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Micromanipulation System for Handling of Biological Molecule and High Throughput Screening of Microbes

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Chapter 1

Introduction

1.1 Micromanipulation

1.1.1 Micromanipulator

Many types of robotic manipulators have been studied, some of which are aiming at handling small objects ranging from millimeter to micrometer order of size. Grasping and clamping are required to handle much smaller objects. Fukuda et. have reported a bilateral control method [1] of such micromanipulators, requiring the contractive position and force controls, and also a visual recognition method [2] of objects for the micromanipulator. It is advantageous to develop a small manipulator itself in size for handling small objects rather than to use the conventional relatively larger size of manipulator. Thus, the actuator employed at such a micromanipulator is required to be much smaller than ever and to have more degrees of freedom. In this sense, it turns out to be difficult to use an ordinary electro-magnetic motors. Therefore, the development of a new actuator [3–5] has been desired.

1.1.2 Needs for Bio-Micromanipulation

Recently, bio-science is progressed so much with the advancement of the bio-technology, such as gene engineering, cell engineering, as shown in Fig.1.1.

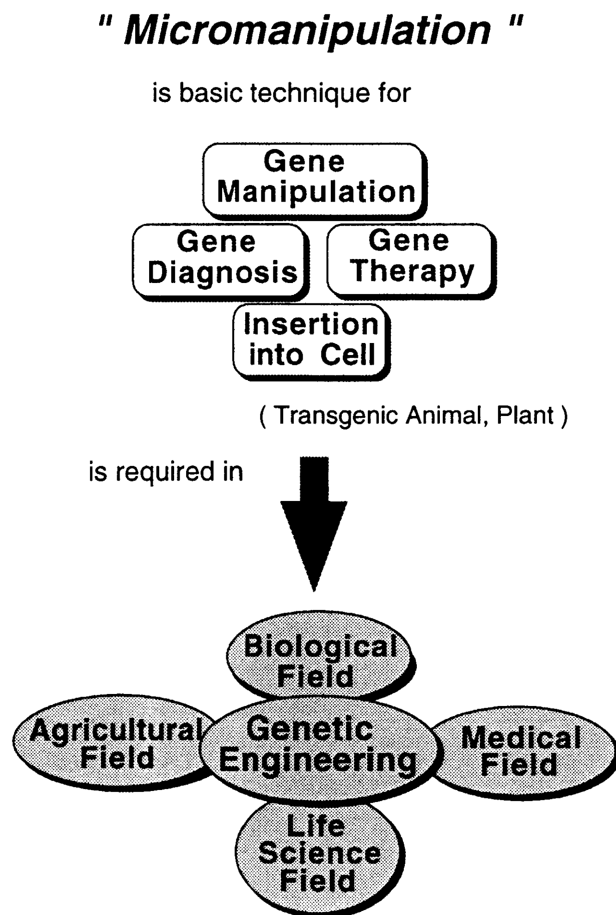

Figure 1.1: Biological Application Field of Micromanipulation

In these research fields, operation of the micro/nano manipulation, mass production, repetitive processing, and high speed and high precision processing are required. For the breakthrough in these fields, integration of the distributed research fields and system technologies is important. At this moment, conven-

tional robotics has potentiality for the breakthrough in the bio-engineering. We should integrate the robotics and bio-engineering. We call this field of study as the bio-robotics [8].

Basic technologies of the bio-robotics are manipulation technology, micro system technology, visualization technology, human interface technology to improve operability, automation technology, and so on. Especially, micro/nano manipulation of a DNA molecule, animal or plant cell, and embryo is important. However, research works are not enough at present.

Micromanipulation can roughly be classified into contact type [6, 7] and noncontact type. We present a new direction and new methods of the noncontact-type bio micromanipulation.

As an example of the contact tasks with the conventional micromanipulator, nuclear transplantation or embryo culture requires those technologies. Most of the bio micromanipulation tasks are performed in water solution. Thus, compared with the general manipulation tasks in the air [6, 7], we have to consider many different kinds of forces acting on the cell in solution such as the gravity, buoyancy, resistance force from viscosity of the fluid, Brownian motion, interactive forces in the micro/nano world, as shown in Fig.1.2, such as Van der Waals force, electrostatic force depending on the surface charges. In the contact manipulation, as shown in Fig.1.3, the internal force is generated at the object. It is important to control the internal force precisely. It is not easy to control its force autonomously. To realize the flexible manipulation, we have to consider an integrated micro force sensor on the microgripper.

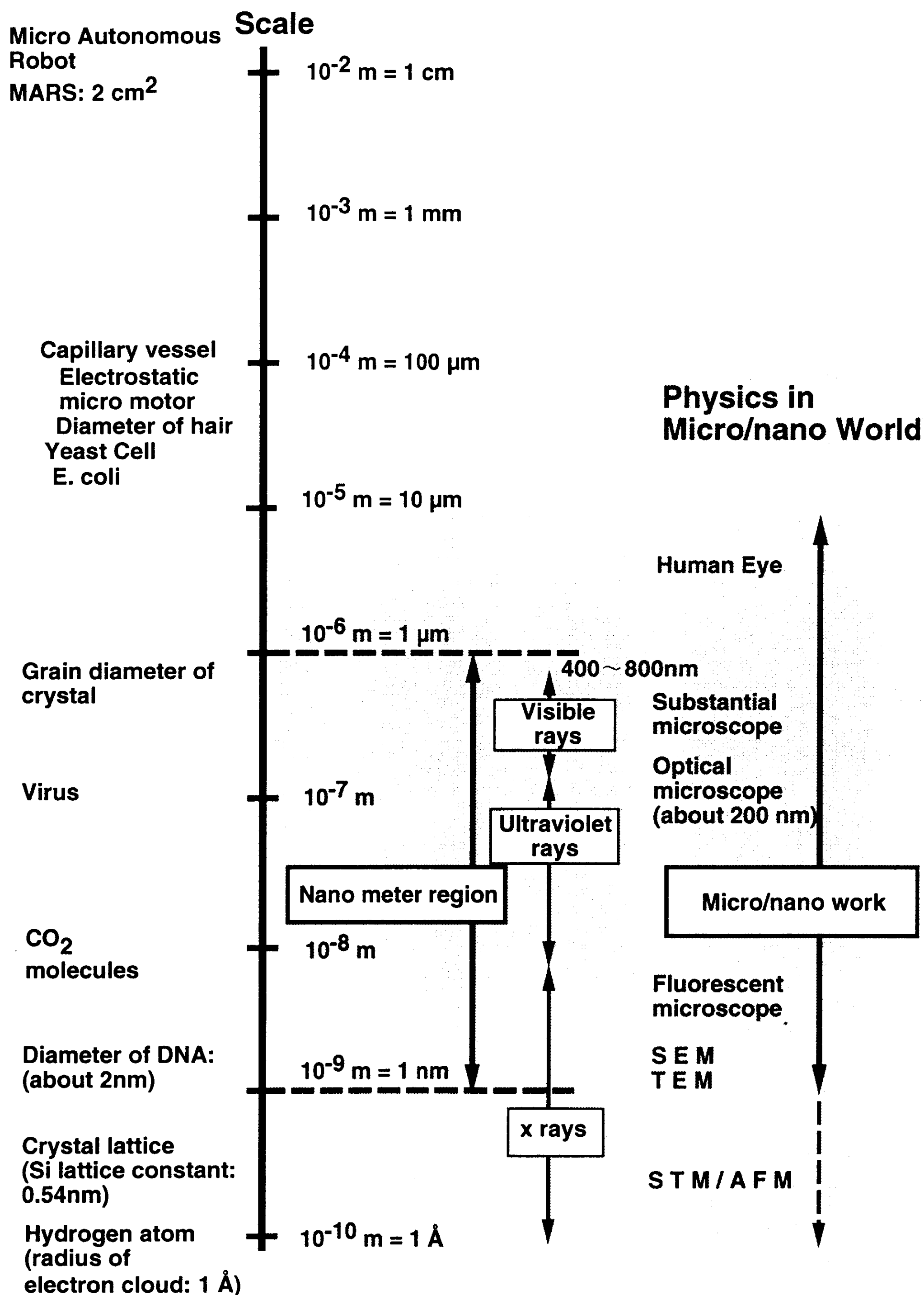

Figure 1.2: Micro/Nano World

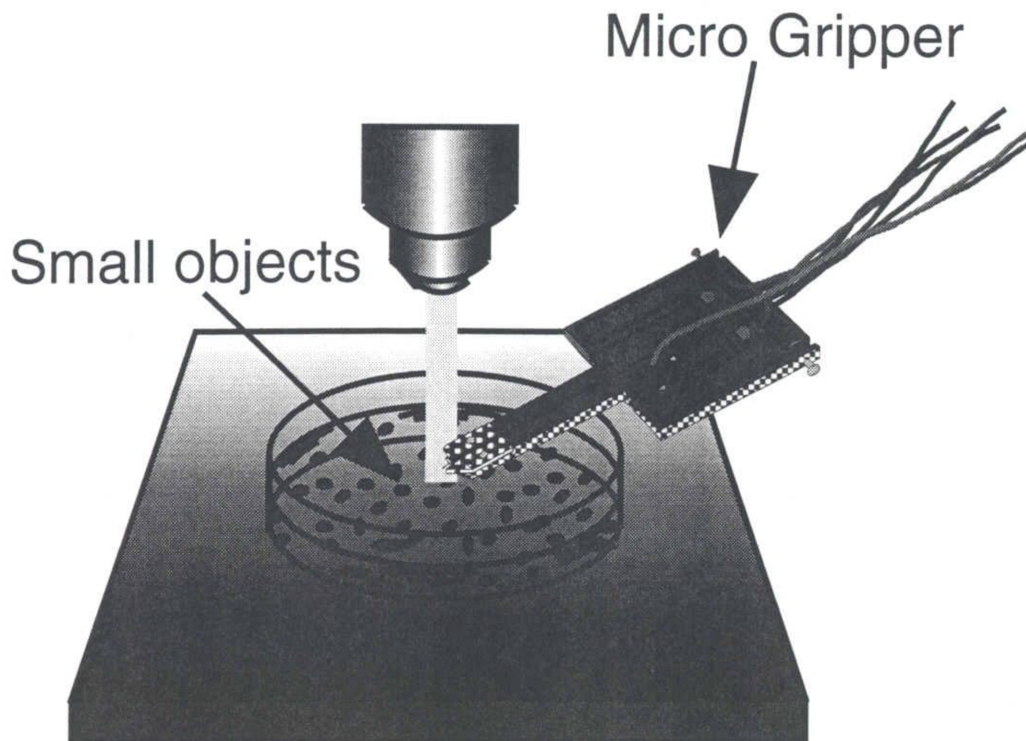

Figure 1.3: Micromanipulation with MicroGripper [7]

Moreover, since size of each manipulation object is in micron order, it is quite difficult to recognize its configuration and to manipulate it freely. Automation of manipulation is also quite difficult and skills of human operator are required in contact tasks. Moreover, if there is a flow in a solution, fluid force will disturb its motion. To reduce the influence on the environmental condition and control their migration, we have to consider more suitable forces acting on them. And also if we operate the conventional micromanipulator in observing biological cells with a microscope and manipulate them with it, it is difficult to handle them sequentially. In this way, as described above, we have difficulties in developing a micromanipulation system in a contact type,

which is more complicated systems and restricted to limited tasks, to realize autonomous function.

On the other hand, if we apply the external force to the biological object in a solution in noncotact ways, not in a cotact way, we can achieve the sequential tasks, such as transportation, manipulation, separation of biological molecules, by controlling its suitable force acting on them. And also, because almost all of the biological materials are soft and flexible materials, it is easier to manipulate them in noncotact ways than in contact ways when we handle biological objects noninvasively.

Then, we consider how to handle a biological molecule in a noncontact way and discuss the methods of controlling the energy field in bio micromanipulation. The methods to apply external force acting on the biological object in a noncontact way are described as follows.

(1) Electric Field

Coulomb Force (D.C. field)

Dielectrophoretic Force (A.C. field) [14, 37, 111, 112]

(2) Laser Beam [46, 47, 77, 78]

(3) Ultrasonic Field [39, 40]

(4) Fluid Flow of Solution

Fig.1.4 depicts the concept of biosynthesis based on field control. Tab.1.1 shows the methods and characteristics of controlling the energy Field in bio

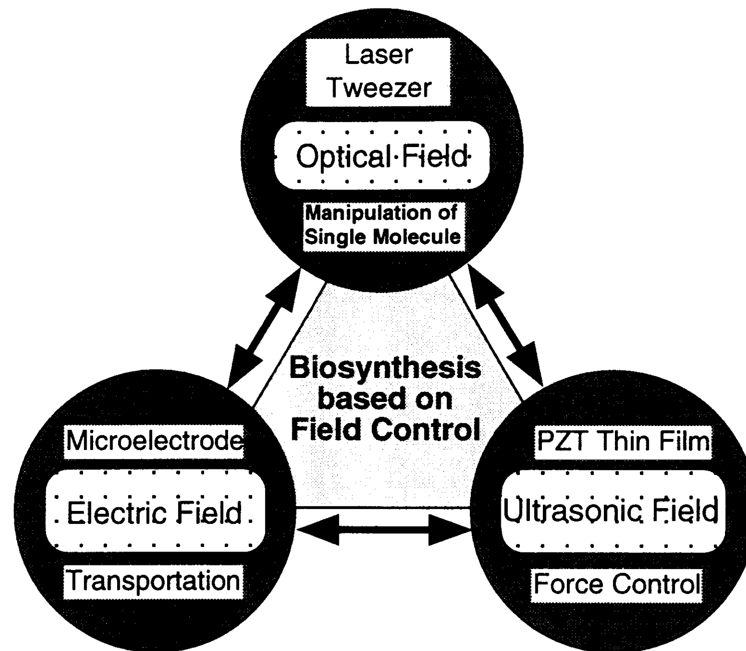

Figure 1.4: Biosynthesis based on Field Control

micromanipulation. Among these for us, Laser Beam and Ultrasonic Field require position information of the object, and the sensing system is needed for automation. When we consider precise and noninvasive handling of micro objects, Laser Beam is suitable tool. Ultrasonic Field will disturb the environment with the fluid flow. Tab.1.2 shows the comparison of the energy field to control the biological object. For these reasons, we can simplify the system by using the electric field and laser tweezers. In case of using coulomb force, convection caused by electroosmosis, polarization of the microelectrode, and electrolysis will occur easily. So, we propose to use dielectrophoretic force to control the biological object, especially giant DNA molecules, and microbes.

Table 1.1: **Methods of Controlling the Energy Field in Bio Micromanipulation**

	Application	Control Method	Selectivity
Electric Field	Transportation Separation	High Frequency Electric Field Patterning of Microelectrode	Difference of Dielectric Properties of Particles
Optical Field	Trapping of Single Object	3D Confocal Scaning of Laser Beam with Mirror	Trapping Particles larger than the wave length of Laser Beam
Ultrasonic Field	Filtration Fixation	Configuration of Micro Resonator	Difference of Size and Density of Particles

Table 1.2: **Comparison of the Energy Field**

	Transportation	Selectivity	Controllability	Fixation	Resolution	3D Operability
Electric Field	○	◎	◎	○	○	◎
Optical Field	△	△	◎	◎	◎	○
Ultrasonic Field	△	○	○	◎	△	△

◎ : Good, ○ : Fair, △ : Poor

1.2 Biomedical Application to Micromanipulation in MEMS

1.2.1 Micro Fluidic Devices and Micro Chip Technology

Basic technologies required for the bio micromanipulation are classified as follows: separation, transportation, and position orientation, control technology, contact, noncontact manipulation with high speed and high precision. Fig.1.5 shows the history of the development of the biotechnology and biomedical study and application to Micromanipulation in MEMS.

Electrophoresis is a powerful separation technique that has been used for years, especially in biochemistry and biology, for the separation of large charged

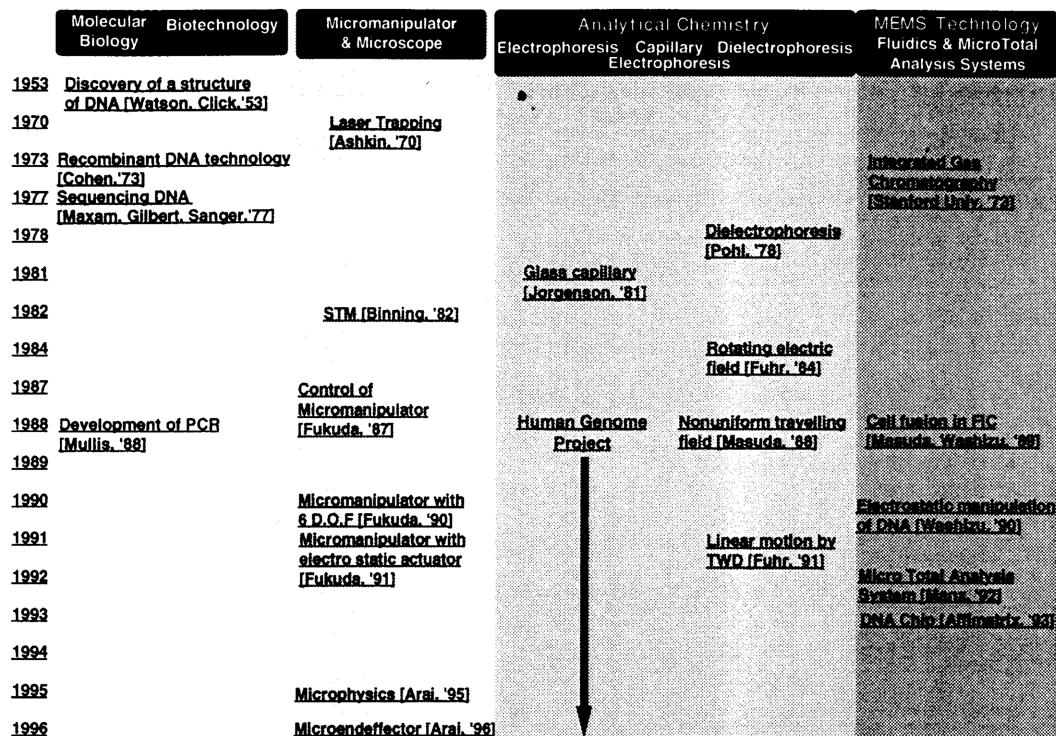

Figure 1.5: History of the Development of the Biotechnology and Application to Micromanipulation in MEMS

molecules such as proteins. It is based on the differential migration of charged species under the influence of an electrical field. Although this concept can be implemented in various ways, it has been mainly used as gel electrophoresis in which separations are performed in slabs or tubes of gel.

In 1981, Jorgenson performed the electrophoresis experiment in a small capillary with an internal diameter of $100\mu m$ or less, extending the concept of electrophoresis and achieving enormous plate counts [9]. At high field strengths, the small dimensions of the capillary provide efficient heat dissipation, prevent convection, and separate with theoretical plates in the millions. This extraordinarily high efficiency has been applied to categories of molecules that

previously had been separated by HPLC, such as organic oligomers and small organic and inorganic compounds.

Recently, micromachining, microlithography, optical fibers, ultramicroelectrodes, and nanotechnology are having a dramatic impact on analytical chemistry. A. Manz proposed "micro total analysis system" [53, 54, 57, 58]. Microchips with micrichannels and reservoirs provide a way to manipulate small volumes for sample handling or separations [48,50,55,59]. Microfabrication and microfluidic techniques [49,52] have been progressed toward small volume handling and integrated microdevices [51,56] capable of chemical and biochemical analysis. The trend in chip technology [60] has appeared to be toward smaller devices. The novel application of microtechnologies and miniaturisation to the pharmaceutical industry provides tools and techniques for all areas of DNA separation [61–63] and the drug discovery process and clinical diagnostics. The study of system integration [64–67] will be much more needed in future.

Compared with capillary electrophoresis by D.C. electric field, the principle of dielectrophoresis, proposed by H. A. Pohl [37], is defined as the lateral motion imparted on uncharged particles as a result of polarization induced by nonuniform electric fields. Many theoretical and experimental studies have been investigated. Microelectrode structures [18, 22], capable of exerting dielectrophoretic force [14, 16, 17, 19, 99, 103, 104], electrorotational force [10–12,109,118] and travelling field force [15,20,23,101,102,105,106] on bioparticles, are being developed to perform a wide range of applications to bioprocessing functions [21, 26], such as manipulation, trapping, transportation, sep-

aration, cultivation [11,13,24,25,100,107,108,117,119]of biological molecules.

1.2.2 Human Genome Project and DNA Sequencing

For the Human Genome Project [28], the key to sequencing thousands of unidentified human genes is to automate and speed up the special equipment, such as microfluidics that prepares and sequences DNA samples. Determining the human genome sequence and finding the genes is really just a first step. Fig.1.6 depicts the history the development of DNA Technology. After sequencing, we still need to determine what proteins the genes produce, and what those proteins do in the cell. Once the genetic code for a disease is broken, gene and drug therapies can follow. We still need to know the structure and function of the protein produced by the gene, and how that protein interacts in the environment of the cell.

Genes and the proteins hold the key to unlocking the mysteries of genetic diseases. Once the genetic code for a disease is understood, researchers can begin developing gene and drug therapies for that particular disease. The ultimate goal of the worldwide Human Genome Project is to find all the genes in the DNA sequence, develop tools for using this information in the study of human biology and medicine, and improve human health. Sequencing involves determining the exact order of the four individual chemical building blocks, or bases, that form DNA.The total DNA in a single human cell has approximately 3 billion pairs of the chemical building blocks adenine, projects to detect biological signatures of collected samples and in bioremediation projects

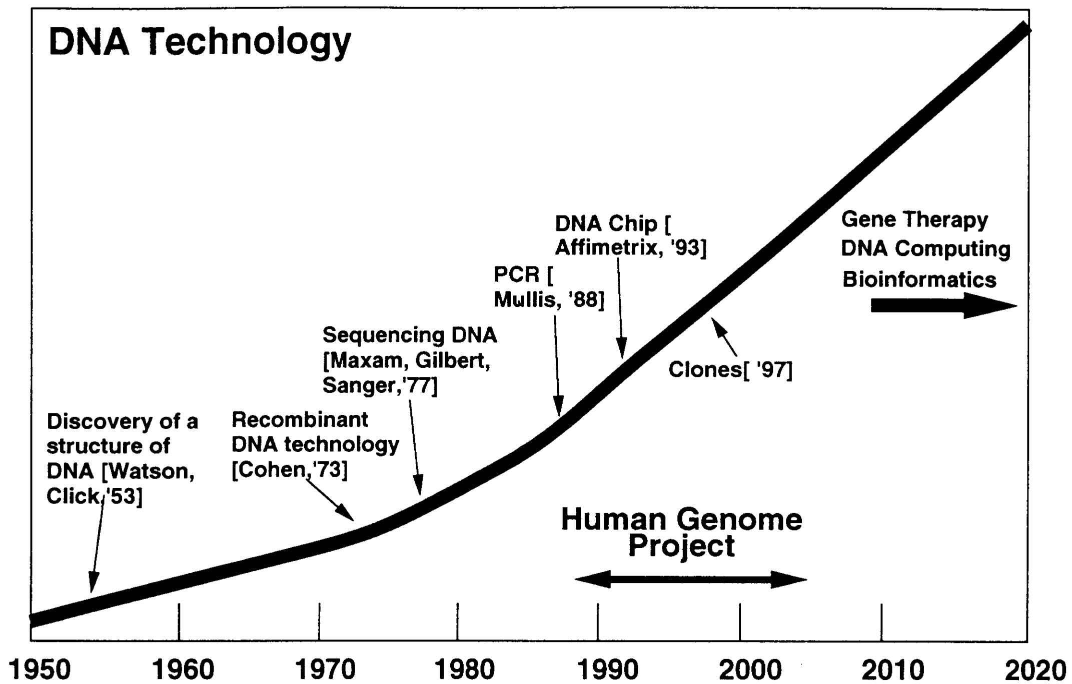

Figure 1.6: History of the Development of the Biotechnology and DNA Technology

to optimize micro-organism action.

