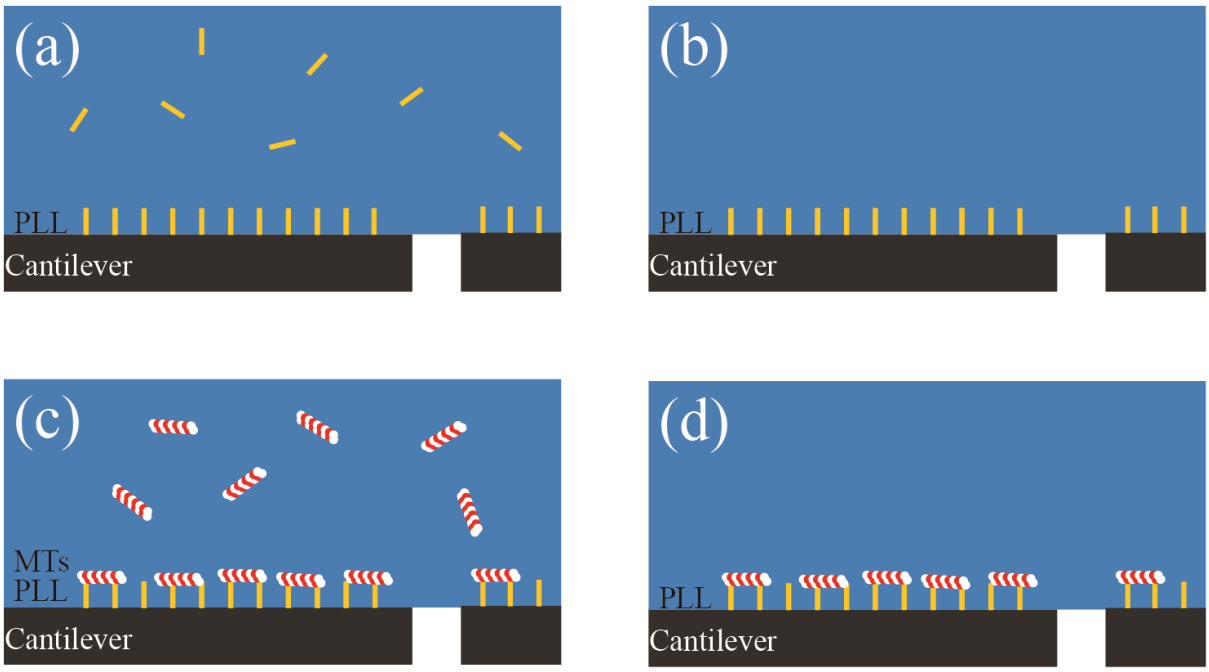

Figure 3-7-1. Schematic of MTs coating on the surface of cantilever biosensor. (a) Injecting PLL solution. (b) Washing microchannel by buffer. (c) Flowing MTs solution to the cantilever. (d) Washing microchannel by buffer.

Cantilever surface coated with PLL attracts MTs and then the surface is coated with MTs. After immobilizing MTs on the cantilever surface, the decrease of resonant frequency is from 2 kHz to 5 kHz (figure 3-7-2).

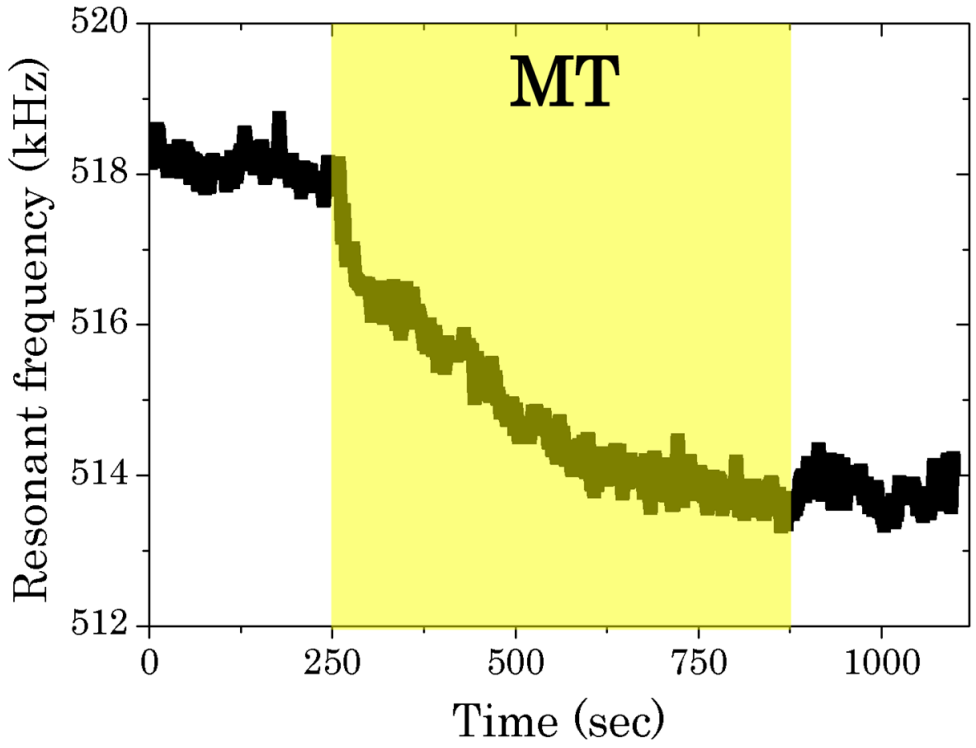

Figure 3-7-2. Real time monitoring of immobilizing MTs on the surface of cantilever.

