論文の内容の要旨

論文題目　Microchamber array for biological analyses in confined spaces

（制限された空間内における生物学的分析のためのマイクロチャンバアレイ）

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Introduction

Traditional bulk experiments represent measurements of ensemble averages by which a vast multitude of duplicate systems are probed and average responses are recorded [6, 7]. Tools comparable in size to the basic compartment of life allow precise understanding of biological functions [1-3]. Single-molecule studies have revealed the molecular behaviors hidden in traditional bulk experiments [8, 9]. These studies rely on sophisticated optical strategies that are necessary to limit the detection volume for single-molecule studies [10, 11] and surface immobilization strategies for direct observation. Rondelez et al. proposed a microchamber array to trap individual molecules for enzymatic studies [4]. The activity of single enzymes was measured with conventional microscope setup by confining products of single enzymes into the ultra-small volume (femtoliter) of the microchambers. This simple and powerful platform can be extended for further biological reactions at single-molecule level. Meanwhile, cell-based biological assays traditionally probe cell ensembles thereby completely averaging over relevant individual cell characteristics and even sometimes misleading the interpretation of cell properties [12-15]. Several methods are proposed to array single cells for interpretation of individual behaviors [14, 16-18]. However, all these approaches focus on how cell behavior arisen from the environmental stimuli [13]. No methodology was proposed for direct analysis of individual intracellular materials to study heterogeneity of single cells.

The ultimate goal of the thesis is to realize further biological analyses for precise understanding of biological functions, which is impossible to achieve with pre-existing methodologies, by using the concepts of microchamber array, confined ultrasmall volumes, statistical manipulation and parallelized analysis of individual differences. A highly efficient protein synthesis system is developed in order to achieve for the first time protein synthesis with the smallest amount of DNA possible: a single molecule which is statistically confined. This required also to specially treating the surface of the device for biocompatibility with cell-free protein synthesis. This ultrasensitive system allows to generate a pure protein sample in each microchambers and will be further developed for new generation high-density protein chips. On the other hand, a novel concept of microchamber array platform, parallelized analysis of individual differences, is proposed for direct analysis of individual intracellular materials. By integrating electrostatic functions into the array, single cells are stably trapped at the floor of microwells. All trapped cells are simultaneously lysed inside the sealed microwells that can lead to parallel manipulation and analysis of populations of cells and their lysate. Quantification of intracellular ATP concentrations among a population of single cells demonstrates the feasibility of this new concept. The approach should be of interest for many biological studies about heterogeneity of single cells.

Microchamber array for biochemical reaction at single-molecule level

Protein synthesis with single-DNA molecules has significant advantages in that we can get the pure proteins from individual DNA molecules. Kinpara et al. performed protein synthesis with micro-size chamber array (minimum volume of the chamber was 1 pL) [20]. With this method, a minimum of 10 molecules of DNA per chamber was necessary in order to observe the signal from protein synthesis. The purpose of this section is to develop a novel cell-free protein synthesis platform with sufficient high-performance to achieve protein synthesis from

statistically confined single-DNA molecules. The dense compartmentalization of the array would allow the development of high-density protein chips.

PDMS chip having 190 fL microchamber array is fabricated with conventional molding replica technique and coated with a 2-methacryloyloxyethyl phosphorylcholine (MPC) polymer to prevent protein adsorption [21, 22]. DNA plasmids containing Emerald GFP gene was added into commercially available cell-free synthesis system. MPC coating increases protein synthesis efficiency about 3 times. In order to verifying a number of DNA molecules confined within each microchamber, rate of protein synthesis is investigated statistically. Occupancy distribution is fitted with Poisson distribution. The fitted expected value $\lambda = 1.38$ is comparable with the introduced concentration of the DNA molecules, about 1.25 DNA molecules per chamber. About 40 percent of chambers contain only single DNA molecules.

This is the first report that performs cell-free protein synthesis inside the microchamber array with the minimal quantity of DNA molecules possible as a starting material: a single molecule. A single DNA molecules are trapped into microchamber array statistically. Fine tuning cell-free protein synthesis system and finding the appropriate coating surface reagent for biocompatibility of the system were critical to achieve this result. With this method, pure proteins can be synthesized from individual DNA molecules in an array format, which will allow extreme compaction of the pure protein spots (about 10000 spots in 1 mm^2 area) for high-density protein chips.

Novel microchamber array platform for study of heterogeneity of single cell

The goal of this section is to realize the concept of parallelized analysis of individual differences with new microchamber array platform. The concept is demonstrated by quantifying individual ATP concentration ([ATP]). To understand fully how ATP levels influence cellular functions and faith, a direct measurement of its concentration at the single-cell level is needed. This requires single-cell trapping, cell lysis and readout of the ATP amount contained in each cell. This is a difficult task and so far no methodology exists for this purpose. We have successfully reach this goal described in the section below.

The device for the parallelized analysis of individual cells contains a large number of arrayed microwells that can lead to parallel manipulation and analysis of populations of cells (50 by 70 microwells inside 0.9 mm^2 area). The microwell array was fabricated on inter-digitated ITO electrodes with negative-type photoresist. Electric fields are highly localized at the edge of the electrodes at the bottom of the microwell. This set up efficiently induces dielectrophoresis (DEP) force to trap cells into the microwell with stability and electroporation (EP) for lysis. The microwells physically restrict not only the available space for cells during trapping but also prevent the diffusion of intracellular materials after cell lysis.

Microwells were gradually occupied with single cells by DEP trapping and after 3 minutes almost all of them contained single cells. In order to demonstrate confinement of intracellular materials, the microwell array is closed by pressing the PDMS membrane with rounded plastic tips and electric pulses are applied to lyse trapped cells with EP. 100 percent of cells inside the microchambers are lysed at the same time since we can apply homogeneous and high electric fields to each cell. The intracellular materials are tightly confined inside the closed microchamber array. By lysing trapped cells with luciferin-luciferase (LL) reaction reagents inside the microchabmer array, intracellular [ATP] can be quantified after calibration of light emitted (luminescence) from the microchamber array. Individual intracellular [ATP] is obtained from randomly selected 100 microchambers. The mean value of intracellular [ATP] obtained in device, 1.6 mM, is comparable with the values obtained in bulk measurements with the luminometer, 1.4 mM. Measured individual [ATP] is distributed from 0.8 to 2.2 mM. It is impossible to see this individual difference with traditional bulk measurements which probes cell ensembles thereby completely averaging over relevant individual cell characteristics.

In this section, a novel concept of microchamber array is developed to realize parallelized quantification of absolute intracellular [ATP] individually. To do so, cells are trapped into the microchamber array and lysed simultaneously. Tightly enclosed microchambers confine individual intracellular materials for certain periods of time (30 min). Individual intracellular

[ATP] is quantified by lysing single cells with LL reaction reagents inside the microchambers, which is impossible to realize with the existing methodologies.

Conclusions

Microchamber array platform is desirable for biology analyses because of its ultrasmall confined volumes, statistical manipulation and parallelized analysis of individual differences. In the thesis, further biological analyses, which are impossible to perform with conventional methodologies, are realized by using concepts of microchamber array platform. Microchamber array platform is improved with anti-absorption coating for cell-free protein synthesis with statistically confined single DNA molecules. This platform will allow the fabrication of high-density RNA and protein chips. Moreover, a novel concept of microchamber array platform is realized by integrating additional electrostatic functions, which provides the essential first steps in parallel analysis of individual intracellular materials. Such integrated microchamber array platform promise to answer biological questions about heterogeneity of single cell, difficult to realize with the exiting methodologies.

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