When biological researchers want to sequence a section of DNA (Fig.1.7), they clone fragments of that section and then run four nearly identical reactions on those fragments [27]. In these reactions, the four bases are chemically labeled with four different fluorescent dyes. To sequence a section of DNA, researchers first use special enzymes that act as biological scissors to cut DNA at specific points into smaller fragments. They then clone or make hundreds of identical copies of these fragments. When researchers wish to sequence a fragment, they run four nearly identical reactions using that DNA as a template, in which the four bases are chemically labeled with four different fluorescent dyes [84]. An electric current is then applied to the gel, and, because

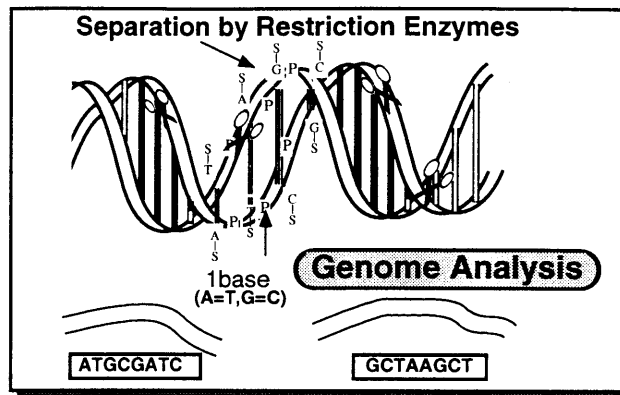

Figure 1.7: Genetic Analysis

the DNA itself has a negative charge, the fragments migrate from the top to the bottom of the plate. The DNA fragments move at different rates depending on their size: smaller ones move faster than larger ones. As the fragments migrate past a certain point in the gel, a laser beam scans back and forth across the plate, exciting the dyes on the DNA bases. As the fragments pass the laser, the bases are separated from smallest to largest. The fluorescent signals generated by the laser are detected by photomultiplier tubes, and a computer captures,

stores, and processes them [68].

Another way to speed up the process is to increase the electric field. The velocity of the DNA increases proportionally. In the current system, however, just increasing the field leads to other problems (Fig.1.8).

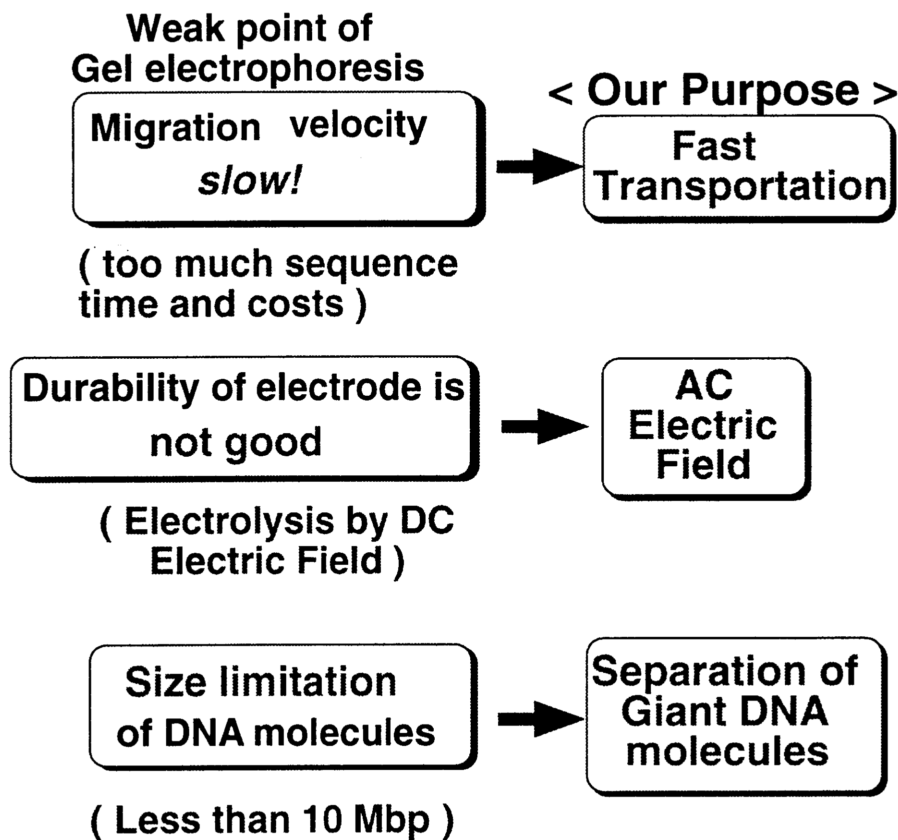

Figure 1.8: Weakpoint of Gel Electrophoresis

A higher electric field increases the power dissipation, which increases the temperature in the sieving media. And when the gel heats up and the DNA samples in it, thermal diffusion causes the fluorescent bands to spread out. The bands run into each other and can no longer be identified as individual and distinct bands. This problem can be significantly reduced by using a

very thin gel (about 50 micrometers thick) or other sieving media in place of the polyacrylamide gel now being used. The thinner gel means that the temperature gradient across the width of the gel is smaller, and the thermal diffusion of the DNA fragment bands is less.

With this thin sieving media, the instrument can run with an electrical field three to four times higher than that used on the conventional instrument. Thus, the speed of the run increases by the same factor.

Recent advances in genetic-engineering technologies then made it possible to examine and sequence DNA faster and more efficiently than ever imagined. For instance, because the DNA fragments are moving at a higher velocity when they come to the laser, the laser has to scan across the plates faster. In addition, given the 96 lanes now and the 384 to come in the future.

Other systems being developed include the laser-induced fluorescent detection system, the fluidic and pumping system for the polymer medium, a temperature control system, and analysis software. These improvements plus the microchannel plates themselves add up to major parts of the high-throughput DNA sequencer. The final production system is still down the road.

1.2.3 High Throughput Screening of Microbes

Many biologists are eager to manipulate and separate a single biological particle for their experiment. With the conventional method, it takes so many times to reculture on plates. This method has such strong probability for pure culture of targeted microbes, such as a Escherichia coli. It will be such

a promising method of pure culture of microbe for the biologists to discover a new microbe. It is possible to manipulate microbes, such as a Escherichia coli and a yeast cell, with the Laser manipulator we used. Once a target Escherichia coli is trapped at the focal point of the laser beam, we can easily realize noncontact manipulation automatically.

1.3 Motivation and Goals

In this study, the manipulation of DNA molecules is defined as "transportation" and "separation". We analyze the transportation of the DNA molecule by dielectrophoretic force. Since a high field gradient can be obtained between the electrode gaps based on the electrode pattern, the dielectrophoretic force, which is in proportion to the magnitude of the field gradient, is generated between the electrode gaps and it is possible to transport DNA molecule along the flow channel. It is considered to be possible to separate a DNA molecule when DNA molecules are continuously transported. By using micromachine technology, it is possible to miniaturize the electrodes and the flow channels, so that the basic technique of manipulation of individual DNA molecules will be advanced. We show that transportation of DNA with the state of compacted globule is profitable in the future practical application for the separation of giant DNAs such as human gene.

Then we propose new methodology of selective transportation system of Escherichia coli by dielectrophoretic force and optical radiation pressure and carried out a preliminary experiment. We demonstrated the experiments.

Thus, the bio-automation will be greatly improved by the proposed micro manipulation technology in the future.

1.4 Organization of this Dissertation

This Thesis consists of 6 chapters. Fig.1.9 shows the organization of this dissertation.

Chapter 2 describes noncontact transportation of DNA molecule by dielectrophoretic force. A simple micro electrode-flow system is designed and experiments were carried out. Experimental demonstration of DNA transportation using dielectrophoretic force and direct observation of the DNA molecule in a non-uniform electric field was carried out with fluorescence microscopy. Motion of the DNA molecule is modeled in terms of the electric field generated by the electrode design.

Chapter 3 describes a new methodology on noncontact transportation of DNA molecules by dielectrophoretic force together with the Micro DNA Flow System. We utilize the conformational transition in the higher order structure of DNA for transportation. We designed a simple micro electrode-flow system. Experimental demonstration of DNA transportation in the globule state using dielectrophoretic force and direct observation of the DNA molecule in a non-uniform electric field were carried out with fluorescence microscopy. We show that transportation of DNA with the state of compacted globule is profitable in the future practical application for the separation of giant DNAs such as human gene.

Chapter 4 describes a new methodology of selective transportation system of Escherichia coli by dielectrophoretic force and optical radiation pressure. We have developed a prototype of Microchannel system for high throughput screening of Escherichia coli. Experimental demonstration of noncontact transportation and manipulation of Escherichia coli by dielectrophoretic force and radiation pressure of laser tweezers were carried out with Laser manipulator system. In experiments, we show that transportation and separation of Escherichia coli with electric field and optical radiation pressure is profitable in the future practical application for the high throughput screening of microbes.

Chapter 5 describes Bio-Micromanipulation system·for high throughput screening and micro surgery in microchannel. We discussed research issues in the bio micromanipulation and presented a new direction in this field. We consider the basic strategies to improve the working efficiency and the operability of the bio micromanipulation. Application of liposome to micro encapsulation delivery and concept of trapping single globular DNA by laser tweezers and its application to Micro encapsulation by liposome is shown.

Chapter 6 summarizes the results presented in this dissertation and discuss research directions for future works.

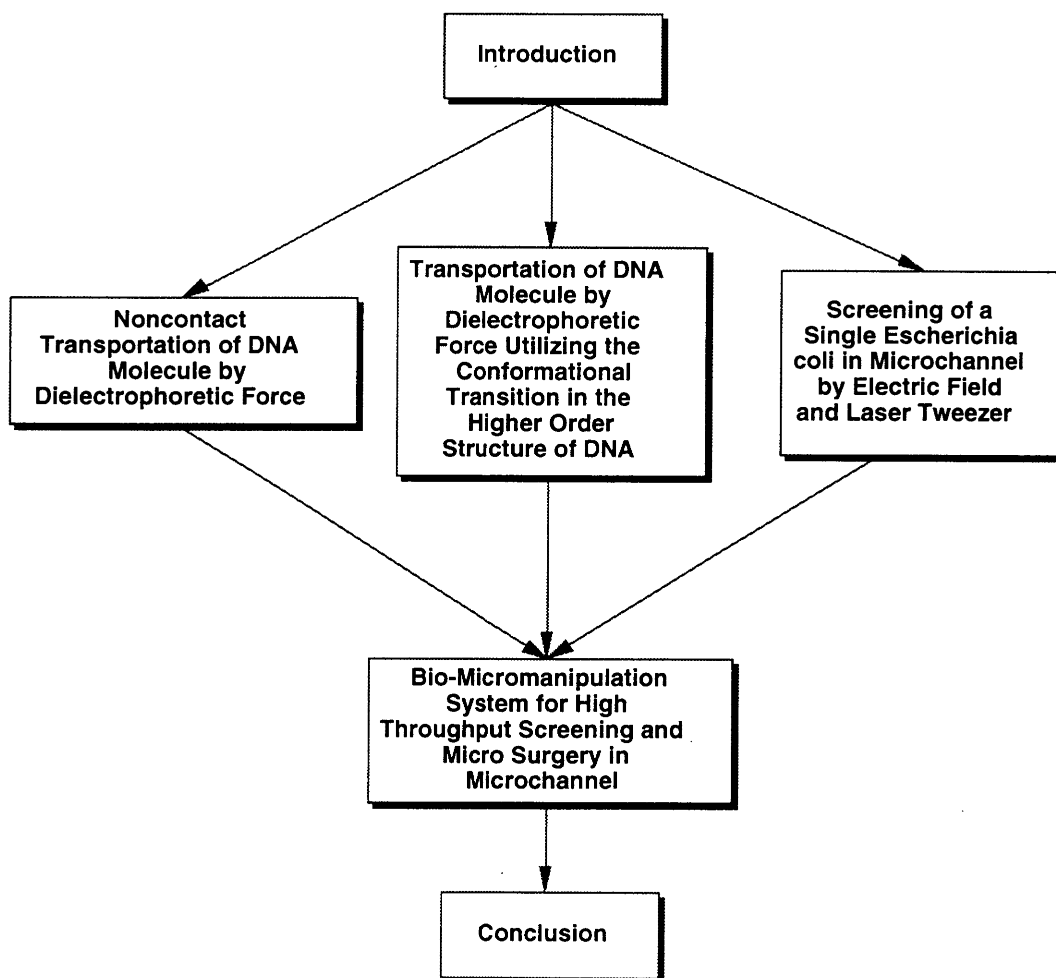

Figure 1.9: Organization of this Dissertation

Chapter 2

Noncontact Transportation of DNA Molecule by Dielectrophoretic Force

2.1 Introduction

Recently, there has been great interest in the sequencing of the genome of various species, especially the human genome. The DNA molecule consists of a sequence of units, called nucleotides, which consist of a phosphate and a sugar group with a base attached. There are four types of nucleotide in the DNA. Physically, the DNA is carried in structures called chromosomes. Each human has 46 chromosomes. The total length of an individual's DNA is about $3 \text{x} 10^9$ bases [27]. At present, only 4% of the sequencing of the human genome has been decoded. It is necessary to develop a technique by which DNA molecules can be separated by the length. It would be expected to separate a giant DNA molecule ($Mbp \sim 10Mbp$) individually [27].

On the other hand, since remarkable progress has been made in the development of micromachining technology, micro devices such as the microelectrode, micropump, and micromotor have been fabricated on Silicon wafer. Micro

electro mechanical systems have been considered for application in the field of biotechnology. For example, the manipulation of many kinds of micro organisms such as a cell, a single DNA molecule, a chromosome etc is a very essential technique. In conventional studies, cell manipulation on a Fluid Integrated Circuit (FIC) [112, 113, 115], and the trapping of biological particles by use of ultrasonic resonance fields [39, 40], and optical tweezer [72, 76–78] have been presented. Micromachine technology makes it possible to fabricate microelectrodes and microflow channels [48, 54] of the same size as biological particles, so that this technology has great potential to separate micro organisms such as DNA molecules.

At present, gel electrophoresis is the basic technique used in the separation of DNA molecules. However, each research group carries out the experiments only to have to search for the best conditions for separating a giant DNA molecule within the limit of separation [27]. In gel electrophoresis, it takes too much time to separate DNA macromolecules and it is very difficult to separate them in gel. As for the point of improving the durability of the electrodes and the controllability of the manipulation of a single DNA molecule, electrophoresis in a DC field causes electrochemical reaction and electroosmosis so often that it is not suitable for manipulation of DNA molecules. To solve these problems, a high frequency AC field is suitable [37, 110, 112, 113, 115]. For this reason, the study of cell manipulation by using the dielectrophoretic force has been reported [14–16, 19, 41, 110, 112, 113, 115]. One of the applications of the dielectrophoretic force to DNA molecules is, for example, the

electrostatic orientation of the DNA molecule [113–116]. The application of noncontact manipulation of a single DNA molecule by the dielectrophoretic force has not been reported so far. In our study, we define the manipulation of DNA molecules as "transportation" and "separation". We analyze the transportation of the DNA molecule by dielectrophoretic force. Since a high field gradient can be obtained between the electrode gaps based on the electrode pattern, the dielectrophoretic force, which is in proportion to the magnitude of the field gradient, is generated between the electrode gaps and it is possible to transport DNA molecule along the flow channel. It is considered to be possible to separate a DNA molecule when DNA molecules are continuously transported, especially in the circle packet (as shown in Fig. 2.4) as we proposed. By using micromachine technology, it is possible to miniaturize the electrodes and the flow channels, so that the basic technique of manipulation of individual DNA molecules will be advanced.

This chapter proposes the concept of Micro DNA Flow System, as shown in Fig. 2.2. In our system, DNA molecules can be manipulated and transported and finally be separated individually by using the dielectrophoretic force. At first, we developed a simple electrode design on a glass plate to show the possibility of transporting DNA molecules based on Micro DNA Flow System. In the experiments, we observed the motion of DNA molecules between the electrodes and measured the velocity of migration of a single DNA molecule. Thus, the possibility of transportation of DNA molecules can be shown.

2.2 Manipulation of DNA Molecules

2.2.1 Micro DNA Flow System

In order to manipulate and separate an arbitrary single DNA molecule in a fluorescence microscopy system, it is essential to have the following points in Micro DNA Flow System:

(1) noninvasiveness

(2) selectivity

(3) noncontact

(4) high sensitivity

(5) high speed

(6) durability

(7) bioautomation process

Although electrophoresis is one of the most useful separation techniques, those points; (2), (4), (5), (6), (7) must be improved.

2.2.2 Gel electrophoresis

At first, we explain the difference between the electrophoretic force and the dielectrophoretic force acting on the DNA molecule.

Gel electrophoresis is a method of distinguishing entities according to their mobility in a DC electric field. The electrophoretic mobility of a molecule is influenced by its size and electric charge, especially in gel [27]. The DNA

molecule moves to the anode in a uniform field, because it has minus charge in gel. By gel electrophoresis, however, it is impossible to separate a giant DNA molecule(more than 10 Mbp). In addition, it takes too much time and it costs too much to sequence it.

2.2.3 Manipulation of DNA molecules by Dielectophoretic Force

The dielectrophoretic force acting on the DNA molecule is generated in a nonuniform field [38]. The polarization is generated by an external electric field per unit volume, so in general, the Equation of the dielectrophoretic force F_d is expressed as follows [37],

$$F_d = 2\pi a^3 \epsilon_m R_e \left[\frac{\kappa_p - \kappa_m}{\kappa_p + 2\kappa_m} \right] \bigtriangledown E^2 \tag{2.1}$$

$$j = \sqrt{-1} \tag{2.2}$$

$$\kappa_p = \epsilon_p - j\frac{\sigma_p}{\omega} \tag{2.3}$$

$$\kappa_m = \epsilon_m - j\frac{\sigma_m}{\omega} \tag{2.4}$$

a: Radius of a DNA molecule (m)

E: External electric strength where DNA molecule exists between electrodes (V/m)

ϵ_p, ϵ_m: Permittivity of DNA molecule and medium

σ_p, σ_m: Conductivity of DNA molecule and medium

κ_p, κ_m: Complex permittivity of DNA molecule and medium

ω: Angular frequency.

The polarization is generated by an external electric field per unit volume, so in general, the Equation of the dielectrophoretic force F_d is expressed as follows,

$$F_d = \frac{1}{2}\alpha v gradE^2 \tag{2.5}$$

where α is polarizability (C/m^3), v is volume (m^3).

In a high frequency field, the DNA molecule is drawn in the direction of the high field gradient because the dielectrophoretic force operates in the direction of $gradE^2$. The force acting on the DNA molecule in a nonuniform field is shown in Fig. 2.1. The conformation of a single DNA molecule in an aqueous solution is in the coil state. In generating a high frequency AC field, however, the field around the DNA molecule becomes a nonuniform field. So, its conformation transforms. Due to this electric field, the positive and negative charges in the DNA molecule generate toward the electric field by its polarization. As a result, the DNA molecule migrates toward the normal to the line of electric line by dielectrophoretic force.

2.2.4 Concept and principle of selective transportation system of DNA molecules

In the future, noncontact manipulation of a single DNA molecule will become a main technique of gene manipulation. In our study, we propose the selective transportation system of DNA molecules for the purpose of transporting and separating a single DNA molecule by use of the dielectrophoretic force

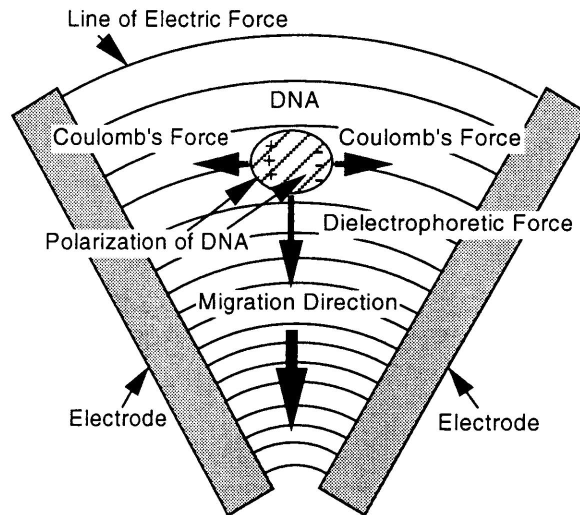

Figure 2.1: Polarization of DNA molecule and dielectrophoretic force

in Micro DNA Flow System, as shown in Fig. 2.2. Micromachine technology has an advantage in mass production of a various kinds of designs of Micro DNA Flow System.

This chapter describes two designs for Micro DNA Flow System. First, in a line packet, as shown in Fig. 2.3, some packets are located along the flow channel, and a single DNA molecule is separated at the point of the packet by controlling the magnitude and the direction of the dielectrophoretic force. They depend upon the electric field generated by the electrodes lying along the line packet.

In the second design, the DNA molecule is injected from the route A into a circle packet as shown in Fig. 2.4 and in order to have DNA molecules

migrated along the circle packet, the electric field between each electrode is generated step by step. Next, at the same time the electric field is turned off, an arbitrary DNA molecule near the packet is drawn into route A or route B. In this way, this circle packet has the advantage of flowing DNA molecules continuously until a target DNA molecule is separated from the aggregation of DNA molecules. And by controlling an electric field between the serial electrodes along it, it can be possible that DNA molecules in aggregation state exhibit conformational change to be be easily separated through the circle packet.

Thus, by using dielectrophoretic force, it is possible to manipulate a single DNA molecule and separate it. In electrophoresis, the total length of the flow channel depends on the electrode gap, but in Micro DNA Flow System it is easier to extend the flow channel.

2.3 Migration Model of DNA Molecule between Micro Electrodes

2.3.1 Structure

We designed a simple electrode design (as shown in Fig.2.5) for the purpose of transporting the DNA molecule in one direction. The electrodes consist of metal tape made by stainless on a slide glass. Its thickness is $30\mu m$. The resin made of polyester was spread on the surface of the electrodes as the insulator. The angles δ between the electrodes are of two kinds; $\delta = 30°$, and $\delta = 60°$.