3-8 Fluorescence results

MTs attached with fluorescence were flowed to cantilever for confirming the

attachment to cantilever surface. The monitoring result is shown in figure 3-8-1.

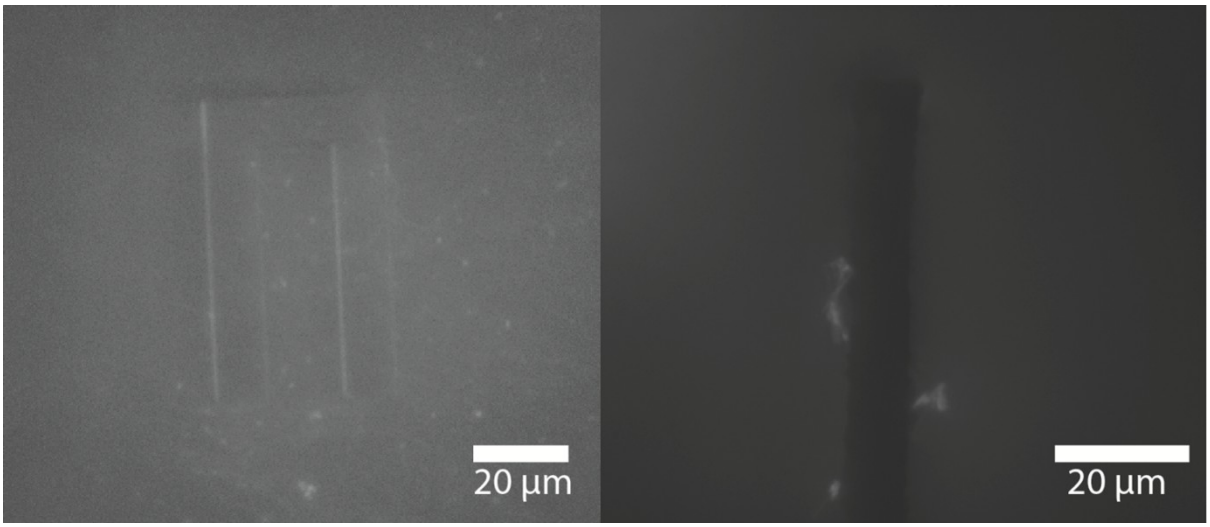

Figure 3-8-1. Fluorescence confirmation of MTs attached on the surface of cantilever. (a) Cantilever resonating at air/liquid interface. (b) Cantilever resonating in liquid.

From figure 3-8-1, I confirmed that MTs are successfully immobilized on the surface of cantilever.

4 Results

4-1 Real time monitoring, MT-Tau

After immobilizing MTs on the surface of cantilever, 1 μg/ml of tau proteins is flowed to the cantilever. The monitoring result of cantilever resonant frequency is shown in figure 4-1-1.

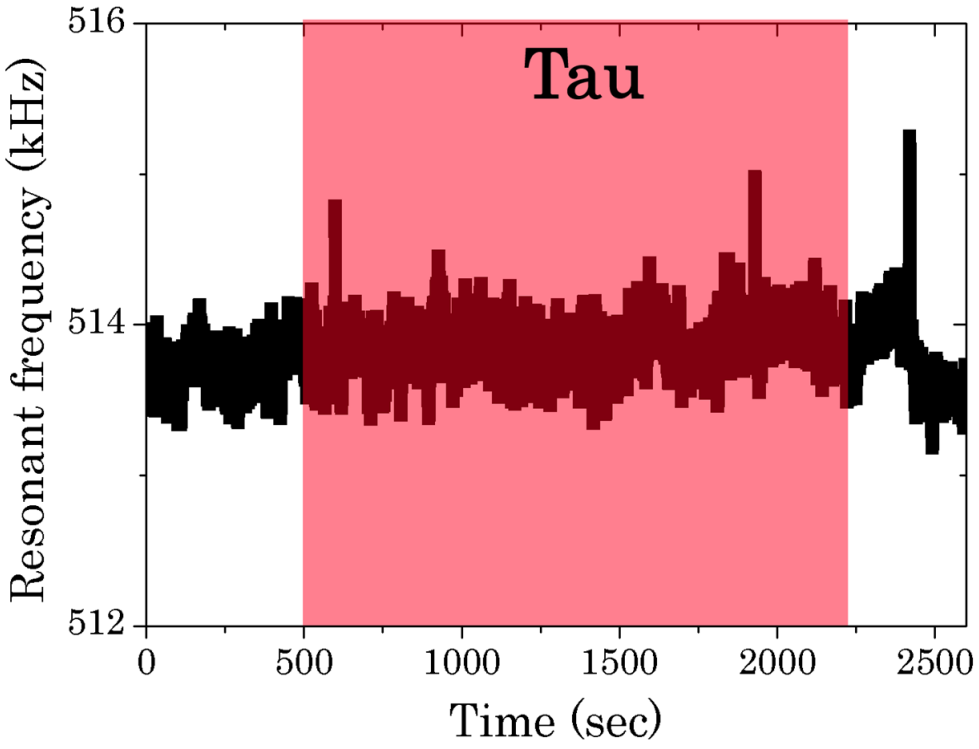

Figure 4-1-1. Real time monitoring results of resonant frequency when tau protein is injected

The decrease of resonant frequency by tau protein attachment to MTs is hard to be detected. The reason why the decrease isn't monitored in this experiment is that mass of tau proteins is comparatively smaller than the mass of MTs.