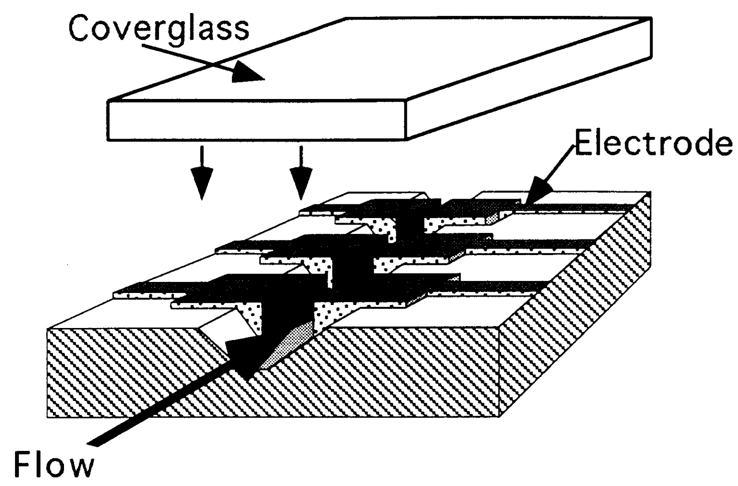

Figure 2.2: Micro DNA Flow System

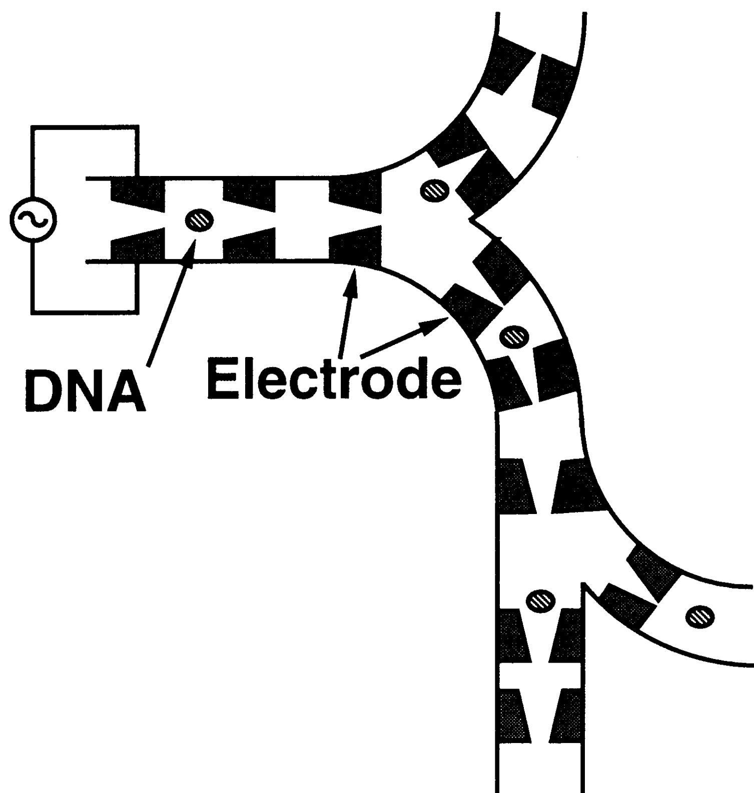

Figure 2.3: Line packet

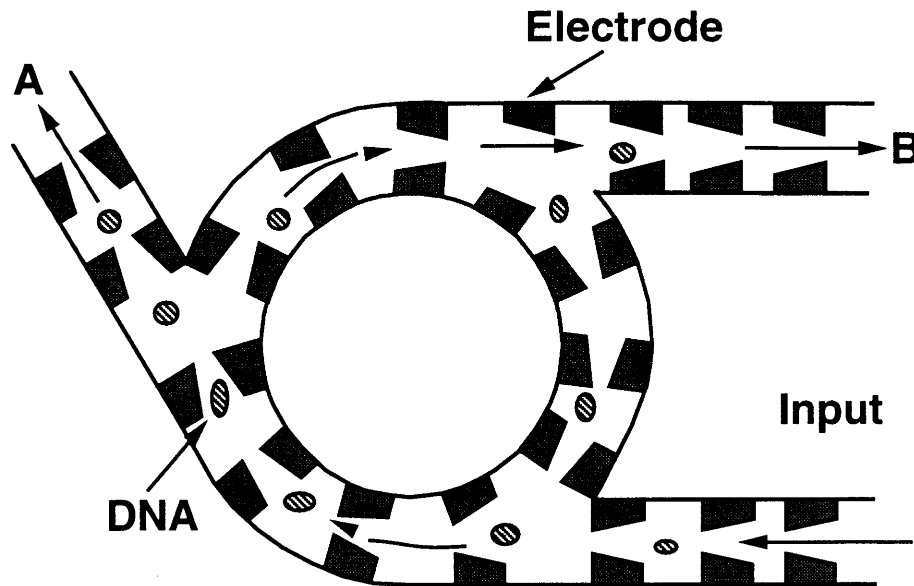

Figure 2.4: Circle packet

2.3.2 Model of electric field

We calculated a model of electric field in a coordinate system as shown in Fig.2.6. Generally, it is quite difficult to solve Laplace's equation, which expresses potential. For simplicity of calculation, we assume the following conditions:

(i) Since the thickness of the electrode ($30\mu m$) is much larger than the size of the DNA molecule(diameter about $2nm$), the electrodes are considered to consist of two infinite planes between an interior angle δ.

(ii) An infinite small gap exists at the origin $r = 0$.

(iii) Potentialf is a function denoted by Laplace's equation in a cylindrical coordinate system.

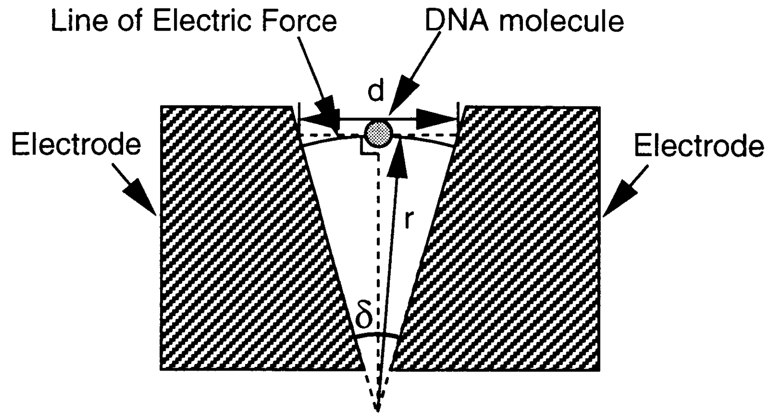

Figure 2.5: Electrode design in an experiment

Here, the absolute coordinates of the DNA molecule (x, y), which exists between the electrodes, is expressed by r, θ.

$$\begin{aligned} x &= r\cos\theta \\ y &= r\sin\theta \end{aligned} \tag{2.6}$$

The field between the electrodes is described by Laplace's equation for the potential ϕ[V],

$$\frac{1}{r^2}\frac{\partial^2\phi}{\partial\theta^2} = 0. \tag{2.7}$$

Since $r \neq 0$, we have

$$\frac{1}{r^2}\frac{\partial^2\phi}{\partial\theta^2} = 0, \theta = 0, \phi = 0; \theta = \delta, \phi = V_0 \tag{2.8}$$

$\theta = 0$, $\phi = 0$; $\theta = \delta$, $\phi = V_0$ We have

$$\phi = \frac{V_0}{\delta}\theta \tag{2.9}$$

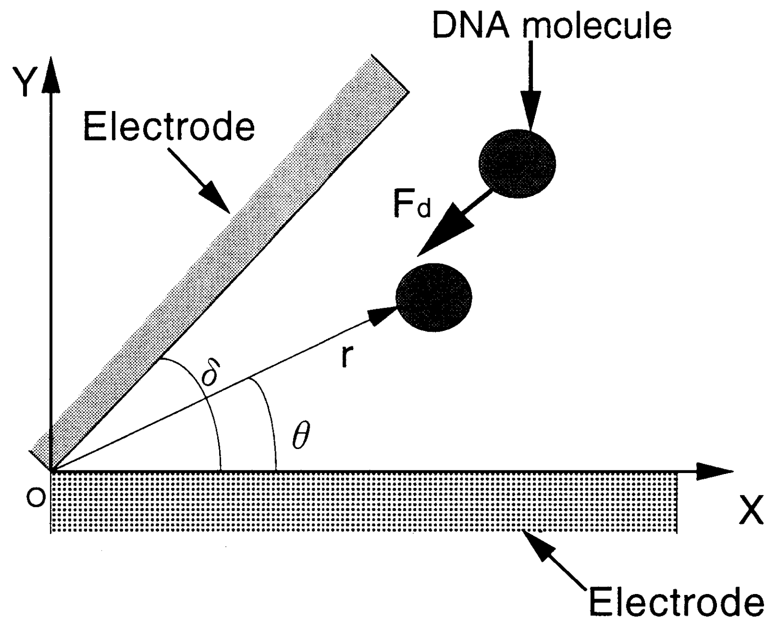

Figure 2.6: The model of the coordinate system for electric field calculation

Since

$$E = - \bigtriangledown \phi \tag{2.10}$$

where $\hat{a}_\theta$: unit vector in θ-direction. We have the magnitude of the electric field:

$$|E| = \frac{V_0}{r\delta} \tag{2.11}$$

Hence, the field gradient is given by;

$$grad|E|^2 = \left(-\frac{2V_0^2}{r^3\delta^2}\right)\hat{a}_r \tag{2.12}$$

where $\hat{a}_r$: unit vector in the r-direction. We have the magnitude of the field gradient:

$$|grad|E|^2| = \frac{2V_0^2}{r^3\delta^2} \tag{2.13}$$

2.3.3 Calculation of Dielectrophoretic Force and model of motion of the DNA molecule

The expression for the dielectrophoretic force F_d is obtained by substituting the Eq. (2.5) into the Eq. (2.13):

$$F_d = -\alpha v \left(\frac{V_0^2}{\delta^2} \right) \frac{1}{r^3} \hat{a}_r \tag{2.14}$$

We have the magnitude of the dielectrophoretic force:

$$|F_d| = \alpha v \left(\frac{V_0^2}{\delta^2} \right) \frac{1}{r^3} \tag{2.15}$$

Next, we derive a dynamic model of a DNA molecule between two electrodes. We assume the following conditions:

(i) The DNA molecule is a spherical particle.

(ii) The Coulomb's force acting on the DNA molecule acts from the center of the DNA molecule in the direction of the tangent to the line of electric force.

(iii) The DNA molecules in the experiment system migrate along the middle direction ($\theta = \delta/2$) between the electrodes, as shown in Fig. 2.5.

Therefore, assume the dynamic model of the DNA molecule is as illustrated in Fig. 2.1. We have the equation for motion as follows:

$$m\frac{d^2r}{dt^2} + 6\pi\eta a\frac{dr}{dt} = F_d(r,t) + F_m(r) \tag{2.16}$$

where r is vector of the DNA molecule, m is mass of the DNA molecule, *eta* is viscosity of the solvent, a is radius of the DNA molecule, $F_d(r,t)$ is the electrostatic force acting on the DNA molecule including dielectrophoretic force except for the Coulomb's force, $F_m(r,t)$ is the external force including gravity except for an electrostatic force.

Let the mass of a single DNA molecule be neglected. The term of the inertial force and the term of the external force in the Eq. (2.16) is negligible. We have as the equation of the motion in the direction of the migration as follows:

$$6\pi\eta aU = F_d(r,t) \tag{2.17}$$

where U is the velocity of migration of the DNA molecules. We can obtain the Eq. (2.18) by substituting the Eq. (2.15) into the Eq. (2.17).

$$6\pi\eta aU = \alpha v\left(\frac{V_0^2}{\delta^2}\right)\frac{1}{r^3} \tag{2.18}$$

$$r = \frac{d}{2\tan\frac{\delta}{2}} \tag{2.19}$$

(as shown in Fig.2.5)

We can get the velocity U of migration of the DNA molecule by substituting the Eq. (2.19) into the Eq. (2.18) as follows:

$$\begin{aligned} U &= \frac{\alpha v V_0^2}{6\pi\eta a}\frac{1}{\delta^2 r^3} \\ &= \frac{4\alpha v V_0^2}{3\pi\eta a}\frac{\tan^3\frac{\delta}{2}}{\delta^2}\frac{1}{d^3} \\ &= k_d\frac{\tan^3\frac{\delta}{2}}{\delta^2}\frac{1}{d^3} \end{aligned} \tag{2.20}$$

$$k_d = \frac{4\alpha v V_0^2}{3\pi\eta a} \tag{2.21}$$

where k_d is the physical characteristic parameter of the DNA molecule in the electric field.

2.4 Experiments

2.4.1 Experimental apparatus

The experimental system is schematically shown in Fig.2.7. With the fluorescence microscopy, it is easier to observe the dynamics of DNA molecule in an aqueous solution and gel, and perform a quantative analysis [29–31,83]. Fluorescence images of DNA molecules were observed by use of a Zeiss Axiovert 135 TV microscope equipped with a 100 x oil-immersed objective lens and recorded on videotapes with the sampling rate of 30 frames/s through the high-sensitive Hamamatsu SIT TV camera. The data were analyzed with an image processor, Arugus 10 (Hamamatsu Photonics). The applied voltage was controlled by an arbitrary waveform generator.

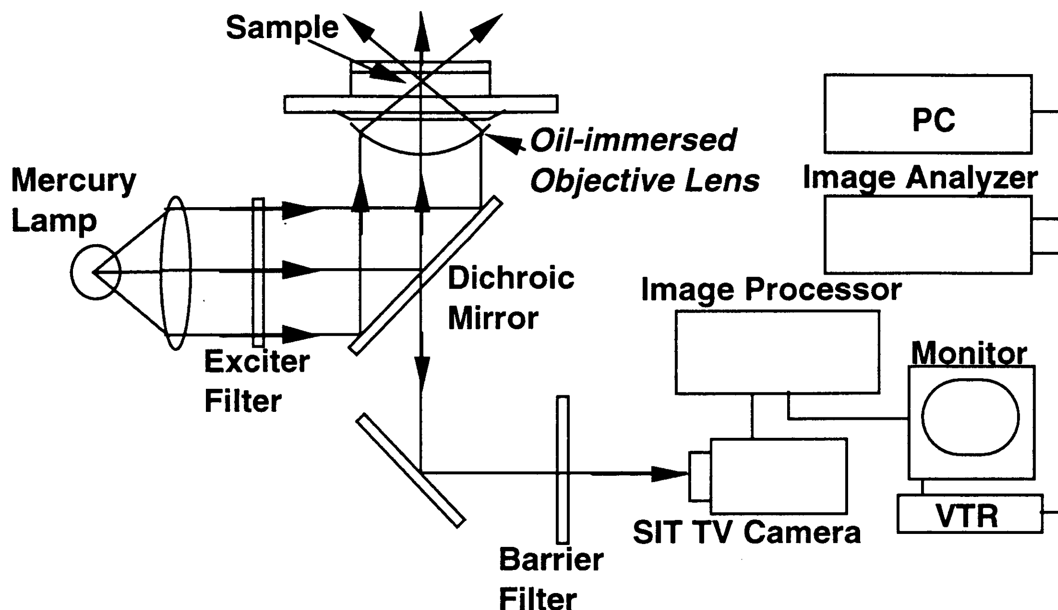

Figure 2.7: Schematic diagram of experimental apparatus

2.4.2 Materials

Bacteriophage T4DNA, 166 kilobase pairs (contour length $55\mu m$), was employed from Nippon Gene. Trisborate(TBE; pH7.8) buffer, T4DNA, YOYO, and 2-mercaptoethanol(2-ME; as an antioxidant) were added in the solution. The final concentrations are as follows: TBE, 45mMTris; 45mM boric acid, EDTA, 1mM; T4DNA, 0.2mM (in nucleotide); YOYO, 0.1mM; 2-ME, $4\%(v/v)$.

2.4.3 Experimental results

We carried out experiments as follows: the applied frequency is constant $1MHz$ and the applied voltage is constant $50V$. By using two kinds of electrode samples, we measured the velocity of migration of DNA molecule between the electrode gaps.

In the experiments, we determined the dependencies of the veocity of DNA molecule on the difference in the electrode gap and the angle between electrodes, which is one of parameters of the electrode design, as shown in Fig.2.8. Fig.2.8 shows the following points:

(i) As the electrode gap becomes smaller, the velocity of migration of DNA molecules is faster.

(ii) As the angle of the electrodes, where the electrode gap is the same size, becomes larger, the velocity of migration of DNA molecules is faster. Because the angle of the electrodes is bigger, the difference in the field gradient is bigger.

Fluorescence images of a T4DNA molecule in an experiment is shown in Fig.2.9. The center of the white circle in Fig.2.9 is a single T4DNA molecule. Fig.2.10 shows the transportation trajectory that the DNA molecule migrates straight between the electrodes.

2.5 Discussion

Using the least square method, we obtained the parameter k_d from the result of the experiments. Though each parameter, related to the physical characteristics of the DNA molecule, is unknown, the coefficient of the term of the dielectrophoretic force acting on a single DNA molecule can be estimated by calculation. The result of the calculation, based on the model and the Eq. (2.20), is shown in Tab.2.1. The solid lines as shown in Fig.2.8 express the

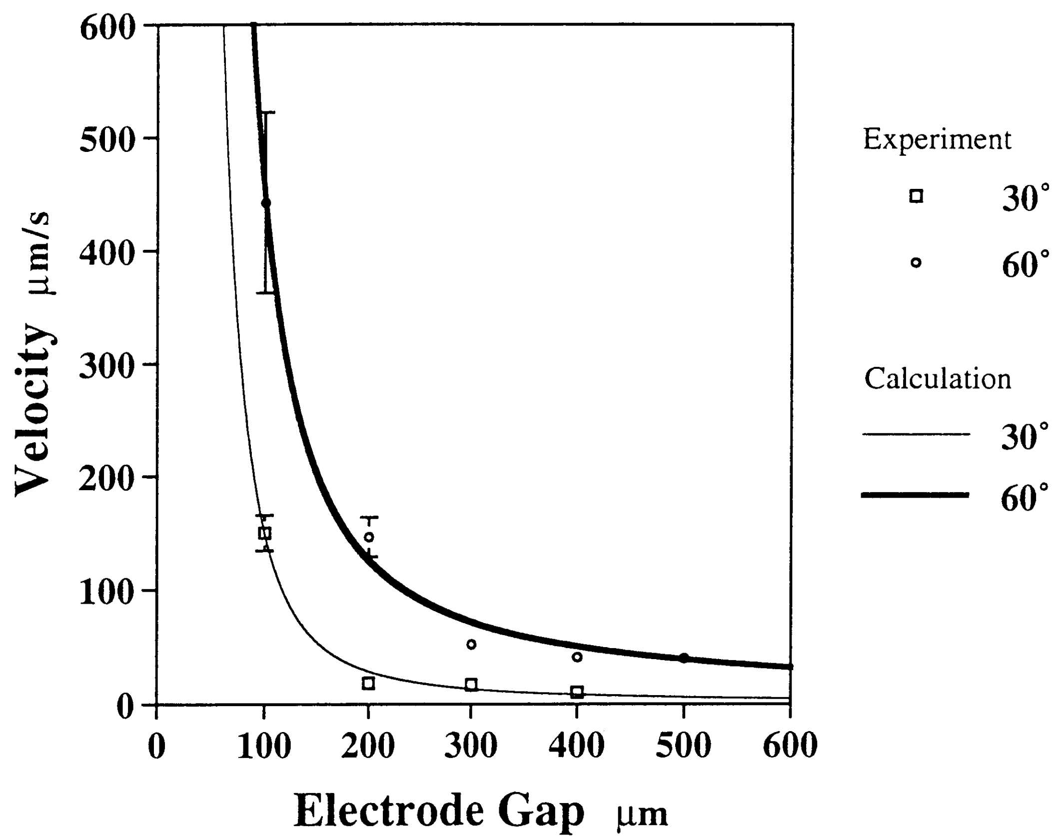

Figure 2.8: The Variation of the Velocity of the Migration of DNA Molecule with the Electrode Gap for the angle $\delta = 30°$ (square) between the electrodes and the angle $\delta = 60°$ (circle) between the electrodes with best fits of Eq.(2.20) (lines)

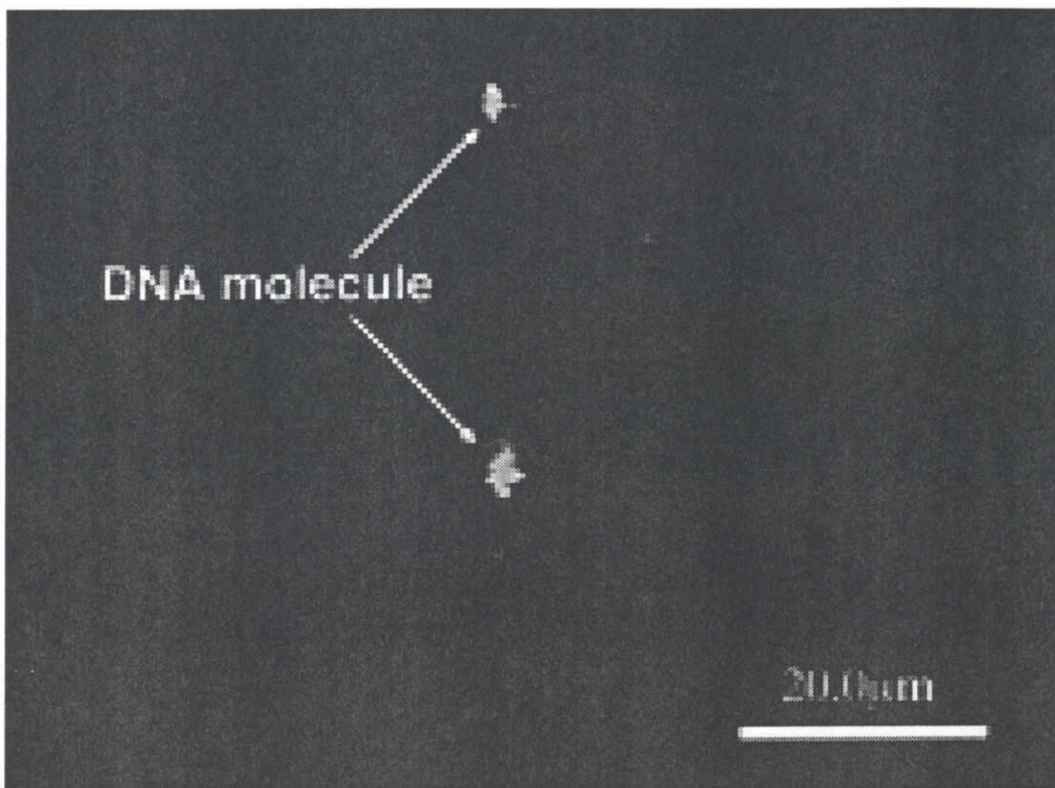

Figure 2.9: Fluorescence Images of T4DNA Molecule Suspending in a Medium (at E=0)

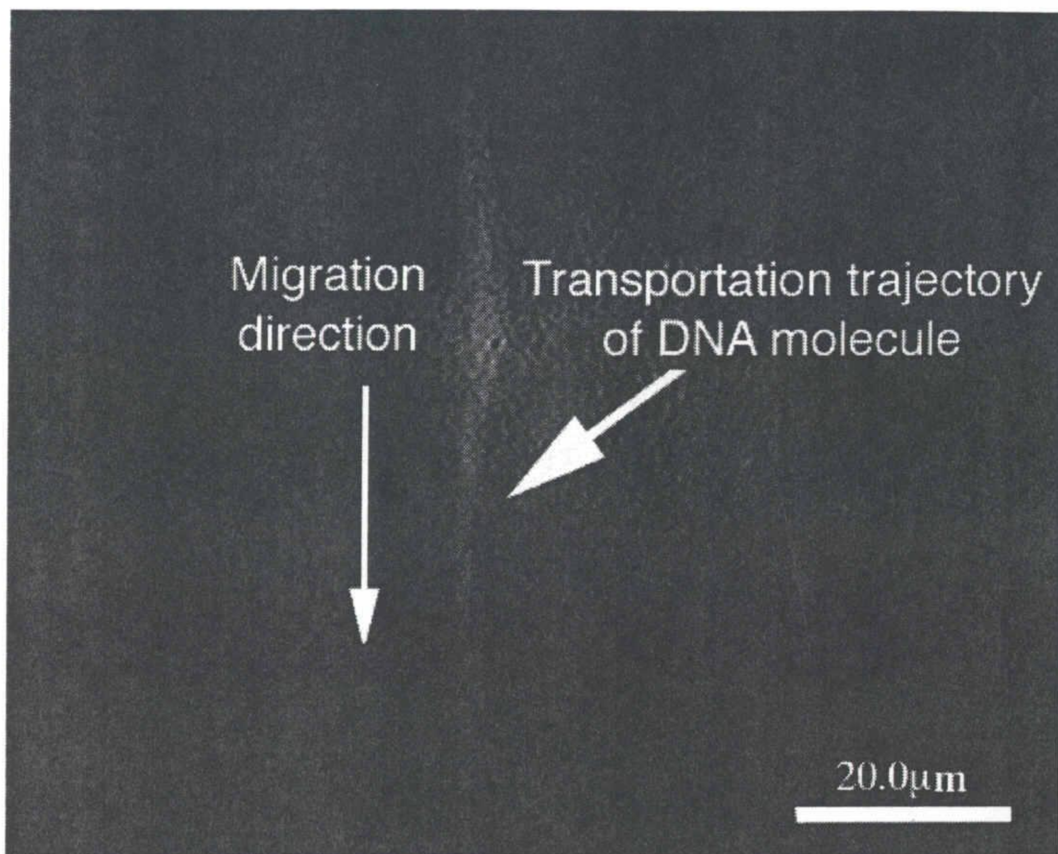

Figure 2.10: Transportation Trajectory that the DNA Molecule Migrates Straight Between the Electrodes

result of the calculation using the least square method. Fig.2.8 shows the variation in the velocity of migration of the DNA molecule with the change in the electrode gap. The dispersion of each parameter k_d between two kinds of the electrode samples in the experiments is appeared. Because the parameter k_d depends only upon the viscosity of the medium and the physical characteristics of the DNA molecule, the values are expected to be equal to each other. The reason why the parameter determined by the experimental results is different is considered in the following points:

Table 2.1: The results of calculation of parameter k_d

Angle(deg)	k_d
30	2.145×10^{-3}
60	2.596×10^{-3}

i) The electric field intensity was not obtained precisely because of a measuring error in the width and the angle of the electrode.

ii) The difference of shape, volume, and polarizability among individual DNA molecules in the experiments.

iii) The physical parameters including k_d may depend upon the magnitude of the electric field.

iv) Measuring error caused by heat convection, ion flow, electrification, vibration etc.

v) The shape of DNA molecule is like a long chain, so it may be impossible to assume it is a spherical particle.

vi) If the polarization of the DNA molecule becomes unbalanced, it may be possible that the Coulomb's force acts on the DNA molecule in the direction of its migration.

Those are hypotheses, so it is necessary to consider measuring the physical parameters of the DNA molecule and establish a dynamic model of DNA molecule. From the qualitative viewpoint, in terms of the experimental model, what we discussed in this chapter can be confirmed from the model we proposed.

Fig.2.11 shows the dependencies of the electric strength upon the difference in the electrode gap and the angle between electrodes. Fig. 2.12 shows the dependencies of the field gradient $gradE^2$ upon the difference in the electrode gap and the angle between electrodes. Compared with Fig.2.20 and Fig.2.11, Fig.2.12, the field gradientE^2 has more influence on the velocity of migration of the DNA molecule than the electric field strength. The theoretical curve, as shown in Fig.2.12, was calculated by the Eq.(2.13). From this result, it is considered that the field gradient E^2 is a very important parameter in designing a precise electrode to control the motion of DNA molecules.

2.6 Summary

In summary, we carried out experiments involving noncontact transportation of DNA molecules by using dielectrophoretic force. Compared with the

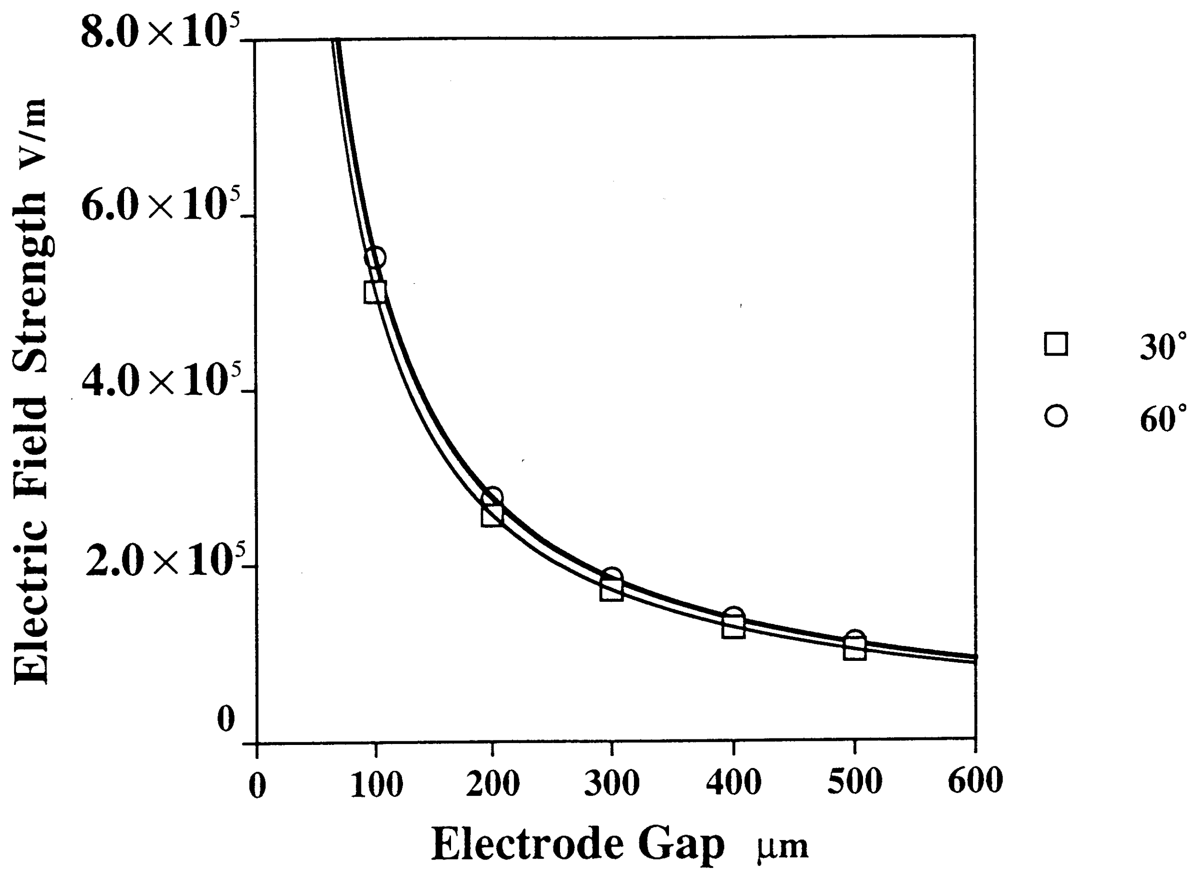

Figure 2.11: The Variation of the Electric Field Strength with the Electrode Gap for the angle $\delta = 30°$ (square) between the electrodes and the angle $\delta = 60°$ (circle) between the electrodes.

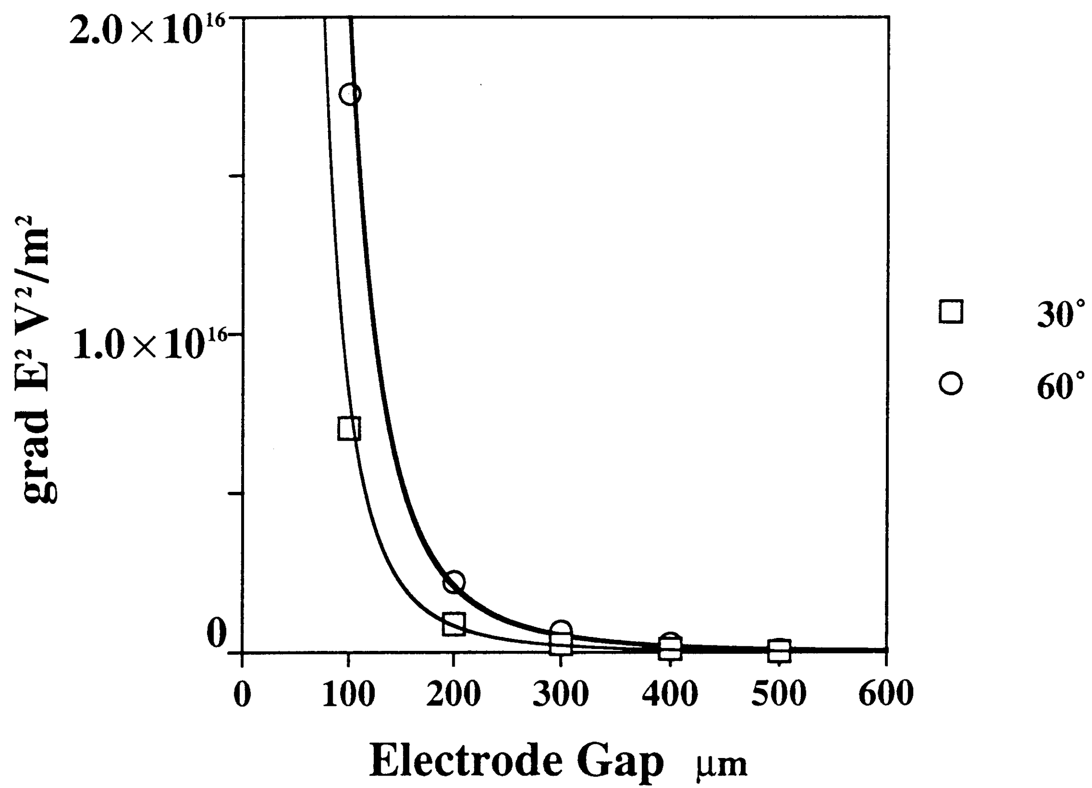

Figure 2.12: The Variation of the Field Gradient gradE2 with the Electrode Gap for the angle $\delta = 30°$ (square) between the electrodes and the angle $\delta = 60°$ (circle) between the electrodes.

method employing electrophoresis, electroosmosis and chemical reaction on electrodes doesn't occur by use of an AC field. In a Micro DNA Flow System it is also easier to extend the flow channel. We designed a simple electrode, carried out the experiments and measured the velocity of migration of the DNA molecule. We observed the dynamics of DNA molecules between electrodes in an AC field. We showed the possibility of noncontact transportation of a DNA molecule by using dielectrophoretic force. We are planning to create a new model, to realize the separation of DNA molecules, and to propose a manipulation system taking into consideration the conformational changes in the DNA molecule. Thus, the bio-automation will be greatly improved by the proposed micro manipulation technology in the future.

Chapter 3

Transportation of DNA Molecule by Dielectrophoretic Force Utilizing the Conformational Transition in the Higher Order Structure of DNA

3.1 Introduction

This chapter analyzes the transportation of the DNA molecule in the globule state [33–36] by dielectrophoretic force [125]. It is very difficult to transport DNA molecule in the coil state stably. Since a high field gradient can be obtained between the electrode gaps based on the electrode pattern, the dielectrophoretic force, which is in proportion to the magnitude of the field gradient, is generated between the electrode gaps and it is possible to transport a giant DNA molecule along the flow channel. Micromachine technology makes it possible to fabricate microelectrodes and microflow channels of the same size as biological particles, so that this technology has great potential to separate

micro organisms such as DNA molecules. In conventional studies, cell manipulation on a Fluid Integrated Circuit(FIC) [112, 113, 115], cell manipulation by using the dielectrophoretic force [14–16, 19, 41, 110, 112, 113, 115], the trapping of biological particles by use of ultrasonic resonance fields [39, 40], optical tweezer [72,76–78], the electrostatic orientation of the DNA molecule [113–116], and optical manipulation of single DNA molecule by binding between the latex bead and the DNA molecule [47] have been presented. The application of noncontact manipulation of a single DNA molecule by the dielectrophoretic force and transportation of DNA molecule utilizing the conformational transition [33–36] in the higher order structure of DNA have not been reported so far.

In our system, DNA molecules can be manipulated and transported and finally be separated individually by using the dielectrophoretic force [125], as shown in Fig. 3.1. At first, we developed a simple electrode design on a glass plate to show the possibility of transporting DNA molecules based on Micro DNA Flow System. In the experiments, we observed the motion of DNA molecules in the globule state between the electrodes. Thus, the possibility of separation of DNA molecules can be shown.

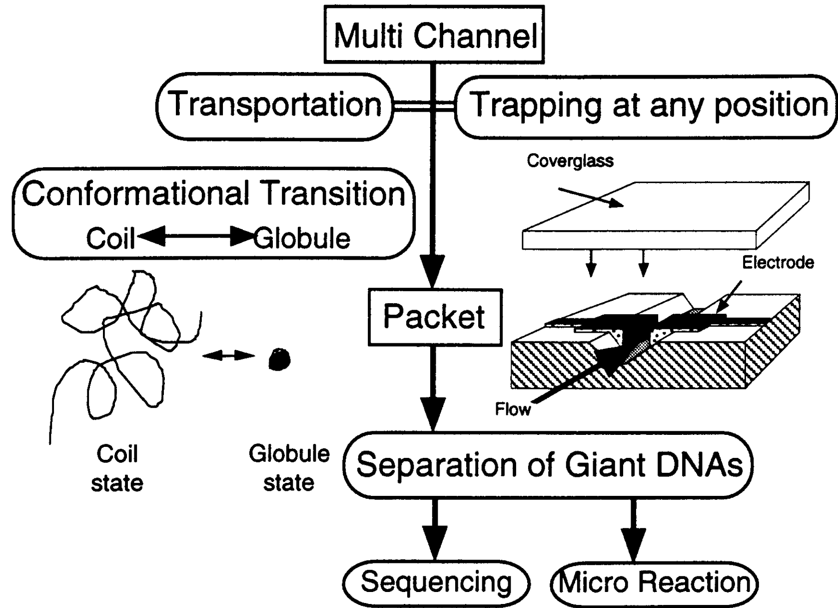

Figure 3.1: Concept of Micro DNA Flow System

3.2 Conformational Transition of DNA and Transportation of DNA Molecule by dielectrophoresis

3.2.1 Dielectrophoretic Force acting on the DNA Molecule in a Nonuniform Field

The dielectrophoretic force acting on the DNA molecule is generated in a nonuniform field. The polarizability of the DNA molecule depends not only upon the dielectric characteristic, but also upon its conductivity. Some cells and DNA molecules etc. have conductivity in an aqueous solution. So, their conductivity as well as the dielectric characteristic is very important. In this case, dielectrophoretic force F_d is given by,

$$F_d = 2\pi a^3 \epsilon_m R_e \left[\frac{\kappa_p - \kappa_m}{\kappa_p + 2\kappa_m} \right] \nabla E^2 \tag{3.1}$$

In a high frequency field, the DNA molecule is drawn in the direction of the high field gradient because the dielectrophoretic force operates in the direction of gradE^2. The force acting on the DNA molecule in a nonuniform field is shown in Fig. 3.2. As a result, the DNA molecule migrates toward the normal to the line of electric line by dielectrophoretic force [125].

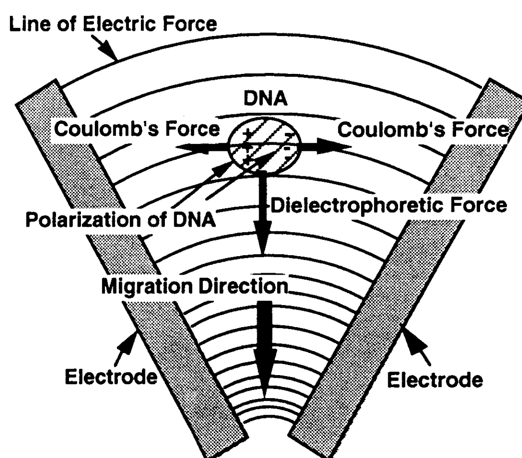

Figure 3.2: Polarization of Globular DNA Molecule and dielectrophoretic Force

3.3 Conformational Transition in the Higher Order Structure of DNA

It has been known that the conformational change of a DNA molecule in an aqueous solution depends on the composition, temperature, and concentration of the solvent and that the condensation of a single DNA molecule is induced by polycation, hydrophile polymer, and surfactant under a suit-

able condition [33]. Generally, the conformation of a polymer chain such as DNA molecule is different from in a good solvent and a poor solvent. The conformation of DNA molecule in a good solvent is in the coil state, and the compactization of a DNA molecule, which is in the globule state, is induced in a poor solvent. Fig. 3.3 illustrates the conformational change of a single DNA molecule and Fig. 3.4 shows fluorescent image of conformational transition of DNA molecule [35,36]. Recently the coil-globule transition has been investigated both theoretically and experimentally [32,34] so it has been possible to produce a DNA molecule in the compact globule state in vitro [36]. It is easy to transform the conformation of a DNA molecule for its transportation since this conformational transition in the higher order structure of DNA is reversible.

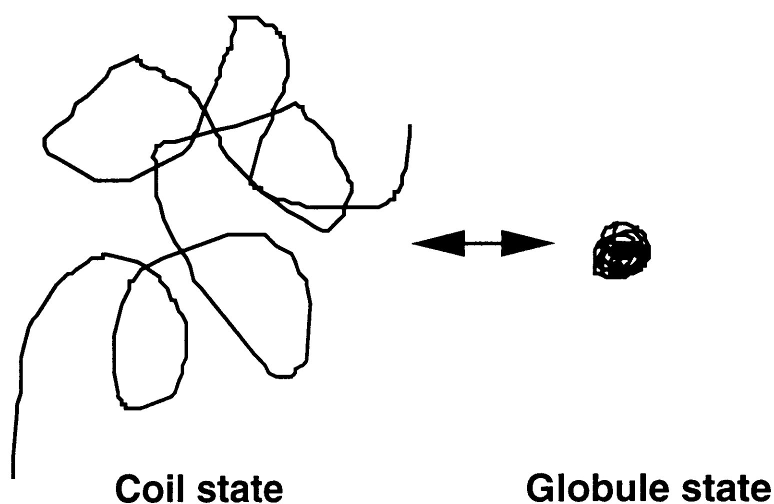

Figure 3.3: Conformational change of DNA molecule

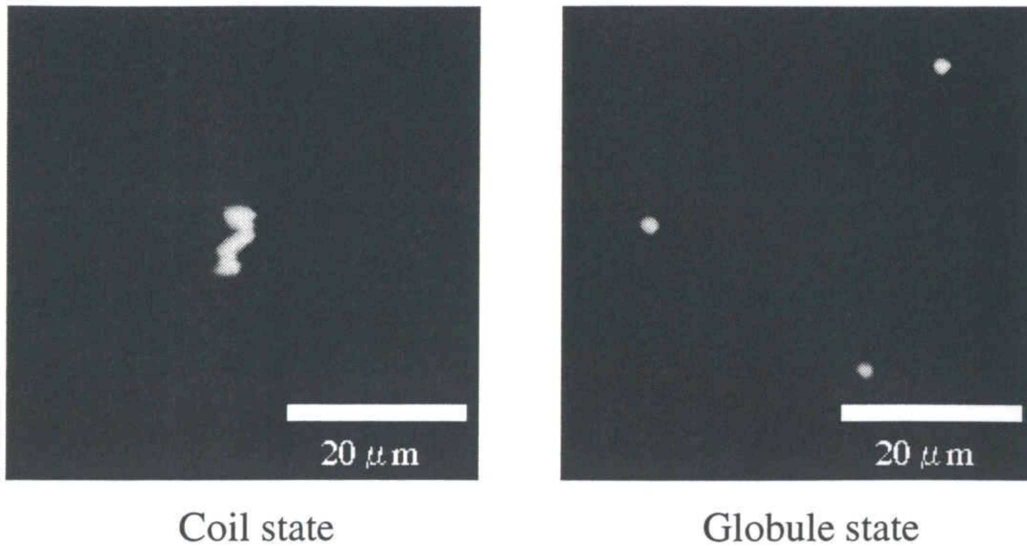

Figure 3.4: Fluorescent image of conformational transition of DNA molecule [35]

3.4 Transportation of DNA Molecules in the Globule State

In the future, noncontact manipulation of a single DNA molecule will become a main technique of gene manipulation. In our study, we propose the selective transportation system of DNA molecules utilizing the conformational transition in the higher order structure of DNA for the purpose of transporting and separating a single DNA molecule by use of the dielectrophoretic force in Micro DNA Flow System[3]. It is easier to manipulate a single DNA molecule if the Brownian motion doe'snt have an influence on the structure of DNA molecule. A DNA molecule in the coil state may be torn off during an observation and the transportation since the DNA molecule in the coil state tends to have a great influence of the Brownian motion and the heat convection. So,

it is considered that the DNA molecule in the coil state is less suitable for the transportation of DNA molecules. On the other hand, a single DNA molecule both in the globule state and in the coil state can not be manipulated and transported by Laser trapping [47, 76–78], since it is too small.

In this chapter, we demonstrate the transportation of DNA molecule in the globule state by dielectrophoretic force. A DNA molecule is polyelectrolyte [95–98]. However, the compactization of a single DNA molecule is considered to induce charge neutralization, so it is not effective to use the electrophoresis that is one of the methods of separation of charged particles. Dielectrophoresis is suitable for transportation and separation of dielectric particles. In the experiments, we observed the transportation of the DNA molecule in the globule state in 2-propanol.

3.5 Experiments

3.5.1 Materials

Bacteriophage T4DNA, 166 kilobase pairs (contour length $55\mu m$), was purchased from Nippon Gene. Trisborate(TBE; pH7.8) buffer, T4DNA, YOYO, and 2-mercaptoethanol(2-ME; as an antioxidant) were added in the solution. 2-propanol was added in order to keep DNA molecules globule state. The final concentrations are as follows: TBE, $45mMTris$; 45mM boric acid, EDTA, 1mM; T4DNA, 30nM (in nucleotide); YOYO, 6nM; 2-propanol, $40\%(v/v)$; 2-ME, $4\%(v/v)$; saccharose, $0.4g/ml$; glucose, $2.3mg/ml$; glucoseoxidase, $0.1mg/ml$; catalase, $0.018mg/ml$.

3.5.2 Experimental apparatus

The experimental apparatus is schematically shown in Fig. 3.5 and Fig.3.6. The observation of DNA molecule with fluorescence microscopy has been carried out [30, 31]. With the fluorescence microscopy, it is easier to observe the dynamics of DNA molecule in an aqueous solution and gel, and perform a quantative analysis [30, 31]. Fluorescence images of DNA molecules were observed by use of a Zeiss Axiovert 135 TV microscope equipped with a 40 x objective lens and recorded on videotapes with the sampling rate of 30 frames/s through the high-sensitive Hamamatsu SIT TV camera. The data were analyzed with an image processor, Arugus 10 (Hamamatsu Photonics). The applied voltage was controlled by an arbitrary waveform generator.

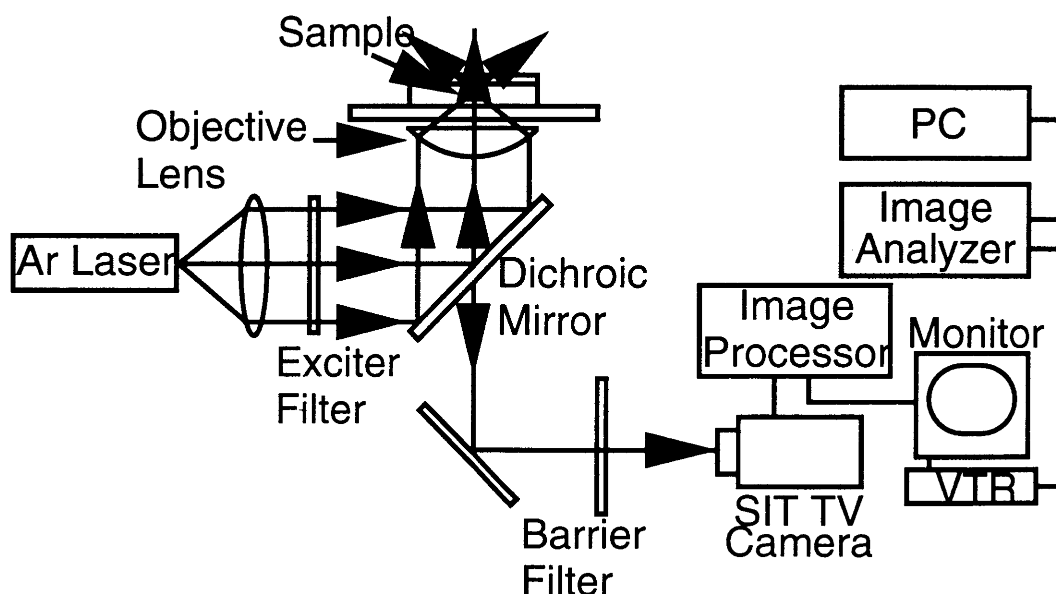

Figure 3.5: Schematic diagram of experimental apparatus

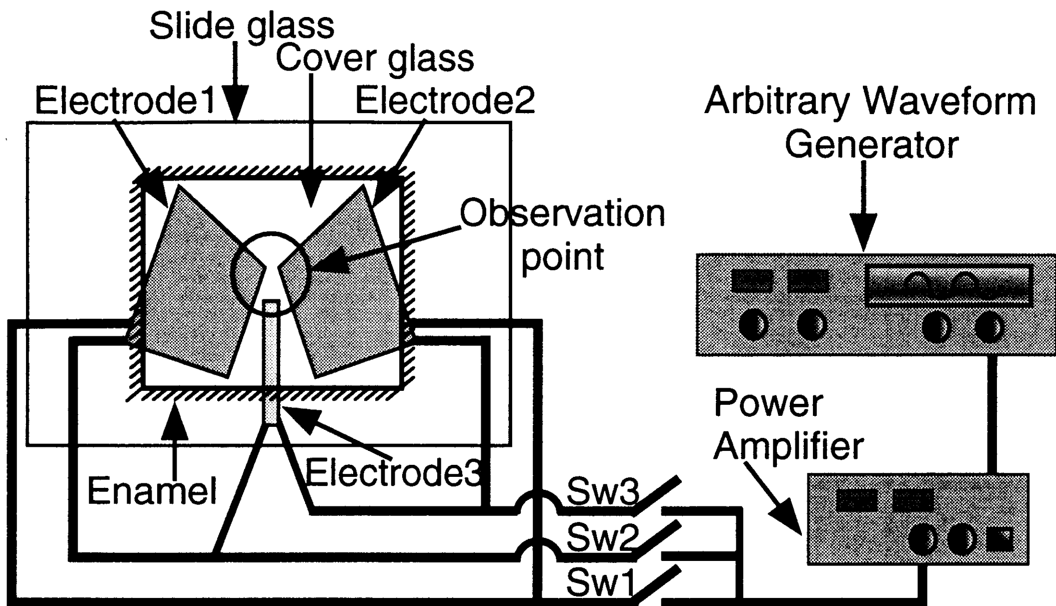

Figure 3.6: Schematic diagram of the observation sample and the experimental system

3.5.3 Experimental condition

We carried out experiments as follows: the applied frequency is constant 1MHz and the applied voltage is constant $100Vp-p$. By using three kinds of electrode samples, as shown in Fig. 3.7, we observed the motion of DNA molecule in the globule state through the packet, as shown in Fig. 3.7, by switching the electric field. We designed three kinds of electrode designs (as shown in Fig. 3.7) for the purpose of transporting the DNA molecule in the globule state. The angles ($\delta 1$, $\delta 2$) between the electrodes are of three kinds; ($\delta 1$, $\delta 2$)=(60°, 60°), (30°, 120°), (120°, 30°). The minimum electrode gap is $100\mu m$.