4-2 MT-Tau-Antibody

To confirm tau proteins are attaching to MTs, larger mass change is necessary for detection of resonant frequency decrease. Tau antibody (InG1, immunoglobulin G) is more than 3 times heavier than tau proteins (Tau proteins: 45 kDa, Antibody: 150 kDa). After tau attachment, flowing antibodies and tau-antibody attachment could be definitely detected by resonant frequency. The monitoring result of cantilever resonant frequency is shown in figure 4-2-1. The resonant frequency decreased gradually from 534.5 kHz to 534 kHz. Then, there was a sudden decrease from 534 kHz to 532 kHz.

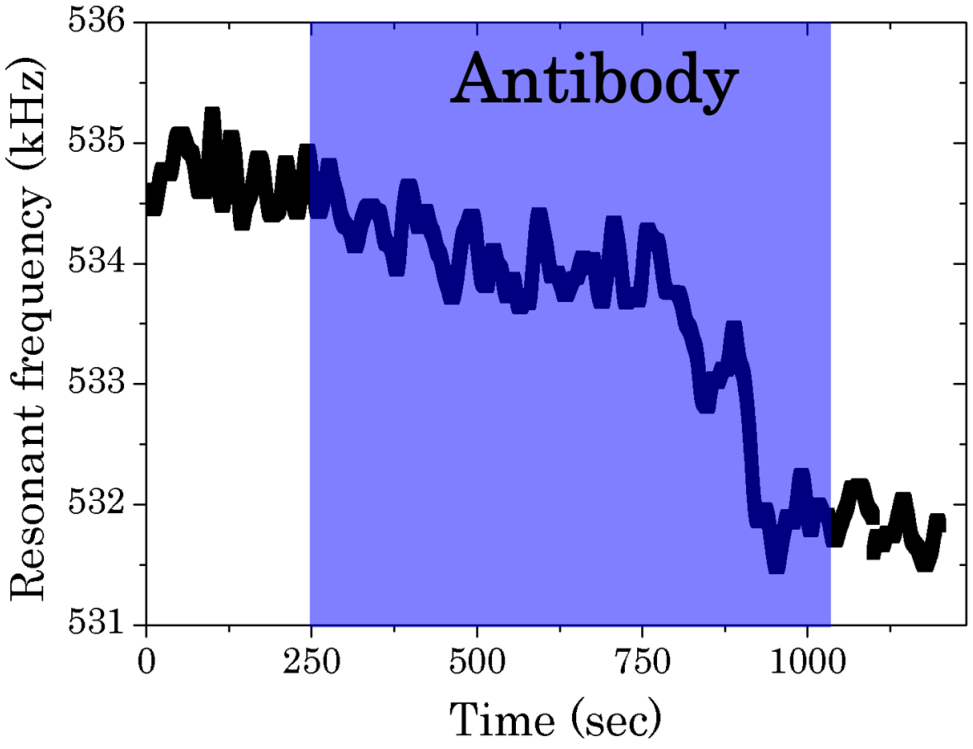

Figure 4-2-1. Real time monitoring results of resonant frequency when antibody is flowed

Before we could conclude that antibodies attach to tau proteins attached with

MTs. There are two issues to be cleaned. One is the reason why the sudden frequency change occurred. The other is antibodies' non-specific attachment; such as attachment between PLL and antibodies, can occur. Attachment between PLL and antibodies should be examined for fully understanding the mechanism of MTs and tau proteins attachment.

4-3 MT-Antibody, Antibody (without tau)

Some control experiments have been done for checking the second issue of the non-specific attachment of antibodies. First, antibodies were flowed after MTs coating on the cantilever surface (without tau proteins). The monitoring result of cantilever resonant frequency is shown in figure 4-3-1.

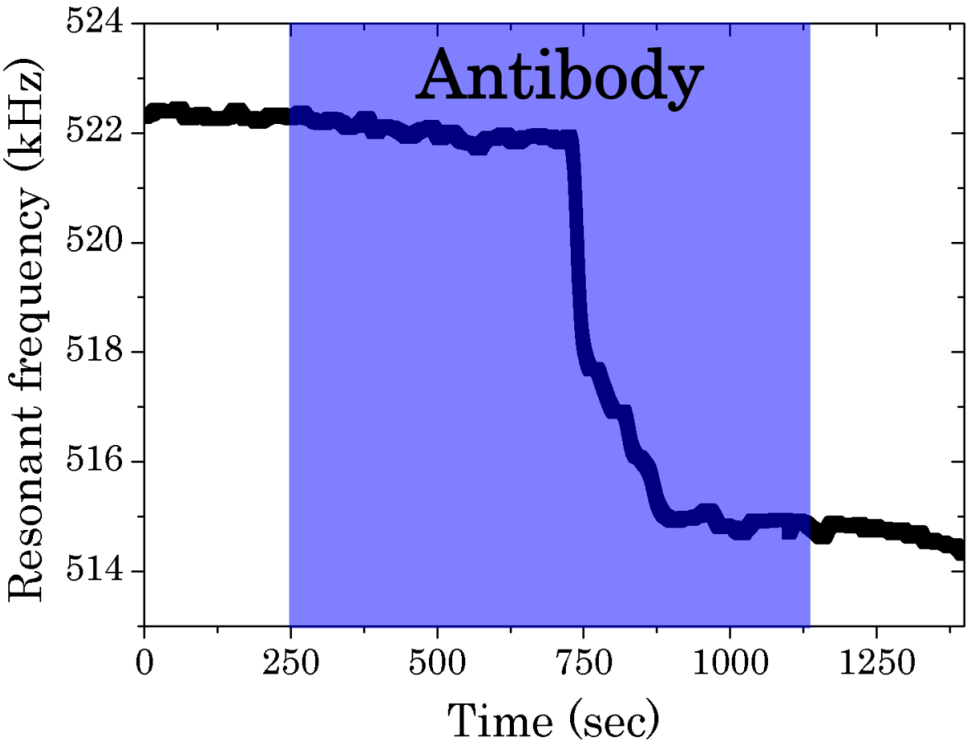

Figure 4-3-1. Real time monitoring results of resonant frequency when antibody is flowed without tau protein.