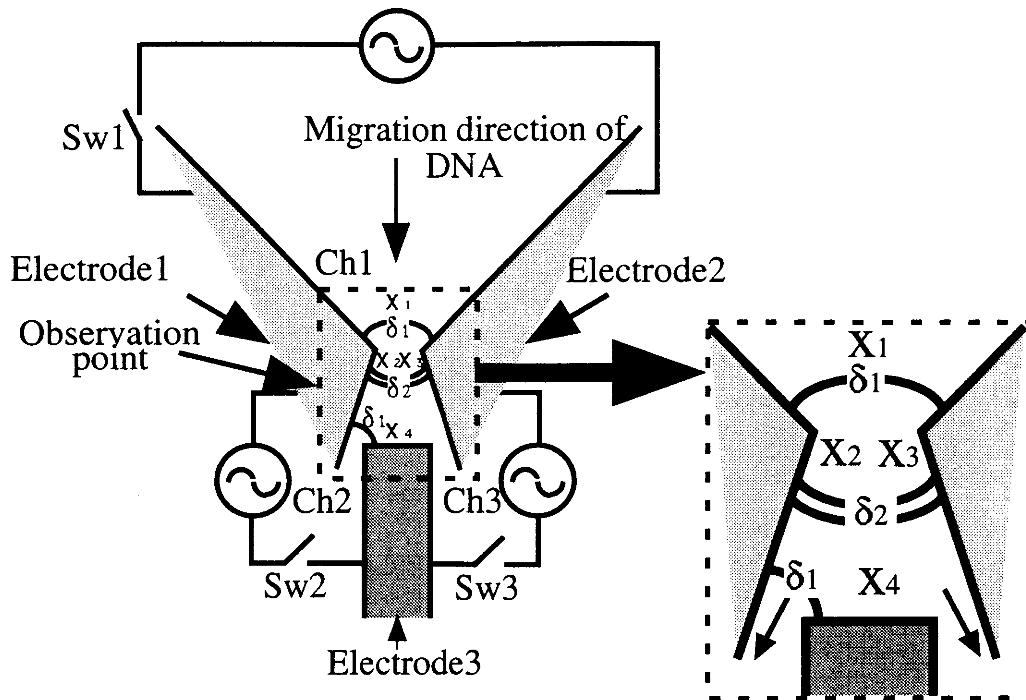

Figure 3.7: Design of Packet in an experiment

3.5.4 Results

In our previous experiments, we have confirmed the dependencies of the velocity of DNA molecule on the difference in the angle between electrodes, which is one of parameters of the electrode design. As the angle of the electrodes, where the electrode gap is the same size, becomes larger, the velocity of migration of DNA molecules is faster. Because the angle of the electrodes is bigger, the difference in the field gradient is bigger.

From this results, we carried out the experiments by using three kinds of designs of packet and determined the suitable electrode design.

It is necessary to pass between the electrodes in order to transport DNA molecules from Ch1 to Ch2 or Ch3, as shown in Fig.3.7. Using the packet which

has an angle ($\delta1$, $\delta2$= (30°, 120°) between the electrodes, we observe that DNA molecules migrate from X4 direction to X1 direction. Using the packet which has an angle ($\delta1$, $\delta2$= (60°, 60°) between the electrodes, it is observed that they aggregate in the middle of the packet between the electrodes. In the case of the packet which has an angle ($\delta1$, $\delta2$= (120°, 30°), DNA molecules migrate from X1 direction to X4 direction, so they are transported from Ch1 to Ch2 or Ch3. In each cases, DNA molecules don't migrate when the electric field is off. These results are in good agreement with our previous results that the dielectrophoretic force acting on the DNA molecule depends upon the field gradient E^2. From this reason, we carried out the experiments and observed the motion of the DNA molecules, using the packet which has an angle ($\delta1$, $\delta2$= (120°, 30°).

Fig. 3.8~Fig. 3.14 shows the transportation trajectory that the DNA molecule migrates between the packet, as shown in Fig. 3.7. Fig.3.8 illustrates that single DNA molecule is in the globule state when the high frequency field is off. It shows that there is no damage in the structure of DNA molecules in the globule state after they are transported between the packet. It is more stable to transport DNA molecule in the globule state than in the coil state and it is suitable for the transportation by dielectrophoretic force.

Fig. 3.10~Fig.3.14 shows the motion of DNA molecules while the high frequency field switches on and off near the packet. They show that by controlling an electric field between electrodes, it is possible that DNA molecules in aggregation state are easily separated through the packet.

Fig.3.15~Fig.3.17 show the results of the electric field calculation around the microelectrode configuration. The arrow in Fig.3.15~Fig.3.17 shows the migrating direction of DNA molecule by dielectrophoretic force. In each cases, the observation results of the transportation trajectory of DNA, which is depicted from Fig.3.9 to Fig.3.14, show good agreement with the electric field calculation.

3.6 Discussion

By using dielectrophoretic force, it is possible to manipulate a single DNA molecule and separate it. In electrophoresis, the total length of the flow channel depends on the electrode gap, but in Micro DNA Flow System it is easier to extend the flow channel. And by controlling an electric field between the serial electrodes along it, it can be possible that DNA molecules in aggregation state exhibit conformational change to be be easily separated through the circle packet. Theoretically, we can get the velocity U of migration of the DNA molecule as follows,

$$\begin{aligned} U &= \frac{2}{3\eta} a^2 \epsilon_m R_e \left[\frac{\kappa_p - \kappa_m}{\kappa_p + 2\kappa_m}\right] \left(\frac{V_0^2}{\delta^2}\right) \frac{1}{r^3} \\ &= \frac{16 V_0^2}{3\eta} \left(\frac{\tan^3 \frac{\delta}{2}}{\delta^2 d^3}\right) \epsilon_m R_e \left[\frac{\kappa_p - \kappa_m}{\kappa_p + 2\kappa_m}\right] \end{aligned} \tag{3.2}$$

Eq. (3.2) shows that the migration velocity of DNA molecules in the globule state depends upon the difference of shape, volume, and polarizability among individual DNA molecules. The migration velocity of DNA molecules is in proportion to the square of the radius of DNA. So, it is possible to separate

DNA molecules by length and polarizability by dielectrophoretic force. It is shown that the transportation of DNA with the state of compacted globule is profitable in the future practical application for the separation of giant DNAs such as human gene. In future we consider the Micro DNA Flow System and our new methodology utilizing conformational transition of higher order strucure of DNA as one of the biomanipulation and automation systems for DNA sequencing is a promising technique.

3.7 Summary

This chapter presented a new methodology on noncontact transportation of DNA molecules by dielectrophoretic force utilizing the conformational transition in the higher order structure of DNA for transportation. Experimental demonstration of DNA transportation in the globule state using dielectrophoretic force and direct observation of the DNA molecule in a non-uniform electric field were carried out with fluorescence microscopy. We show that transportation of DNA with the state of compacted globule is profitable in the future practical application for the separation of giant DNAs such as human gene.

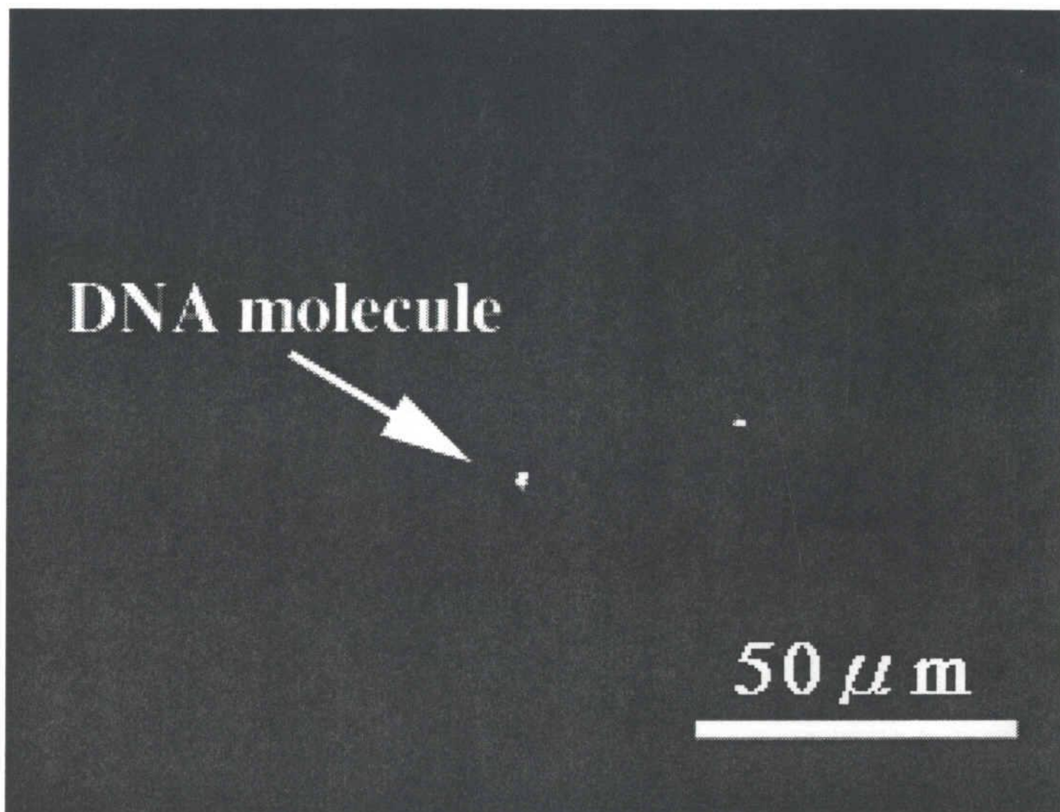

Figure 3.8: Compacted globule DNA molecule suspending in a medium (at E=0)

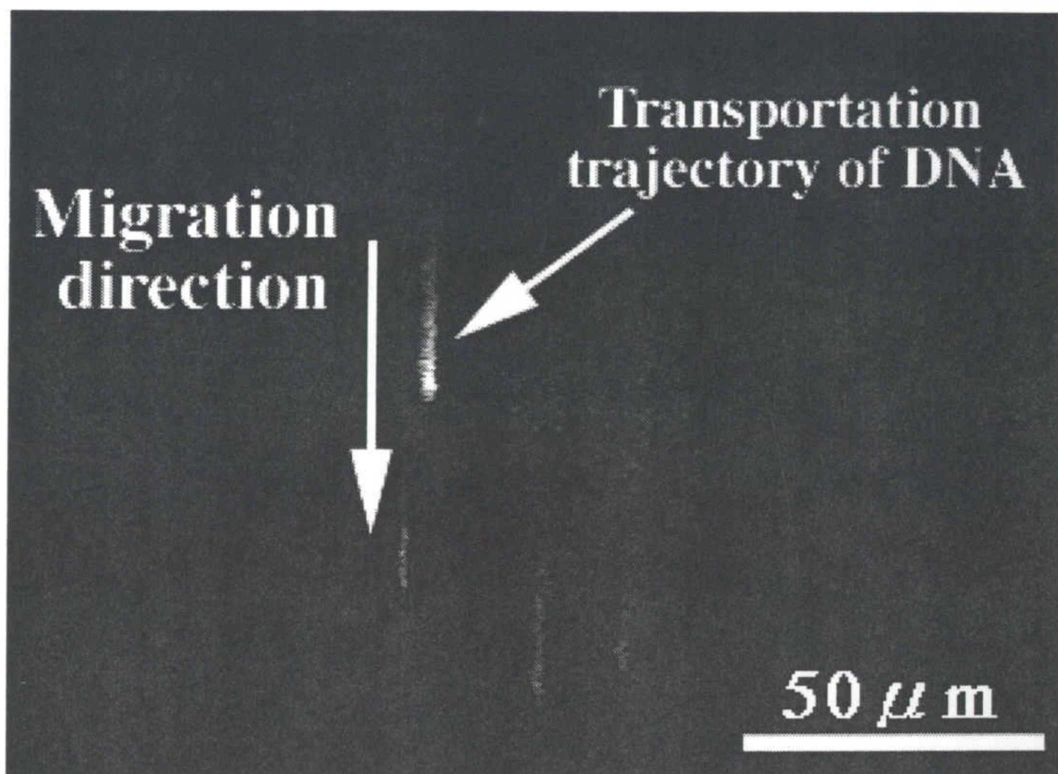

Figure 3.9: Transportation trajectory that the DNA molecule migrates straight between the electrodes near X1 point (as shown in Fig. 3.7)

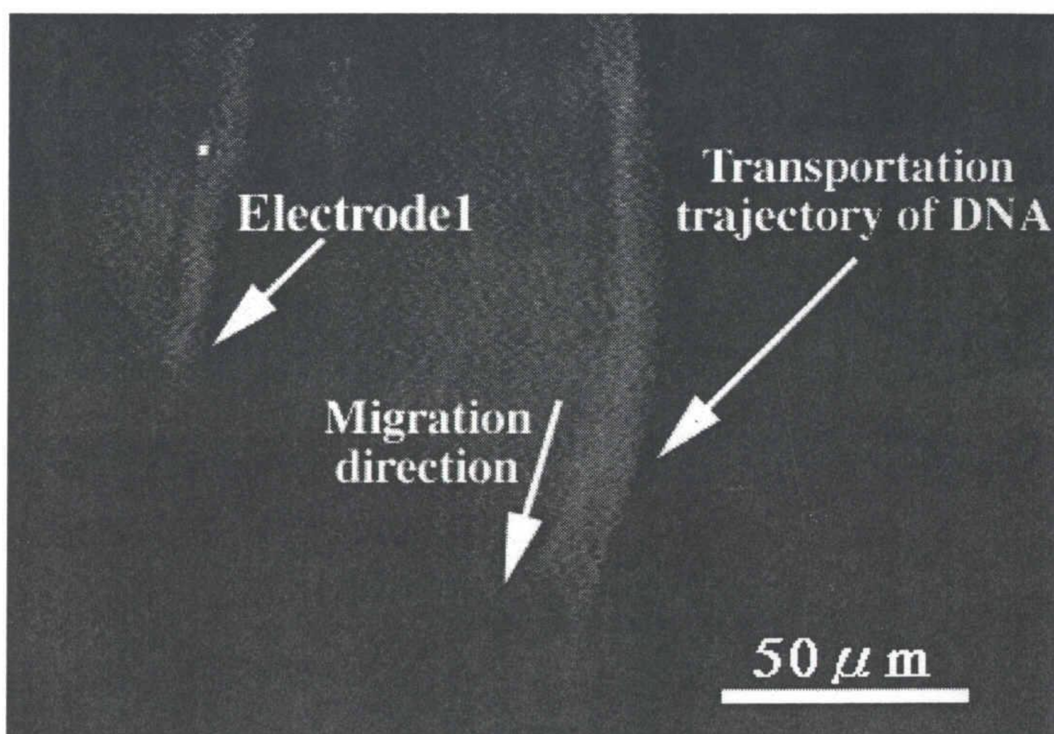

Figure 3.10: Transportation trajectory that the DNA molecule turns to the left near X2 point (as shown in Fig. 3.7)

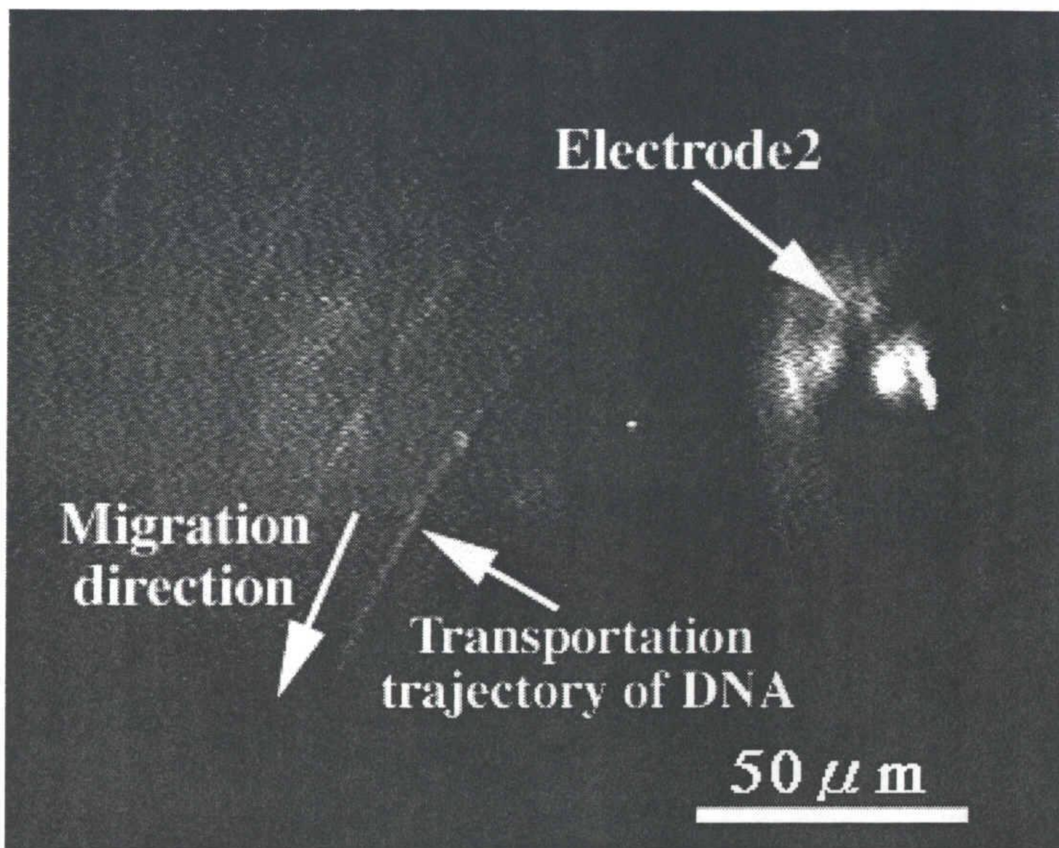

Figure 3.11: Transportation trajectory that the DNA molecule turns to the right near X3 point (as shown in Fig. 3.7)

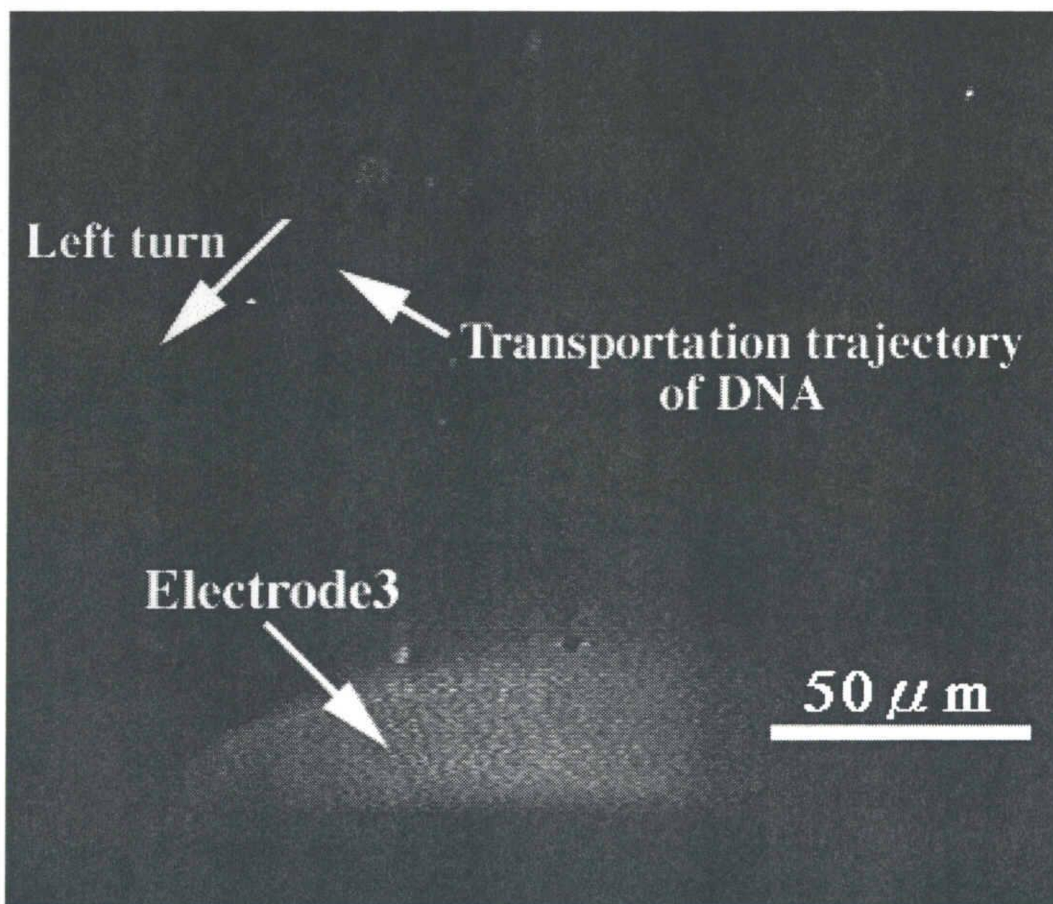

Figure 3.12: Transportation trajectory that the DNA molecule near X4 point (as shown in Fig. 3.7) migrates to the left toward the Ch2

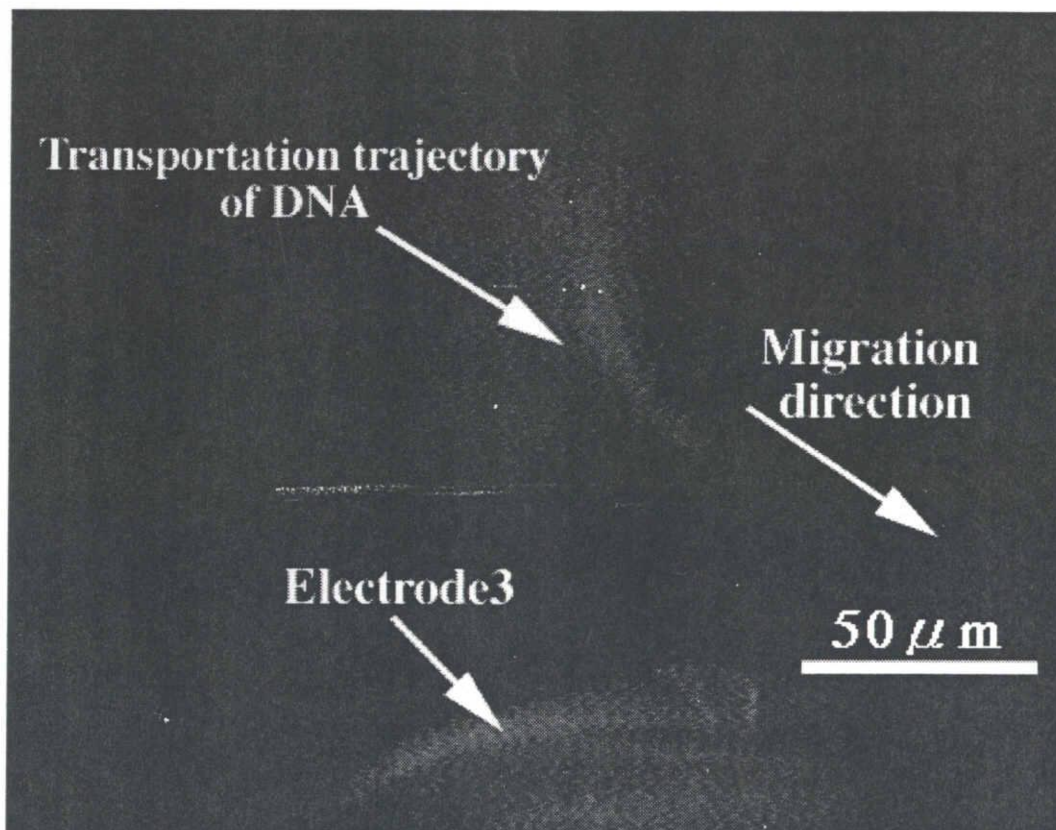

Figure 3.13: Transportation trajectory that the DNA molecule near X4 point (as shown in Fig. 3.7) migrates to the right toward the Ch3

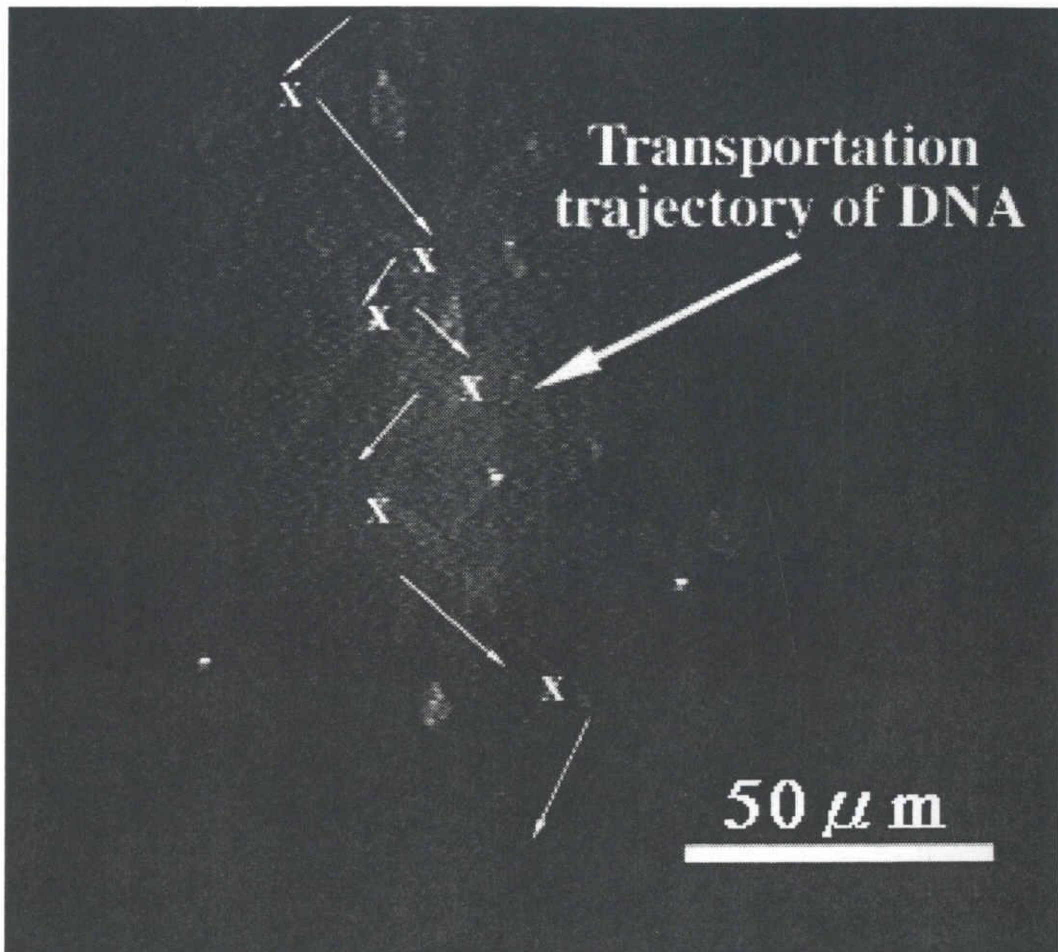

Figure 3.14: Transportation trajectory that the DNA molecule migrates in a zigzag through X4 point (as shown in Fig. 3.7) by switching the electric field (X: switching point)

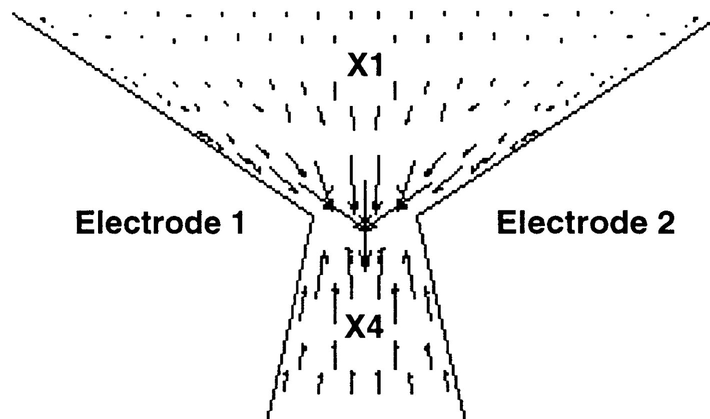

Figure 3.15: Calculation of transportation trajectory of DNA molecule around the micropacket ($\delta 1$, $\delta 2$)=(120°, 30°) from X1 point to X4 point

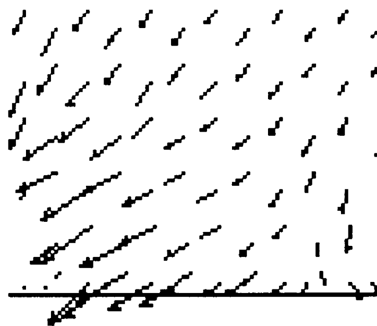

Figure 3.16: Calculation of transportation trajectory that the DNA molecule near X4 point migrates to the left toward the Ch2 in the micropacket ($\delta 1$, $\delta 2$)=(120°, 30°)

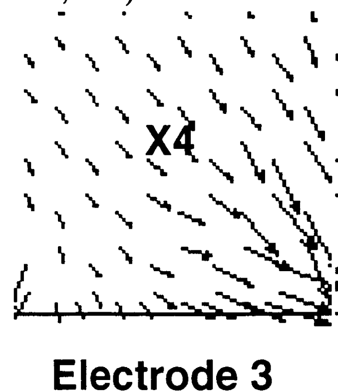

Figure 3.17: Calculation of transportation trajectory that the DNA molecule near X4 point migrates to the right toward the Ch3 in the micropacket ($\delta 1$, $\delta 2$)=(120°, 30°)

Chapter 4

Screening of a Single Escherichia coli in Microchannel by Electric Field and Laser tweezers

4.1 Introduction

In this chapter, we present a new high throughput screening method in microchannel. We have proposed the selective transportation system of DNA molecules for the purpose of transporting and separating a single DNA molecule by use of the dielectrophoretic force in Micro DNA Flow System [127, 128].

In this study, we propose a microsystem combined with optical trapping and dielectrophoretic force and carried out a preliminary experiment of manipulation of a single Escherichia coli. So, the technique, which isolates a single Escherichia coli speedingly and reliably, is needed. The methodology, that an arbitrary biological particles in the aggregation state can be manipulated, is needed because there are a number of Escherichia colis in an aqueous solution.

4.2 High Throughput Screening of Escherichia coli

4.2.1 Operation Improvement by Dielectrophoretic Force and Laser tweezers

Generally, many different kinds of forces are acting on the cell in solution such as the gravity, buoyancy, resistance force from viscosity of the fluid, Brownian motion, interactive forces in the micro/nano world such as Van der Waals force, electrostatic force depending on the surface charges. Moreover, if there is a flow, fluid force will disturb its motion. To control the biological object, we need to control the external force. To apply external force acting on the biological object, the following methods can be used.

(1) Electric Field

Coulomb force (D.C. field)

Dielectrophoretic force (A.C. field) [14,37,111,112]

(2) Ultrasonic Field [39,40]

(3) Laser Beam [46,47,77,78]

(4) Fluid Flow of Solution

(2) and (3) require position information of the object, and the sensing system is needed for automation. However, when we consider precise and noninvasive handling of micro objects, (3) is suitable tool. (4) will disturb the environment with the fluid flow. So, we can simplify the system by using the electric

field and laser tweezers. In case of using coulomb force, convection caused by electroosmosis, polarization of the microelectrode, and electrolysis will occur easily. So, we propose to use dielectrophoretic force to control the biological object.

4.2.2 Dielectrophoretic dispersion of Escherichia coli

In a high frequency field, the Escherichia coli is drawn in the direction of the high field gradient because the dielectrophoretic force operates in the direction of $gradE^2$. The force acting on the Escherichia coli in a nonuniform field is shown in Fig.4.1. Fig.4.2 shows the schematic diagram of the observation sample in the experiment. Fig.4.3 shows the schematic diagram of the electrode design. The force F_{die} and F_d, as shown in Fig.4.3, represent the dielectrophoretic force and the viscous force, when the high frequencyelectric field is applied between the electrode 3 and the electrode 4. In generating a high frequency AC field, the field around the Escherichia coli becomes a nonuniform field. Due to this electric field, the positive and negative charges in the Escherichia coli generate toward the electric field by its polarization. The polarizability of the Escherichia coli depends not only upon the dielectric characteristic, but also upon its conductivity. Some cells and Escherichia colis have conductivity in an aqueous solution. So, their conductivity as well as the dielectric characteristic is very important. As a result, the Escherichia colis migrate toward the normal to the line of electric line and aggregate in the state of the pearl chain formation by dielectrophoretic force. Once the target

Escherichia coli is trapped at the focal point of the laser beam, we can easily realize noncontact dispersion of unneeded Escherichia colis automatically by controlling the magnitude of the electric field.

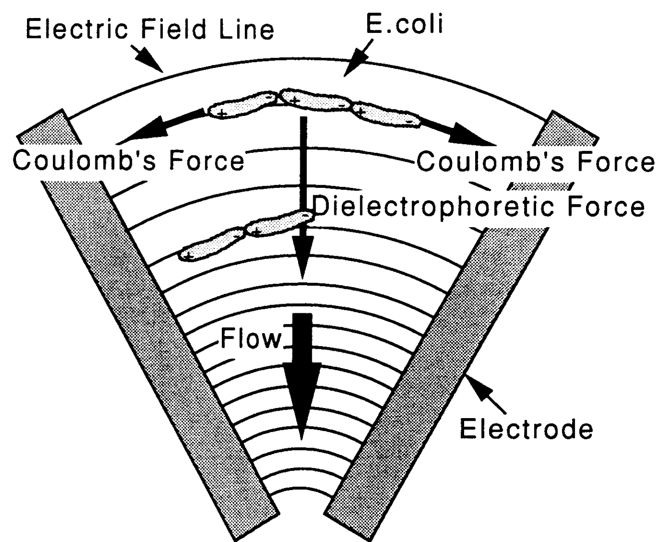

Figure 4.1: Electrical dispersion of Escherichia coli by dielectrophoretic force

4.2.3 Optical Trapping of Escherichia coli by Laser Tweezers

Many biologists are eager to manipulate and separate a single biological particle for their experiment. With the conventional method, it takes so many times to reculture on plates. This method has such strong probability for pure culture of targeted microbes, such as a Escherichia coli. It will be such a promising method of pure culture of microbe for the biologists to discover a new microbe. It is possible to manipulate microbes, such as a Escherichia

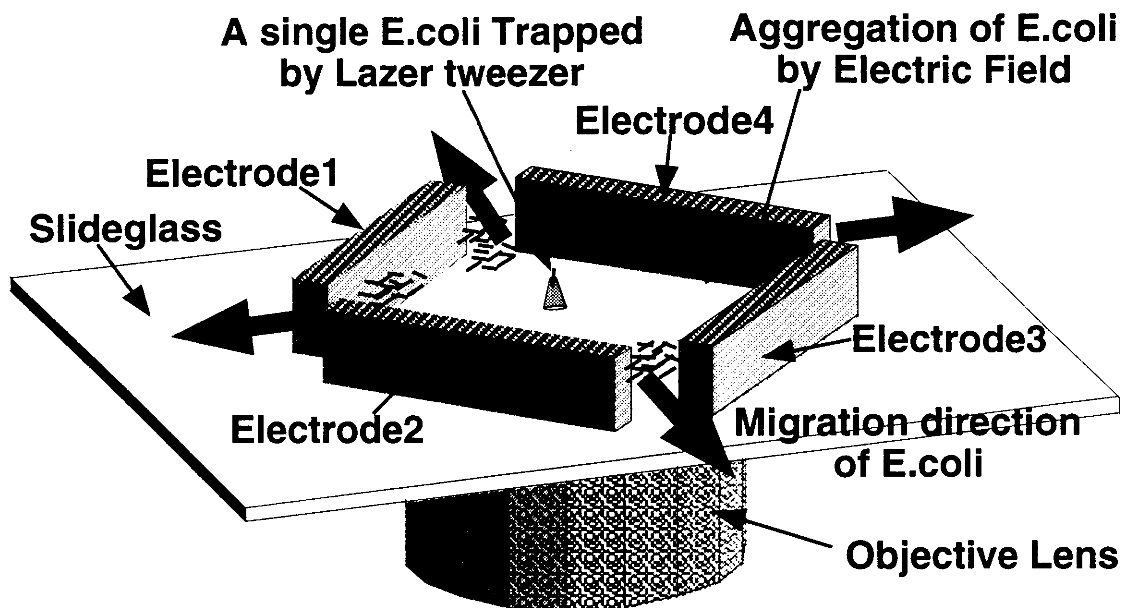

Figure 4.2: Schematic diagram of the observation sample

coli and a yeast cell, with the Laser manipulator we used. Once a target Escherichia coli is trapped at the focal point of the laser beam, we can easily realize noncontact manipulation automatically.

4.3 Experiments

The cultured Escherichia colis diluted with a distilled water were centrifuged. We showed the transportation and dispersion of the Escherichia colis and the observation of the migration of the Escherichia colis when the high frequency electric field switches on and off. We carried out experiments as follows: the applied frequency is constant 1MHz and the applied voltage is constant 10 100Vp-p. By using the electrode samples, as shown in Fig.4.3, we observed the motion of the Escherichia coli by switching the electric field. We designed the electrode (as shown in Fig.4.3) for the purpose of transporting

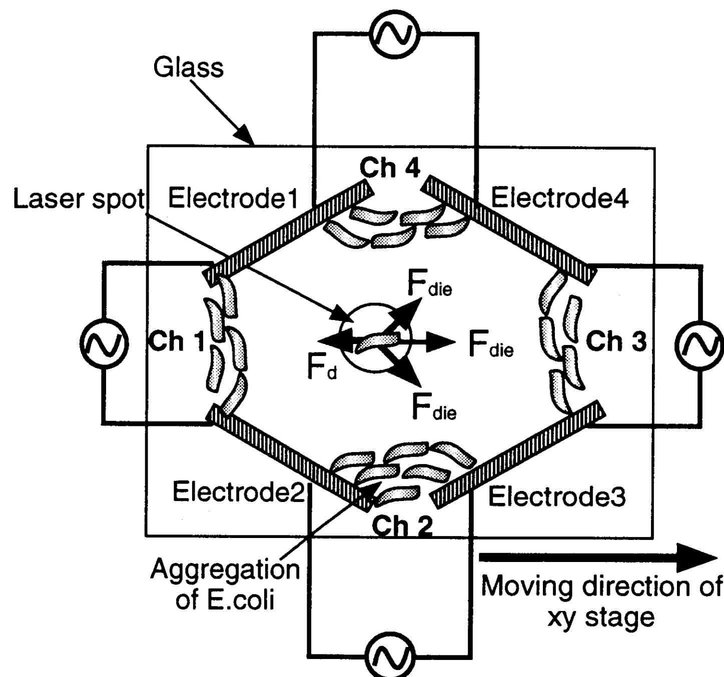

Figure 4.3: Schematic diagram of the electrode design

the Escherichia coli. The thickness of the electrode is $40\mu m$. The minimum electrode gap is $50\mu m$.

The experimental apparatus is schematically shown in Fig.4.4. This laser manipulator consists of the inverted microscope and two high power laser diodes (wave length = 690nm). It is possible to manipulate two micro objects simultaneously. With one of the laser diodes, the focal point in a microscopic view can be moved arbitrarily with two degrees of freedom by controlling two galvano mirrors in the optical system. The trapped objects follows the focal point. The other laser diode is fixed, so the trapped objects cannot be moved without moving the XY stage. The resolution of the XY stage, which is controlled by micro stepping motor, is $1\mu m$. The observation of Escherichia

coli has been carried out with a microscope, as shown in Fig.4.4, equipped with a 100 x oil-immersed objective lens and recorded on videotapes with the sampling rate of 30 frames/s through CCD camera. The applied voltage was controlled by an arbitrary waveform generator.

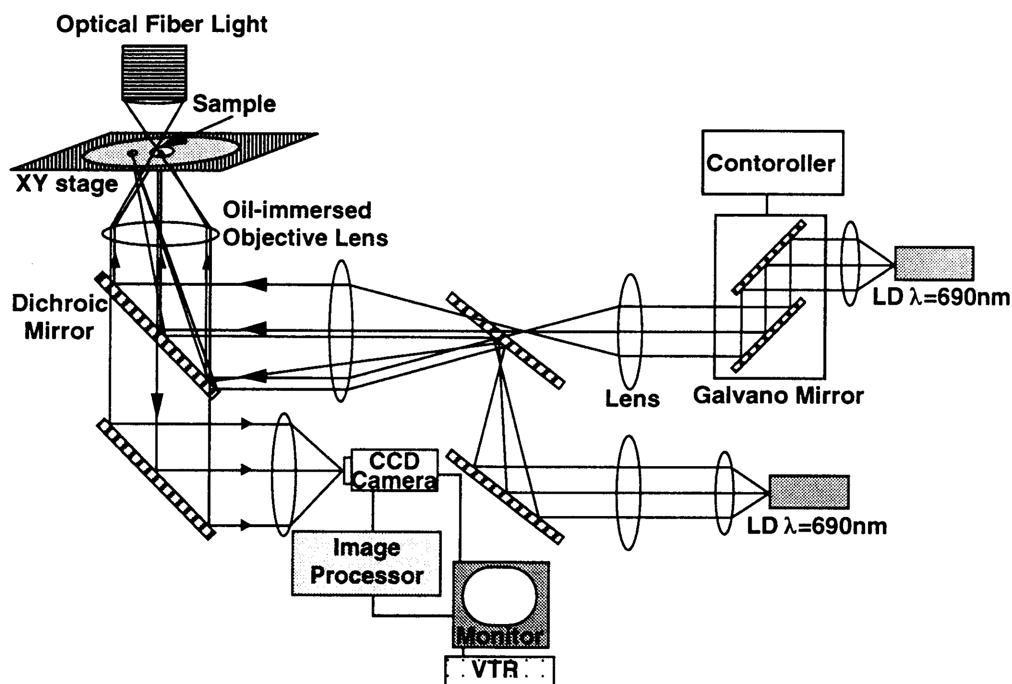

Figure 4.4: Schematic diagram of experimental apparatus

4.4 Results and Discussion

In our previous experiments [8-9], we observed the dynamics of DNA molecules between microelectrodes in an AC field. We showed the possibility of noncontact transportation of a DNA molecule by using dielectrophoretic force. We have confirmed the dependencies of the velocity of DNA molecule on the difference in the angle between electrodes, which is one of parameters of the electrode design. As the angle of the electrodes, where the electrode gap

is the same size, becomes larger, the velocity of migration of a DNA molecule is faster. Because the angle of the electrodes is bigger, the difference in the field gradient is bigger.

From these results, we determined the observation sample design and carried out the experiments by using the observation sample chamber, as shown in Fig.4.2.

It is necessary to disperse unnecessary Escherichia colis toward each channel, as shown in Fig.4.2, in order to manipulate single Escherichia coli by laser tweezers at the center of the observation sample chamber.

Fig.4.5 Fig.4.7 show the motion of the Escherichia colis while the high frequency field switches on and off near the packet. They show that by controlling an electric field between electrodes, it is possible that the Escherichia colis are easily trapped by laser tweezers and transported and dispersed by high frequency electric field through the packet. Fig.4.5 illustrates the microscopic image of the handling of Escherichia coli in Microchannel by optical trapping while the high frequency field was off near the packet. Fig.4.6 and Fig.4.7 show the sequence images while the high frequency field switches on, and the pearl chain formation of Escherichia coli when the high frequency electric field generated between the microelectrodes. In each cases, Escherichia colis don't migrate when the electric field is off.

Fig.4.8 and Fig.4.9 show the motion of the Escherichia colis while the high frequency field switches on and off immediately near the packet. These sequence images depicted that the response of the movement of the Escherichia

colis to the high frequency field is very fast.

Fig.4.10 and Fig.4.11 show the motion of the Escherichia colis while the high frequency field switches on and off between the parallel electrodes. Between the parallel electrodes, the electric field is an uniform field. These images illustrates that the Escherichia colis don't migrate through the microelectrodes and make the pearl chain formation and keep the aggregation state between the parallel electrodes.

Fig.4.12 shows the pearl chain formation of the Escherichia colis near the entrance of channel 2 while the high frequency field switches on. They show that by controlling an electric field between electrodes, it is possible that Escherichia colis in aggregation state are easily separated through the packet.

4.5 Summary

In this chapter, we proposed a novel methodology on high throughput screening and micro surgery. We have developed a prototype of Microchannel system for high throughput screening of Escherichia coli. Experimental demonstration of Escherichia coli transportation using dielectrophoretic force and direct observation of the Escherichia coli in a non-uniform electric field were carried out with Laser manipulator system. We show that transportation and separation of Escherichia coli by dielectrophoretic force and optical trapping is profitable in the future practical application for the high throughput screening of microorganisms.