Even tau proteins din not exist, resonant frequency decreased when antibodies were flowed to cantilever. This means that antibodies attach to PLL or MTs. To know which materials antibodies attach to, antibodies were flowed after PLL coating on the cantilever surface (without MTs and tau proteins). The monitoring result of cantilever resonant frequency is shown in figure 4-3-2.

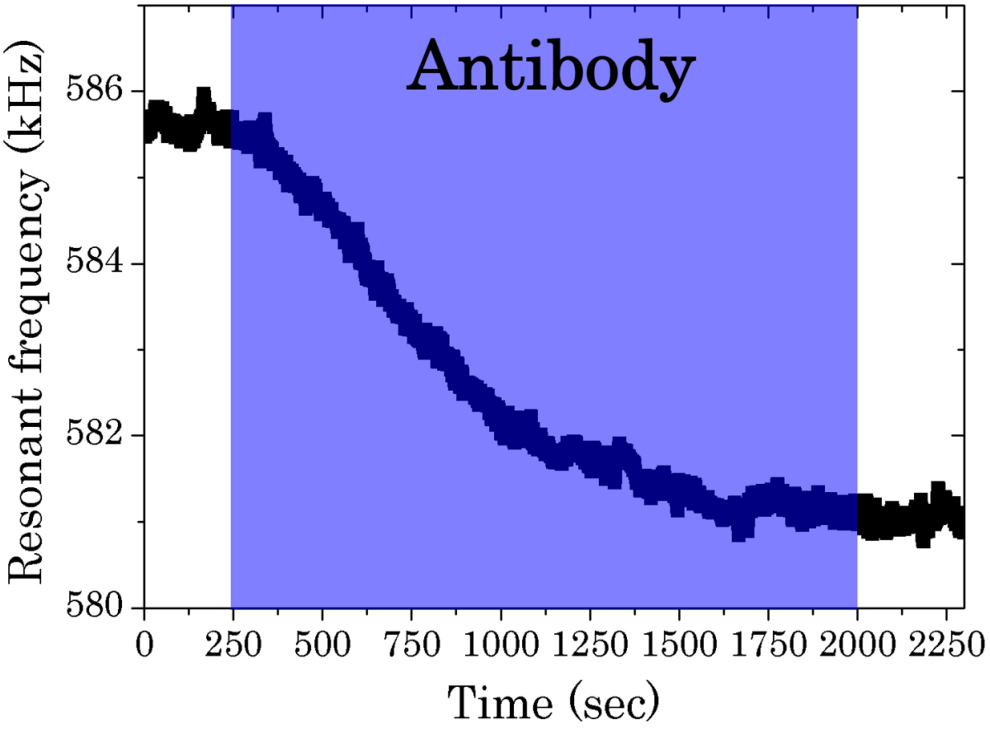

Figure 4-3-2. Real time monitoring results of resonant frequency when antibody is flowed without MTs and tau protein.

From figure 4-3-2, I confirmed that antibodies attach to PLL. Additional chemical coating is necessary for specific bonding of antibodies to tau proteins attached to MTs. Bovine serum albumin (BSA) is protein widely used for blocking biochemical reactions. BSA is also expected to block the PLL area where MTs are not coated. BSA was flowed to cantilever after coating with MTs. To confirm BSA coating, resonant frequency change when flowing antibodies after BSA coating was checked. The monitoring result of cantilever resonant frequency is shown in

figure 4-3-3.

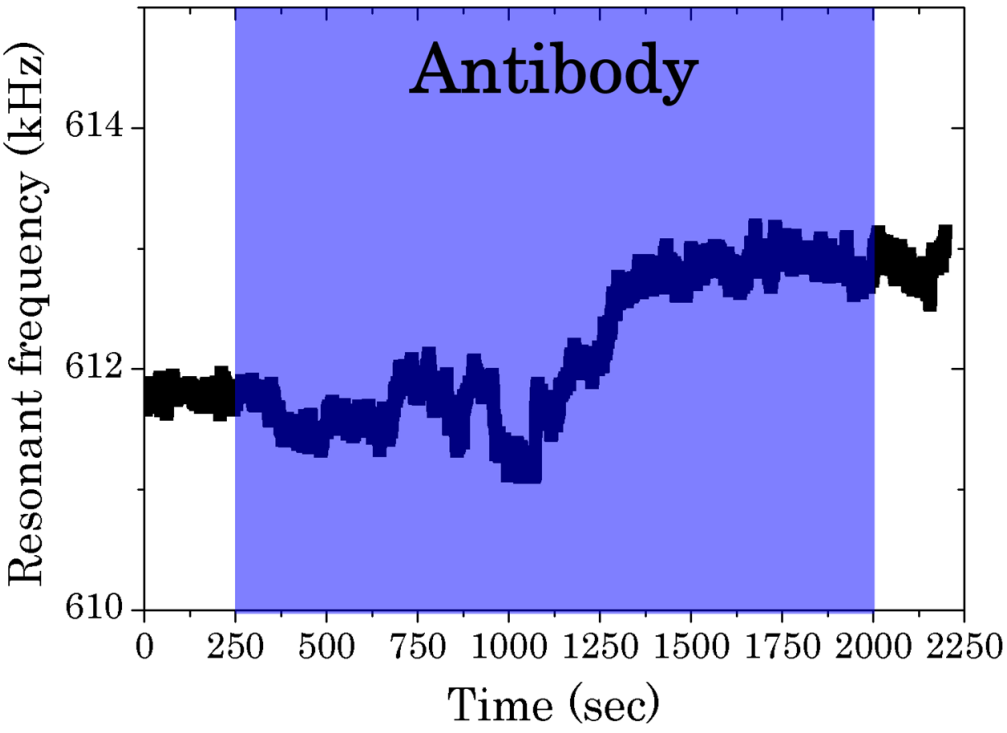

Figure 4-3-3. Real time monitoring results of resonant frequency when antibody is flowed after BSA coating.