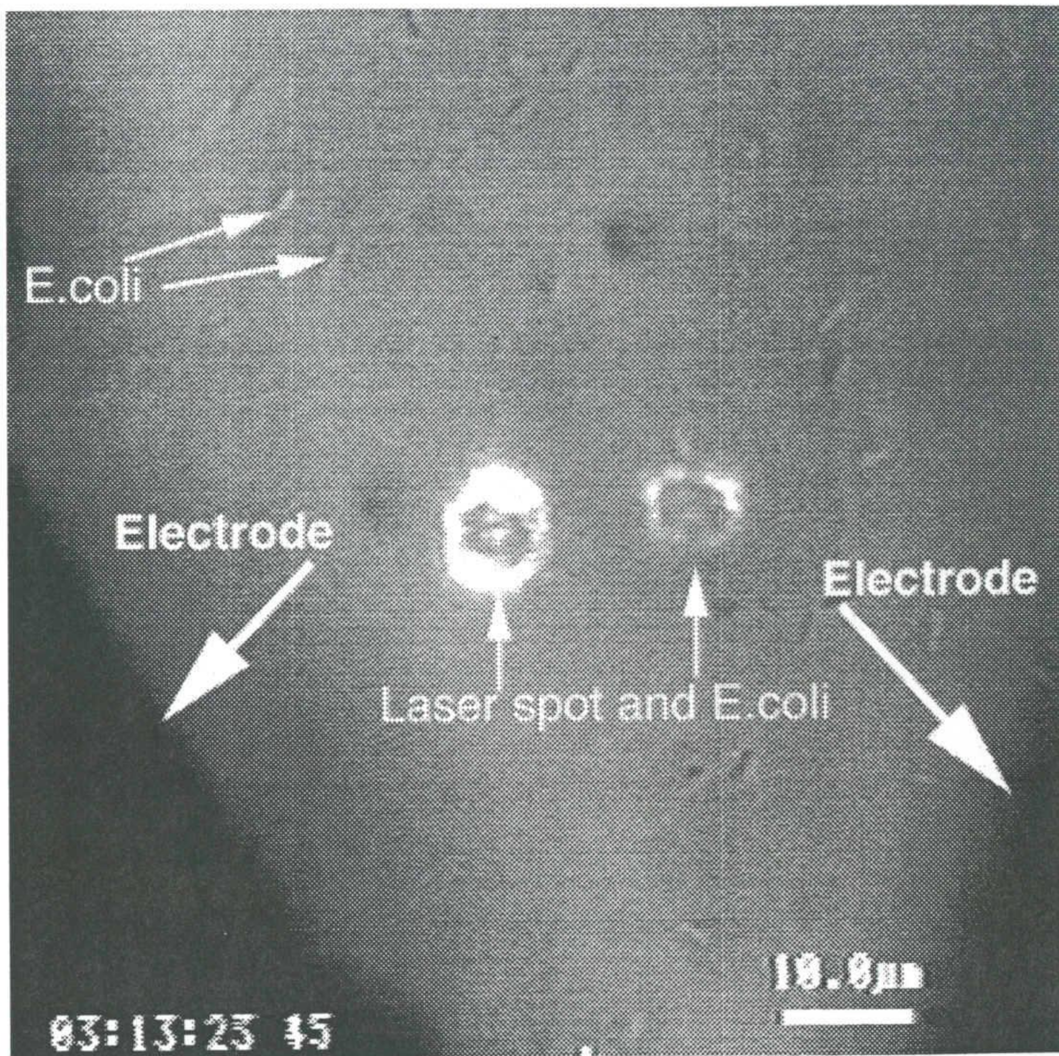

Figure 4.5: Microscope image of Escherichia coli trapped by Laser tweezers in an experiment, while the high frequency field switches off (E=0)

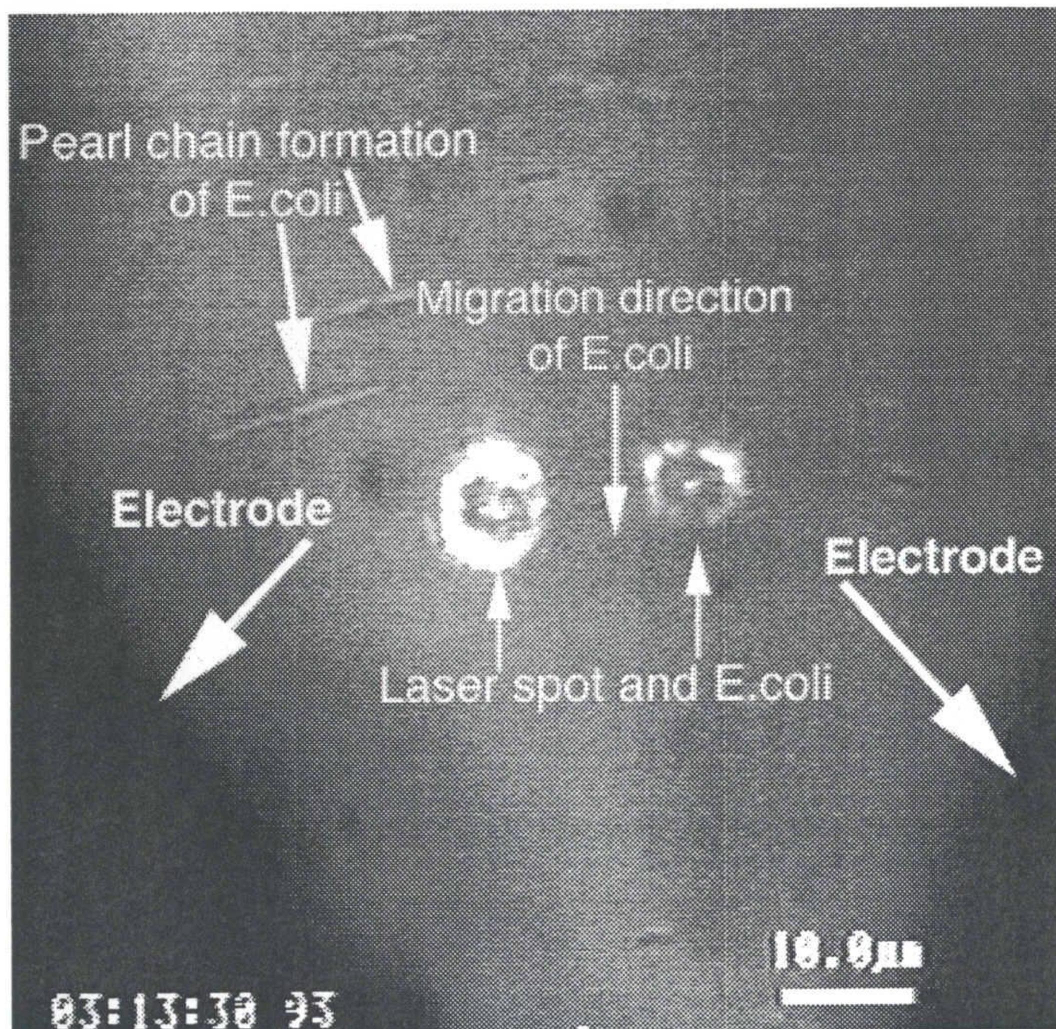

Figure 4.6: Sequence picture 1 that the Escherichia coli in the state of pearl chain formation between the electrode2 and the electrode3 migrate toward the Ch2, while the high frequency field switches on

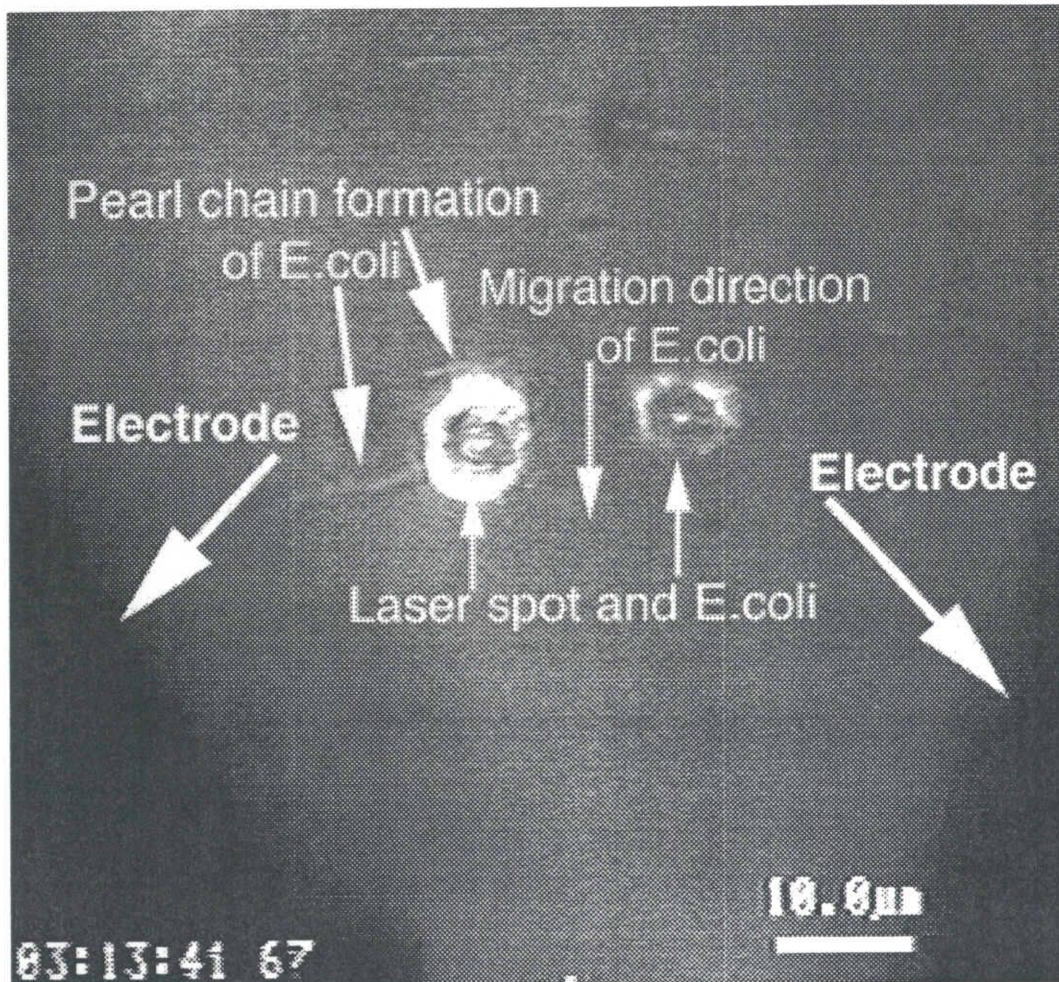

Figure 4.7: Sequence picture 2 that the Escherichia coli in the state of pearl chain formation between the electrode2 and the electrode3 migrating toward the Ch2,while the high frequency field switches on

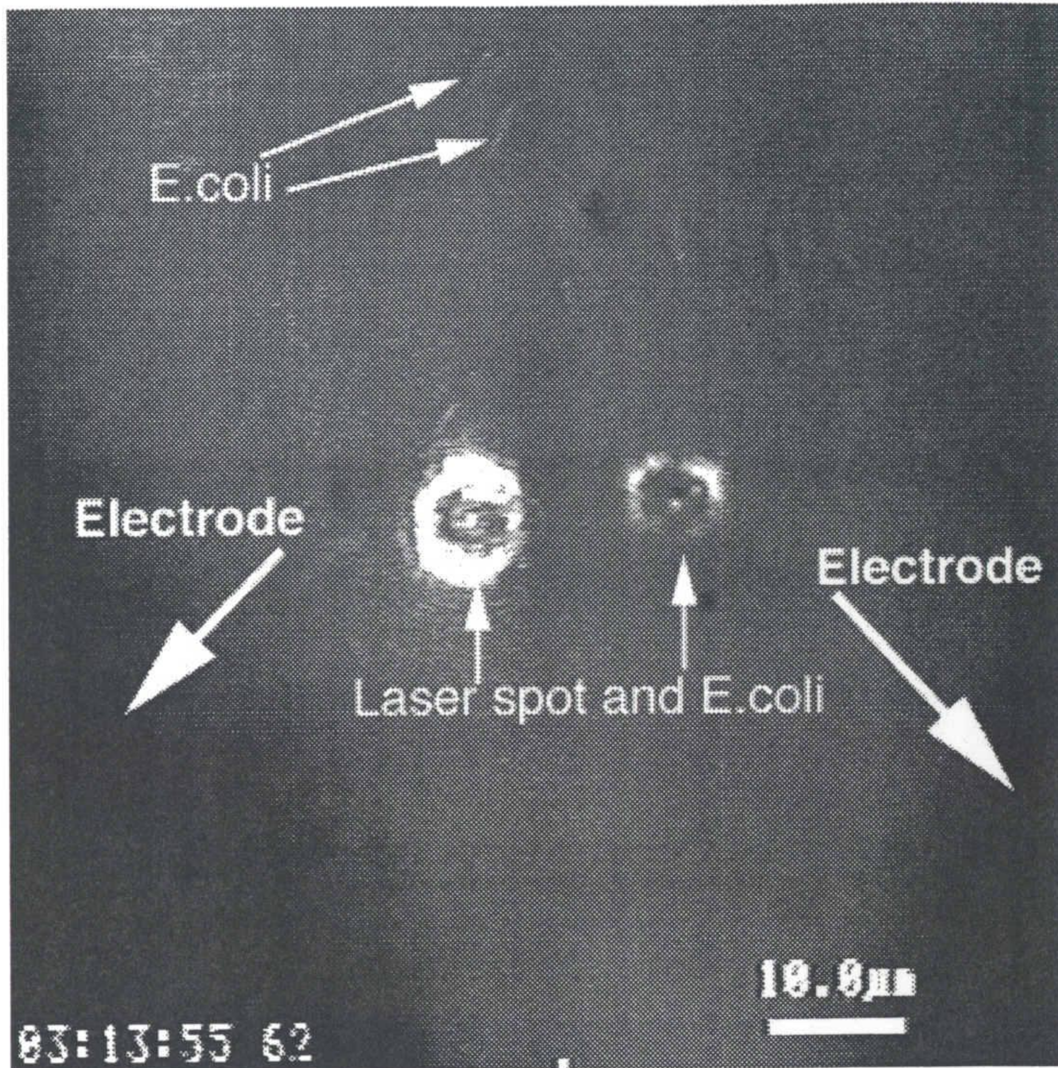

Figure 4.8: Microscope image of Escherichia coli trapped by Laser tweezers in an experiment, while the high frequency field switches off (E=0)

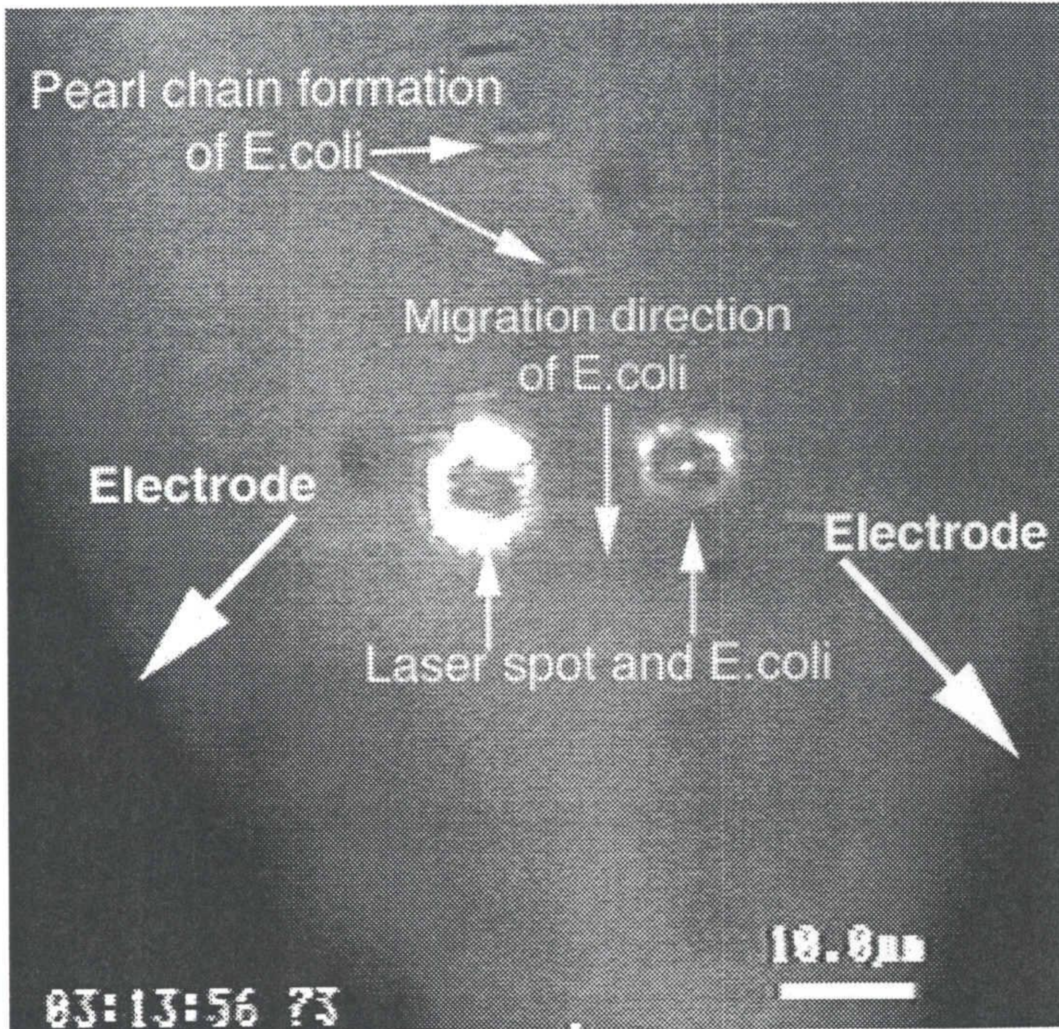

Figure 4.9: Transportation of the Escherichia coli in the state of pearl chain formation between the electrode2 and the electrode3 migrating toward the Ch2

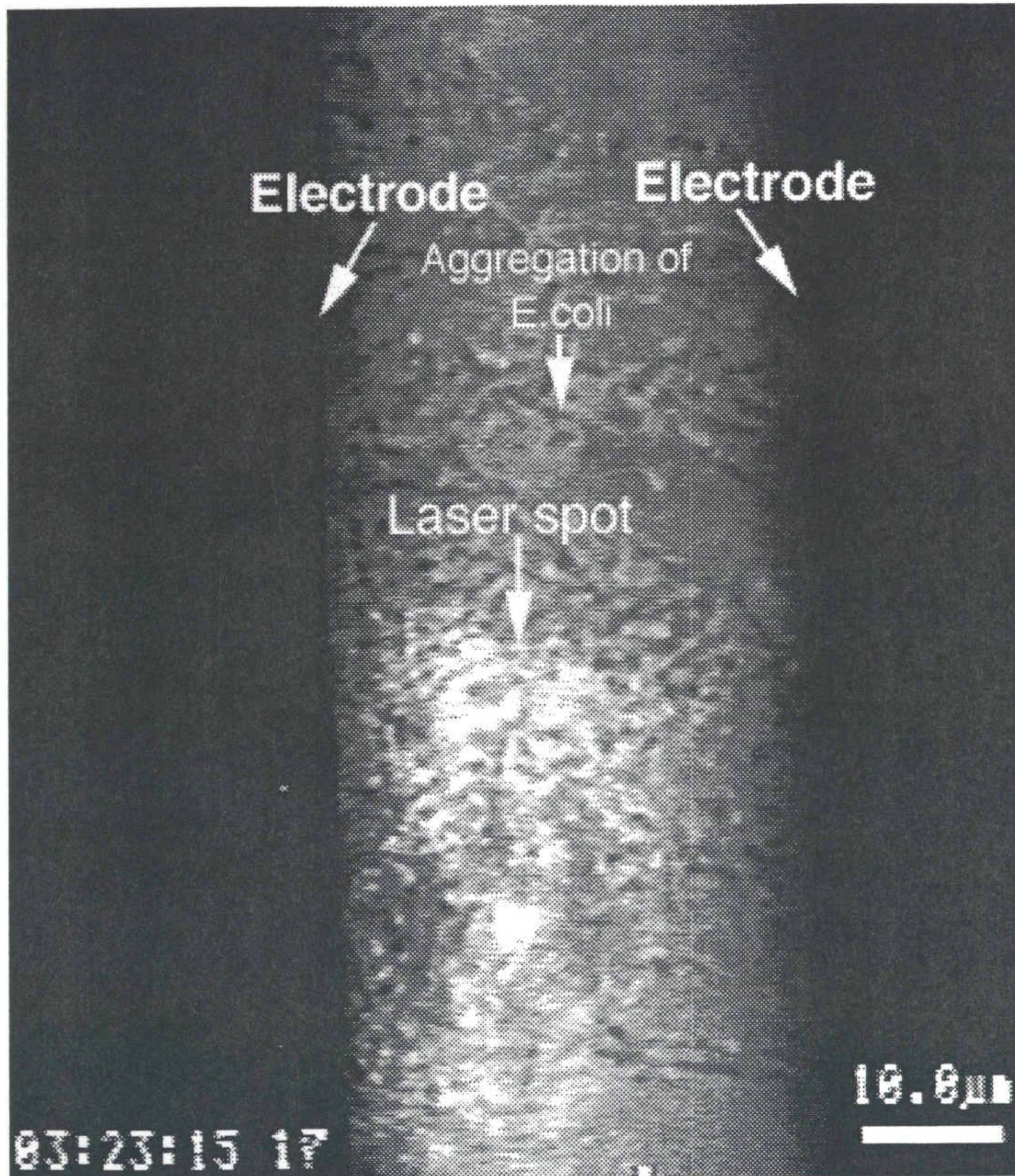

Figure 4.10: Aggregation state of the Escherichia coli while the high frequency field switches off between the parallel electrodes

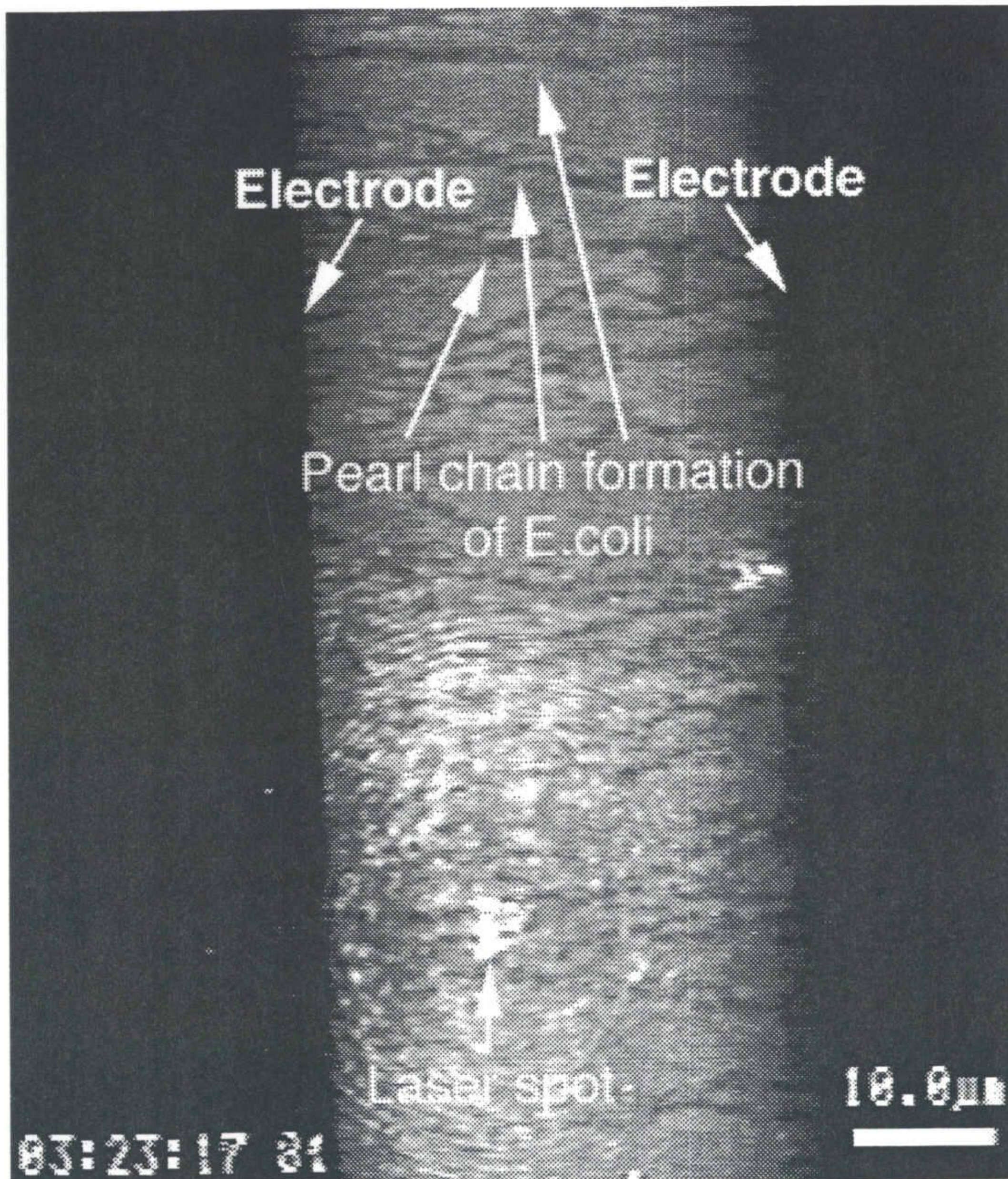

Figure 4.11: Aggregation state of the Escherichia coli in the state of pearl chain formation, while the high frequency field switches on between the parallel electrodes

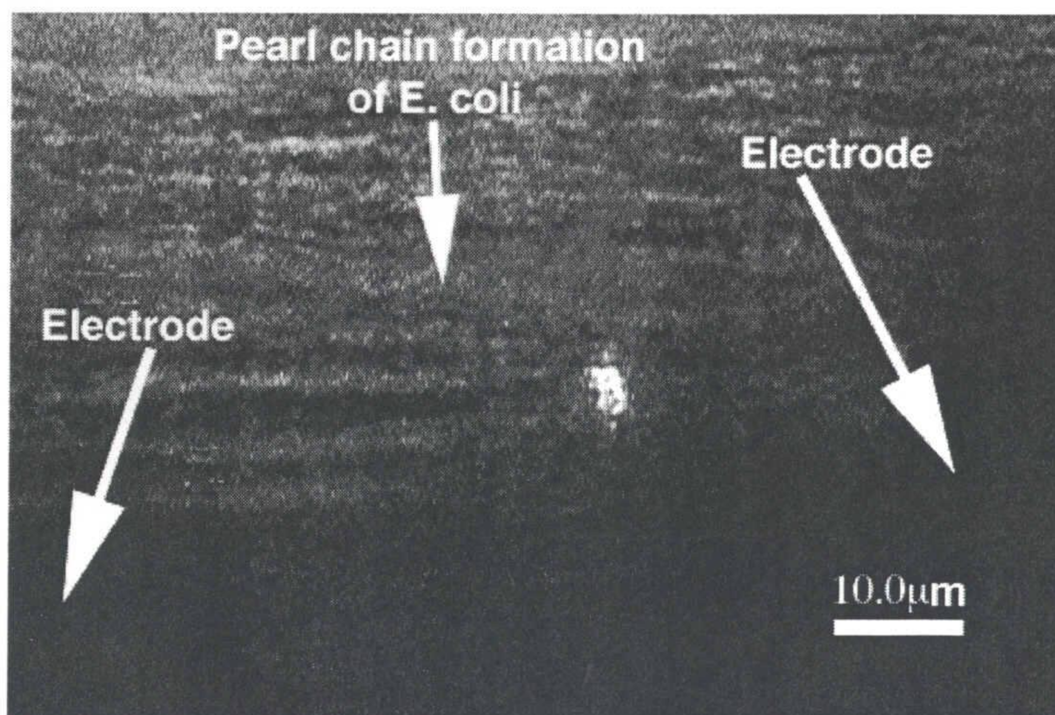

Figure 4.12: Aggregation state of the Escherichia colis in the state of pearl chain formation near the entrance of channel 2 while the high frequency field switches on

Chapter 5

Bio-Micromanipulation System for High Throughput Screening and Micro Surgery in Microchannel

5.1 High Throughput Screening System of Microbes

Recently, there has been great interest in the high throughput screening and micro surgery of microorganisms for the finding of the novel microbes and the development of drug discovery process. Biotechnology has progressed in genetic engineering, life science, medicine and agriculture. For remarkable advancement of bioscience, it is important to develop the new technology, which manipulate and separate microorganisms with high speed and high purity and noninvasive method. In these research fields, operation of the micro/nano manipulation of cells and DNA, mass production, sequential processing, and high speed and high precision processing are required. However, the working efficiency and operability is dependent on the skills of the human operator. For the breakthrough in these fields, integration of the distributed research fields and

system technologies is important. At this moment, micro electro mechanical systems technology and micro robotics has potentiality for the breakthrough in the bioengineering. For these multidisciplinary research areas, there are big challenges to improve the conventional method and develop the new system integration . We have considered the basic strategies to improve the operability of the bio micro manipulation. We should integrate the robotics and bioengineering. We call this research field as the bio-robotics [8].