From figure 4-3-3, BSA coating was successfully done and blocking the attachment between PLL and antibodies. However, sometimes resonant frequency was not stabilized and became out of range to monitoring after BSA coating to the cantilever surface. This phenomenon can be explained as follows: the chemical structure of BSA makes surface tension of solution decrease, and the position of the meniscus at the cantilever slit is lowered. To prevent instability of resonant frequency by meniscus, traditional cantilever detecting biomaterials in liquid state is fabricated. The size of cantilever is 160 $\mu m^{L} \times 10\ \mu m^{W} \times 5\ \mu m^{T}$. This cantilever is long enough to detect a resonant frequency at second mode for high sensitivity [47]. Resonant frequency at second mode has higher frequency of cantilever, and changes more sensitively by mass change (equation 1).

5 Discussion

5-1 Proteins mass and its resonant frequency change

One of tau protein attaches over two tubulins. The mass of two tubulins is 110 kDa and the mass of tau protein is 45 kDa. Consuming that all tau proteins are attached to MTs immobilized on the surface of cantilever, the mass ratio of two tubulins: one tau proteins is about 2.5:1. But the ratio of tubulin part is higher than this ratio. Two main reasons are that, some parts of MTs contacted with cantilever surface can't be bonded with tau proteins, and not all tau proteins attach to tubulins. From figure 4-4-1 and figure 4-4-2, the ratio of frequency decrease between MTs and tau proteins is almost 1.7:1. The ration of tau proteins is quite bigger than calculation results.

The ratio problem is more critical in mutant tau experiment. Mutant tau proteins have tendency not to attach to MTs. But the resonant frequency change is almost same at MTs and mutant tau bonding. This means, tau proteins are expected to attach not only MTs but also other area where no MTs surface. Surface process for specific protein bonding has to be developed more for correct and specific detection

5-2 Problems and proposed solutions

Before BSA coating, tau proteins are not detected by resonant frequency change. But after BSA coating, the decrease by tau proteins is detected. The reason how to detect tau proteins after BSA coating can't be fully explained. The relationship between BSA and tau proteins must be researched at the next step.

Step shaped decrease of resonant frequency is detected in experiments. It is expected that proteins are gathering each other and making some lumps. These lumps attach to cantilever and making sudden drop of resonant frequency. Mixing process such as pipetting is necessary for preventing protein lumps.

The state of BSA blocking must be confirmed again for specific bonding of tau proteins to MTs. Even BSA blocking the attachment between PLL and antibodies, attachment between PLL and tau proteins is not confirmed. If BSA blocking is not suitable for tau protein detection, other blocking materials such as casinein have to be researched in the future.

Developing more sensitive sensor is also one solution. Cantilever biosensor resonating at air state has smaller damping than air-liquid interface cantilever. If microfluidics are embed inside cantilever, cantilever can be resonated in air or vacuum state.

6 Conclusion

Detecting the attachment of proteins by using cantilever biosensors has been successfully accomplished by this research. Detected proteins at this research, such as MTs and tau proteins, are thought to be biomarkers of Alzheimer's diseases. In this research, possibility of new method to diagnose Alzheimer's diseases is proposed.

MTs length control for cantilever immobilization

For coating MTs on the cantilever surface, MTs are cut shorter than the length of slit for preventing bridge over cantilever slit. Some methods have been done, and cutting MTs less than 6 μm by vortexing was succeeded.

Functionalization of cantilever surface for tau proteins detection

Cantilever made by silicon has to be functionalized for detecting attachment of proteins. Sputtering for making cantilever surface hydrophilic, chemical processes for immobilizing MTs on the cantilever surface have been succeeded. After chemical process, MTs are immobilized on the surface of cantilever. Mass change by MTs immobilization is successfully monitored by resonant frequency change and fluorescence.

Detecting tau proteins attachment

After MTs immobilization, attachment of tau proteins is also monitored by resonant frequency change of cantilever biosensors. But tau proteins are thought to be not only attached with MTs but also BSA blocking proteins. In this research, it is not completely understood to detect specific protein attachment. To realize this, proper surface functionalization is developed in the future.

As population aging dramatically in the future, possibility of early diagnosis of Alzheimer's diseases proposed in this research will have great impact in the

future. Early diagnosis of Alzheimer’s disease will decrease patients need care in everyday and make everyone in the society healthy.

7. Appendix: Chemical blocking for antibody's specific bonding

7-1 MT-BSA-Tau-Antibody

Cantilever immersed in liquid was used for tau detection including BSA coating process. After MT immobilization, BSA is flowed to coat an area where MTs are not immobilized. BSA is expected to block an area where MTs are not immobilized and prevents the attachment between PLL and antibodies. The monitoring results of cantilever resonant frequency are shown in figure 7-1-1 to figure 7-1-3. Figure 7-1-1 shows the result of flowing MTs for tau protein detection. Figure 7-1-2 shows the result of flowing tau proteins after MTs immobilization and BSA coating. Figure 7-1-3 shows the result of flowing antibodies after MTs immobilization, BSA coating, and tau protein attachment.

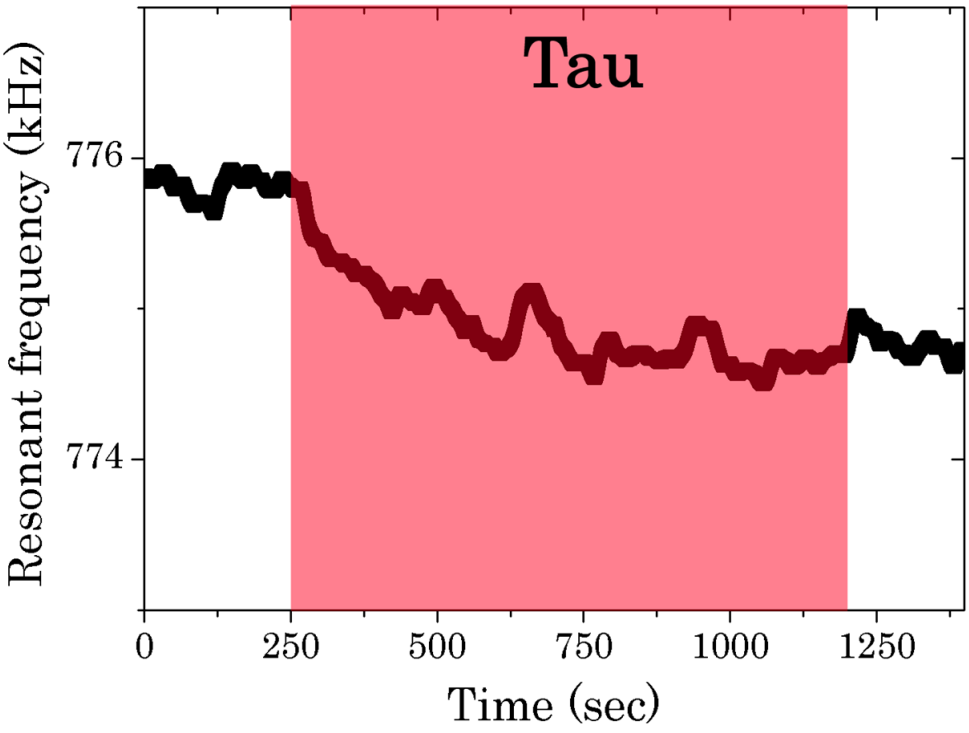

Figure 7-1-1. Real time monitoring results of resonant frequency when flowing MTs.

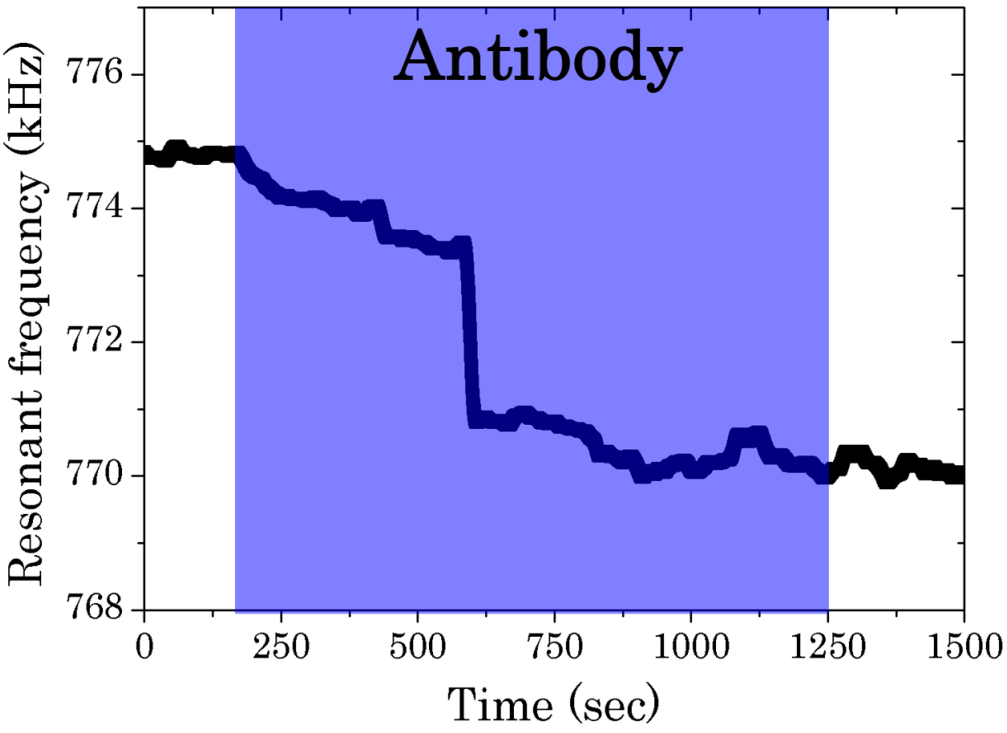

Figure 7-1-2. Real time monitoring results of resonant frequency when flowing tau proteins after MTs immobilization and BSA blocking.