Basic technologies of the bio-robotics are manipulation technology, micro system technology, visualization technology, human interface technology to improve operability, automation technology. Especially, micro/nano manipulation of a DNA molecule, animal or plant cell, and embryo and high throughput screening of microbes is important. However, research works is not enough at present.

5.2 Important Technologies for Bio Micro Manipulation

In this section, at first, we classify basic technologies required for the bio micro manipulation and we apply our methodology and strategies to high throughput screening of Escherichia coli in microchannel as follows:

i) Separation, transportation, and position / orientation control technology

   Contact, non contact, High speed, high precision

ii) MEMS technology

   Fabrication, microchannel, micro valve, micro physics, micro sensor

iii) Measurement / control technology

Sensor, Image processing, teleoperation

iv) Visualization technology

Static, dynamic, 2D, 3D, fluorescence imaging

As an example of the contact tasks, nuclear transplantation or embryo culture requires those technologies. Most of the bio micro manipulation tasks are performed in an aqueous solution. Thus, compared with the general manipulation tasks in the air [6, 7], we have to consider not only the gravitational force but also the buoyancy, fluid force from flow, Brownian motion, interactive forces in the micro/nano world such as Van der Waals force, electrostatic force depending on the surface charges. Moreover, the size of each object to manipulate is in micron order, it is quite difficult to recognize its configuration and to manipulate it freely. Automation of manipulation is quite difficult, so the skills of the human operator are required. From these point of view, we summarize the basic strategies to improve the working efficiency and the operability of the bio micro manipulation for high throughput screening and micro surgery of microbes as follows:

i) Utilization of optical trapping by the laser tweezers [46, 47, 77, 78]

At present, polylysine or micro pipette is used for fixation. Fixation by the polylysine is easy, but this method is not reversible. Automation of the micro pipette operation is difficult. So, we need to develop a new method in a different way. Once the biological object is trapped

at the focal point of the laser beam, we can easily realize noncontact manipulation automatically. In this chapter, we show the system flow diagram utilization of optical trapping by the laser tweezers.

ii) Utilization of dielectrophoretic force generated between the microelectrodes [14, 37, 111, 112]

To improve working efficiency and operability in solution and to realize high speed separation, the technique for sequential processing is needed. Once the object is trapped at the focal point of the laser beam, we can easily realize noncontact dispersion of unneeded microbes automatically by controlling the magnitude of the electric field.

iii) Realization of autonomous function

Micro manipulation tasks are versatile. So, it is difficult to realize a full automation system. We should start from classifying the basic operation for high throughput screening. Most of the contact manipulation, such as nuclear transplantation, is performed by the human operator by hand with the microscope. We cant expect quick response nor precise and repetitive operation by hand. So, we should realize shearing of the autonomous function and manual operation.

5.3 New methodology of selective transportation system of Escherichia coli

We have developed a prototype of Microchannel system (Fig.5.1) for high throughput screening of Escherichia coli. Experimental demonstration is shown.

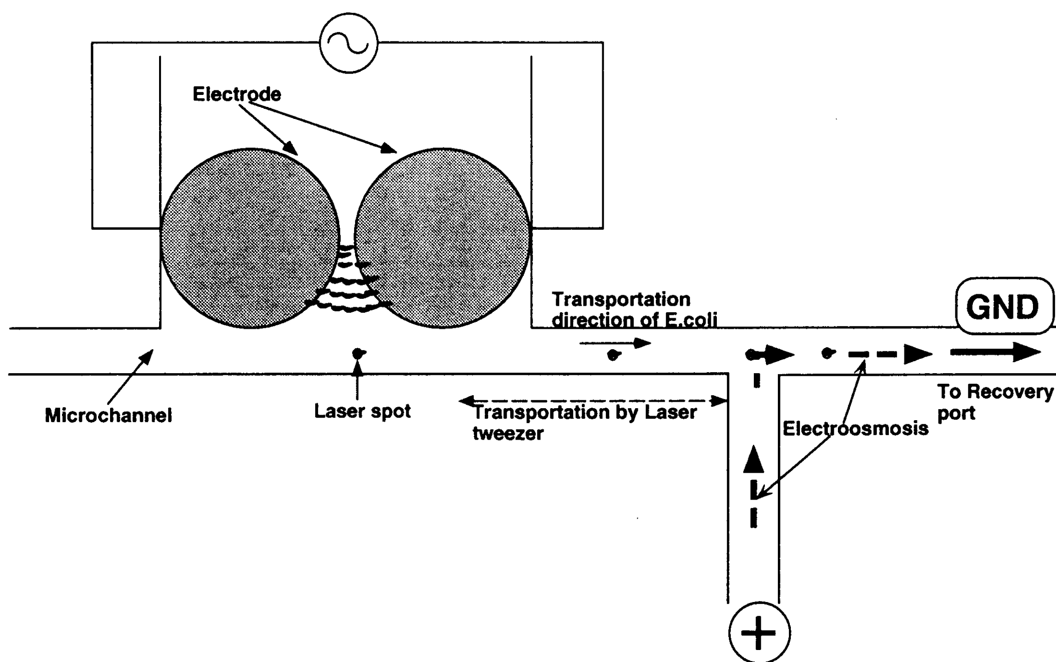

Figure 5.1: Micro Glass Chip for High Throughput Screening of Microbes

Fig.5.2 shows the sequence flow chart and the system flow diagram for our proposed high throughput screening of Escherichia coli. At first, in order to specify a target Escherichia coli, we propose the detection of the target Escherichia coli by image recognition. The characteristics of each Escherichia coli is dependent on the fluorescent color, the shape, the mobility, and the migration ability etc. Using the fluorescence microscopy, it is possible to detect

the fluorescence image of the Escherichia coli. The sequence flow chart we propose is as follows:

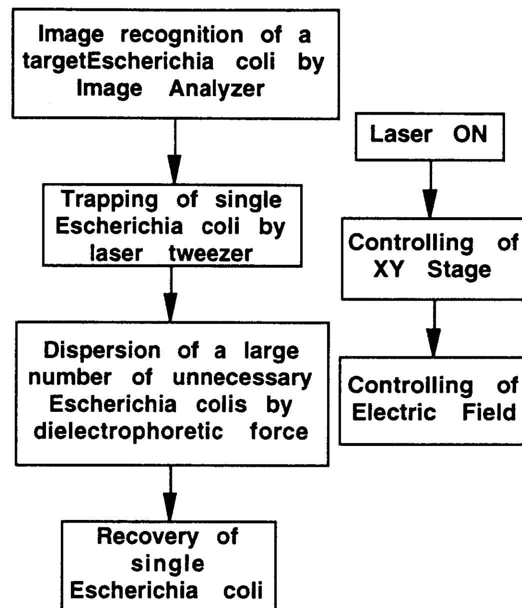

Figure 5.2: System flow diagram for high throughput screening of Escherichia coli

i) Image recognition of a target Escherichia coli by Image Analyzer

ii) Setting the coordinates which the laser beam radiates by controlling of XY Stage or galvano mirror

iii) Laser beam ON

iv) Trapping of the target Escherichia coli by laser tweezers

v) Dispersion of unneeded Escherichia colis in an aqueous solution by dielectrophoretic force while controlling of the magnitude of the electric field

vi) Recovery of single Escherichia coli

vii) Finally, a single Escherichia coli is separated.

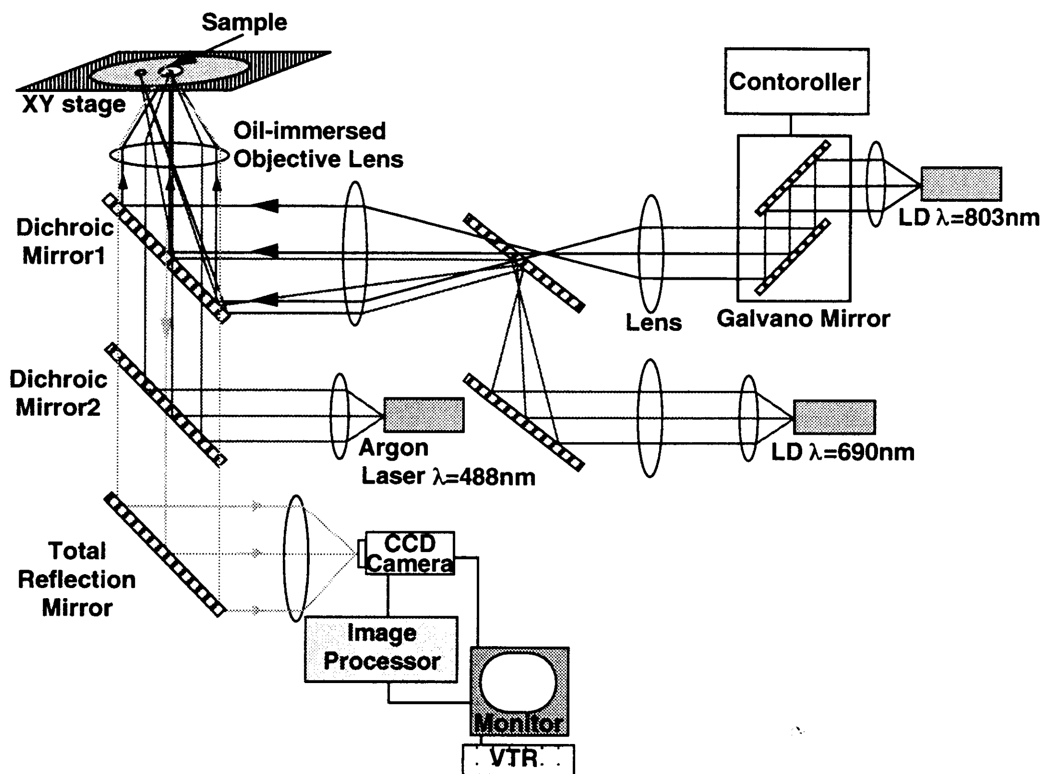

Figure 5.3: Schematic diagram of Fluorescence Microscopy with Laser Manipulator

In this chapter, we propose new methodology of selective transportation system of Escherichia coli by dielectrophoretic force and optical radiation pressure and carried out a preliminary experiment. We demonstrated the experiments through the sequence flow chart (2) to (5), as shown in Fig.5.2.

5.4 Application of Liposome to Micro Encapsulation Delivery of Escherichia coli

5.4.1 Giant Liposome as Micro Capsule

Liposomes are the simplest artificial structures, resembling natural membrane-delimited domains [85, 88, 89]. Fig.5.4 shows the schematic diagram of liposome. When Escherichia coli and DNA molecule are entrapped in liposomes [90,91], the resulting supramolecular structures exhibit very interesting properties from both a fundamental and a practical point of view. Fig.5.5 shows the concept of trapping single globular DNA molecule by Laser tweezers and its application to micro encapsulation by liposome. DNA molecule-loaded liposomes can be regarded as simplified models of biomembranes and they can be used as highly specific microreactors for exploiting the genetic information in a variety of applications. This compartmentation contributes significantly to the entrapment of microbes and single globular DNA molecule and to the optimization of the cell behaviour.

5.4.2 Optical Trapping of Liposome by Laser tweezers

Neutral phospholipids were obtained from Sigma, and inclueded soybean phosphatidylcholine (soybean PC) and dioleoylphosphatidyl-choline (DOPC). These lipids were dissolved in pure methanol or in a $1:1$ (v : v) chloroform-methanol mixture. Next, $0.1\mu mol$ of dry lipid film was prepared by evaporation of the organic solvent in a $10-mm\phi$ test tube under a nitrogen stream, and then stored for at least 2 h under aspiration. The resulting lipid film

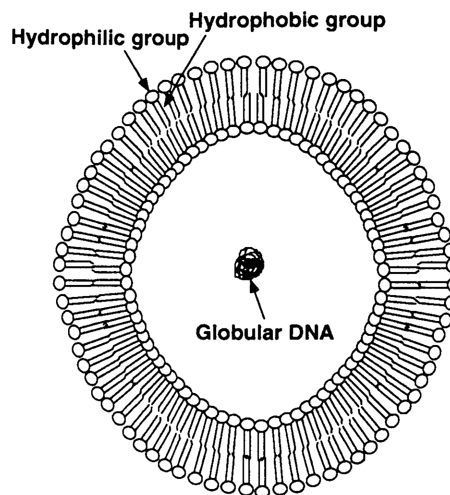

Figure 5.4: Schematic diagram of Liposome

was swollen with $100\mu ml$ of TBE buffer ($90mM$ boric acid, $2mM$ EDTA, pH adjusted to 8.0 by HCl) or aqueous solution containing $MgCl_2$, where the final concentration of phospholipid was $1mM$. Fig.5.6 shows microscopic image of liposome in Microchannel trapped by Laser tweezers. We can control the conformation of liposome and apply it to the micro encapsulation delivery of microbe.

5.5 Summary

In experiments, we show that transportation and separation of Escherichia coli and Liposomes with electric field and optical radiation pressure. Isolation of Escherichia coli by fluorescence microscopy with Laser Manipulator was successful. Using this system, it is profitable in the future practical application for the high throughput screening of microbes and drug delivery and drug discovery.

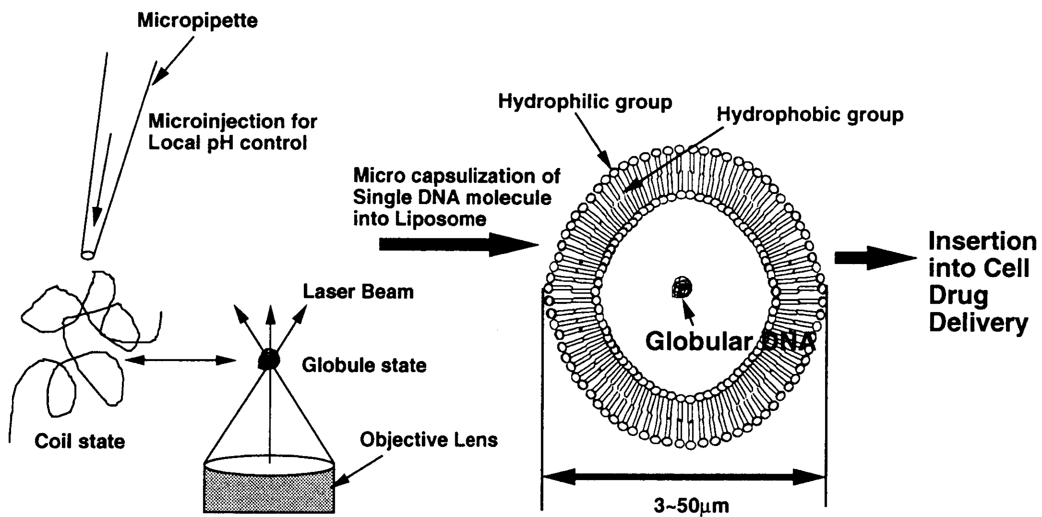

Figure 5.5: Concept of trapping Single globular DNA by Laser tweezers and its Application to Micro encapsulation by Liposome

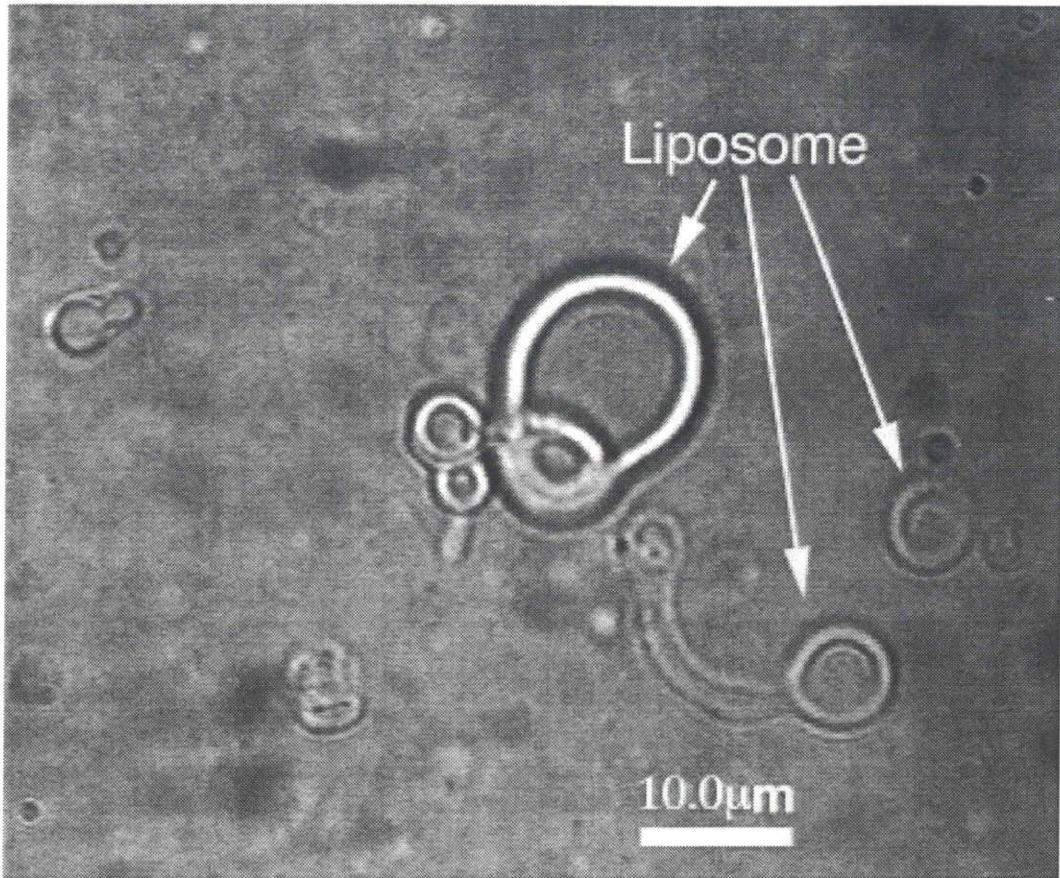

Figure 5.6: Microscopic Image of Liposome trapped by Laser tweezers

Chapter 6

Conclusion

6.1 Summary

In this chapter, I summarize the results obtained in the previous chapters systematically.

Chapter 2 described experiments involving noncontact transportation of DNA molecules by using dielectrophoretic force. Compared with the method employing electrophoresis, electroosmosis and chemical reaction on electrodes doesn't occur by use of an AC field. In a Micro DNA Flow System it is also easier to extend the flow channel. We designed a simple electrode, carried out the experiments and measured the velocity of migration of the DNA molecule. We observed the dynamics of DNA molecules between electrodes in an AC field. We showed the possibility of noncontact transportation of a DNA molecule by using dielectrophoretic force.

In Chapter 3, we demonstrated the transportation of DNA molecule in the globule state by dielectrophoretic force. A DNA molecule is polyelectrolyte. However, the compactization of a single DNA molecule is considered to induce charge neutralization, so it is not effective to use the electrophoresis that is one

of the methods of separation of charged particles. Dielectrophoresis is suitable for transportation and separation of dielectric particles. In the experiments, we observed the transportation of the DNA molecule in the globule state stably. We show that transportation of DNA with the state of compacted globule is profitable in the future practical application for the separation of giant DNAs such as human gene.

Chapter 4 proposed a novel methodology on high throughput screening of Escherichia coli. Experimental demonstration of Escherichia coli transportation using dielectrophoretic force and direct observation of the Escherichia coli in a non-uniform electric field were carried out with Laser manipulator system. By controlling an electric field between electrodes, it is possible that Escherichia colis in aggregation state are easily separated through the packet. We showed that transportation and separation of Escherichia coli by dielectrophoretic force and optical trapping is profitable in the future practical application for the high throughput screening of microorganisms.

Chapter 5 proposed a novel methodology and system on high throughput screening of microbes. We have developed a prototype of Microchannel system for high throughput screening of Escherichia coli. We discussed research issues in the bio micro manipulation and presented a new direction in this field. We consider the basic strategies to improve the working efficiency and the operability of the bio micro manipulation. In experiments, we show that transportation and separation of Escherichia coli with electric field and optical radiation pressure, and application of liposome to micro encapsulation delivery

is profitable in the future practical application for the high throughput screening of microbes. We discuss the application of liposome to micro encapsulation delivery of biomaterials.

6.2 Future work

For these multidisciplinary research areas of biotechnology, there are big challenges to improve the conventional method and develop the new system integration for Bio-Micromanipulation. We have to consider the basic strategies to improve the operability of the bio micro manipulation. We should integrate the robotics and bioengineering.

For example, in the case of DNA sequencing, determining the human genome sequence and finding the genes is really just a first step. After sequencing, we still need to determine what proteins the genes produce, and what those proteins do in the cell. We still need to know the structure and function of the protein produced by the gene, and how that protein interacts in the environment of the cell. Bioinformatics is a necessary field in these studies. When we design the system for a various kinds of research in bioscience field, we have to continue to consider system integration and new methodology for the progress of lifescience and bioscience.

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III Review

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