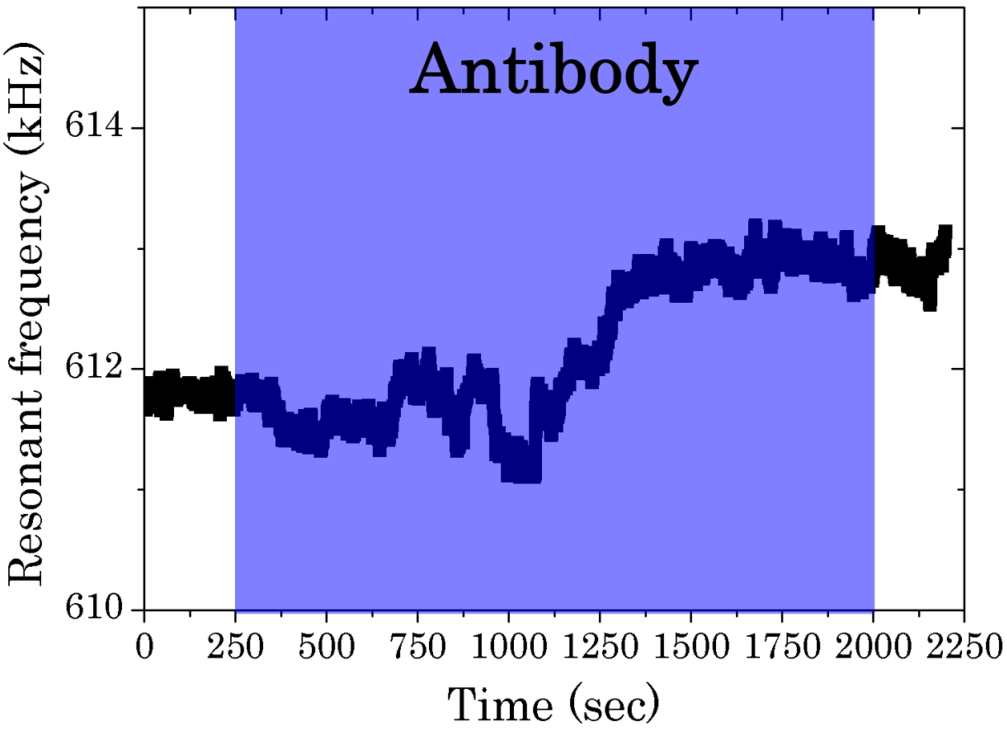

Figure 7-1-3 Real time monitoring results of resonant frequency when flowing antibodies after MTs immobilization, BSA blocking, and tau proteins attachment.

The attachment between tau proteins and MTs was successfully monitored from figure 7-1-1 and 7-1-2. Also from figure 7-1-3, antibodies attachment to tau proteins bonding with MTs could be checked.

7-2 MT-BSA-Mutant tau-Antibody (cantilever in liquid)

The attachment ability of mutant tau proteins, that are expected to have lower attachment ability compared with healthy tau proteins, is monitored by cantilever biosensor. Mutant tau proteins and antibodies are flowed to cantilever biosensor after MTs attachment and BSA blocking. The monitoring result of cantilever resonant frequency is shown in figure 7-2-1 to 7-2-3.

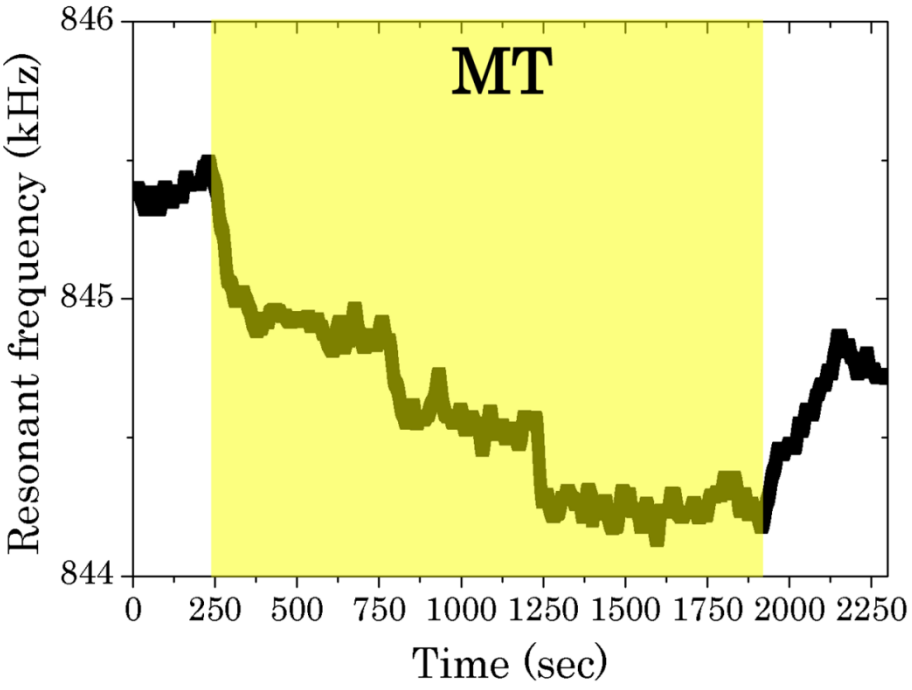

Figure 7-2-1. Real time monitoring results of resonant frequency when flowing MTs.

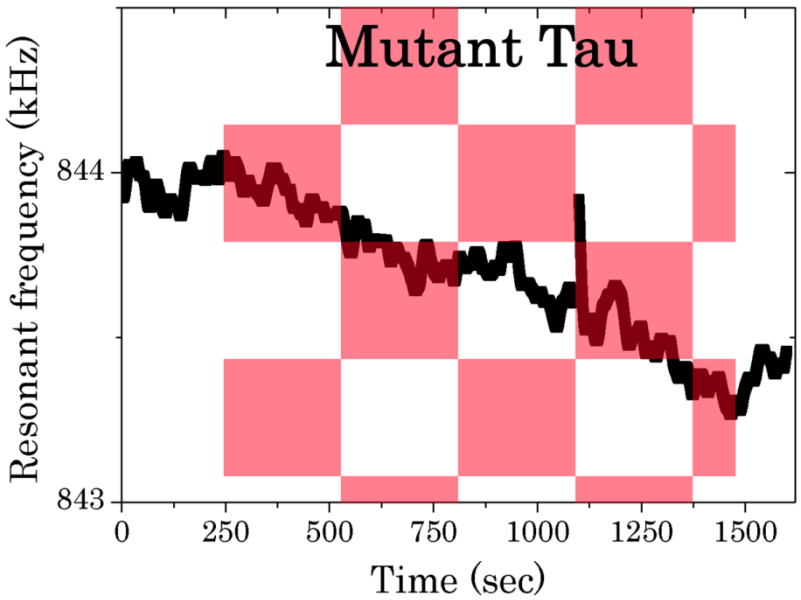

Figure 7-2-2. Real time monitoring results of resonant frequency when flowing mutant tau proteins after MTs immobilization and BSA blocking.

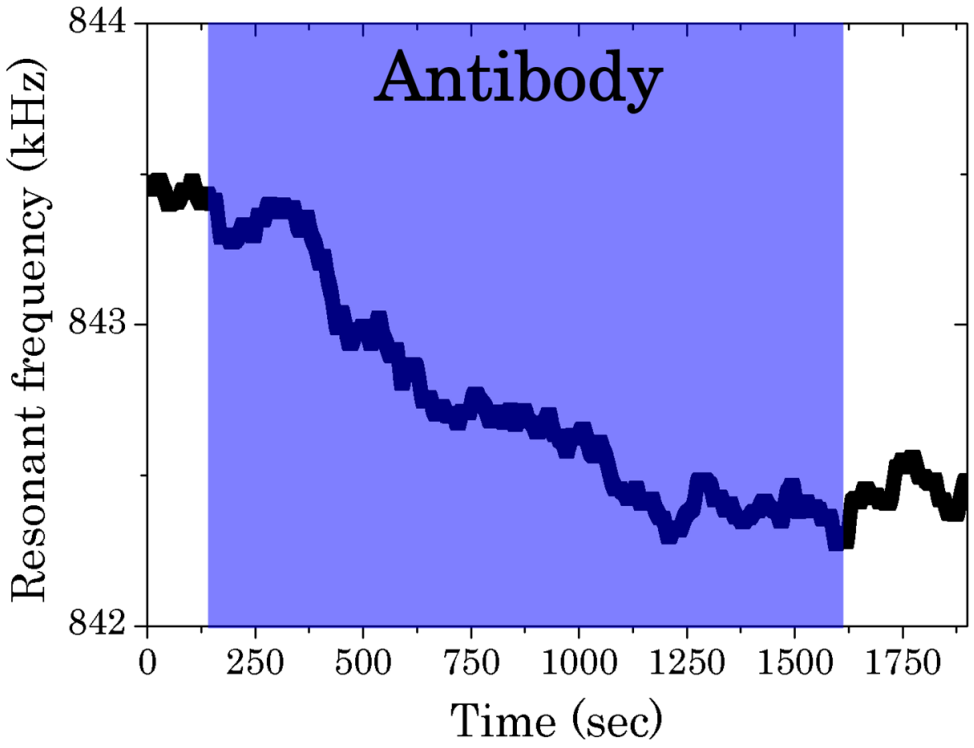

Figure 7-2-3 Real time monitoring results of resonant frequency when flowing antibodies after MTs immobilization, BSA blocking, and mutant tau proteins attachment.

The attachment between mutant tau proteins and MTs was monitored from figure 7-2-1 and 7-2-2. Though resonant frequency decreases as some of mutant tau proteins attach to MTs, the amount of decrease is much smaller than the decrease by healthy tau proteins in figure 7-2-2. Also from figure 7-2-3, antibodies attachment to mutant tau proteins bonding with MTs is smaller than antibodies with healthy tau proteins in figure 7-2-3.

Publication

International conference

[1] Jisu Lee, K. Yagi, M. Kumemura, T. Sato, L. Jalabert, N. Lafitte, D. Collard, H. Houjou, and H. Fujita, "Characterization of Π-conjugated metallopolymer's mechanical stiffness by using silicon nanotweezers", The 17th International Conference on Solid-State Sensors, Actuators and Microsystems (IEEE Transducers 2013), Barcelona, Spain, Jun 16-20, 2013, Oral Presentation.

Domestic conference

[1] Jisu Lee, Jungwook Park, Stanislav L. Karsten, Hideki Kawakatsu, and Hiroyuki Fujita, "Continuous monitoring of protein attachment and its enzymatic digestion using a biosensor resonating at air-liquid interface", The 29th SENSOR SYMPOSIUM, Kitakyusyu Japan, Oct 22 - 24, 2012, Oral presentation.

Workshops

[1] Jisu Lee, Jungwook Park, Stanislav L. Karsten, Hideki Kawakatsu, and Hiroyuki Fujita, "Cantilever biosensor resonating at air-liquid interface", NAMIS Marathon Workshop, National Tsing Hua University, Taiwan, Dec 14-16, 2012, Oral presentation.

[2] Jisu Lee, K. Yagi, M. Kumemura, T. Sato, L. Jalabert, N. Lafitte, D. Collard, H. Houjou, and H. Fujita, "Silicon nanotweezers for characterizing stiffness of metallopolymer", NAMIS Marathon Workshop, National Tsing Hua University, Taiwan, Dec 13-16, 2013, Oral presentation.

Others

[1] Jisu Lee, T. Odera, M. Kobayashi, T. Iida, A. Chikamoto, T. Nakamura, A. Takayanagi, K. Nishikawa, T. Miyatake, N. Wake, Y. Nishiwaki, "DNS Shell by Team UT-Hongo", BIOMOD, Wyss institute, Harvard University, Boston, USA, Nov 3-4, 2012, Oral presentation.

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