Development of Innovative Bio-Measurement Technology by Micro-Flow Light-Induced Acceleration

Dr. IIDA Takuya

In the measurement of biological samples (proteins, nucleic acids, microorganisms, etc.) on the medical treatment, pharmaceutical and public health area, the conventional method is often complicated and requires long-term operation, and there is room for improvement in sensitivity. The authors have developed a microflow-type light-induced acceleration system (LAC-SYS) to realize rapid and highly sensitive measurement, where the measurement targets and probe particles are condensed by synergistic utilization of the "optical pressure" and "light-induced convection" in a narrow space of the microchannel for the acceleration of reaction when photoresponsive materials (substrate, particles) in liquid are irradiated with light. As a result, we achieved tens to hundreds of times higher sensitivity than the conventional method, and succeeded in quantitatively evaluating a very small amount (femtogram-level) of proteins in a few minutes. This technology innovates a wide range of bio-measurement such as drug development, personalized medicine, and food inspection.

Introduction

In recent years, a rapid, highly sensitive, low-cost, and compact biological sample detection system has been eagerly desired for the establishment of tailor-made drug discovery for personalized medical care, telemedicine using ICT technology, preventive medical care, and smart healthcare. Specifically, the cost reduction of preventive medical care and drug discovery, which leads to the reduction of medical expenses in Japan, which is increasing due to the declining birthrate and aging population, including the diagnosis of cancer and adult diseases, has become an important issue. In addition, due to the recent rapid growth of industrial activities in emerging countries and the problem of liberalization of imports and exports, there is also a need for technology such as genetic testing of food and beverage production areas and the rapid and highly sensitive analysis and detection of foreign substances such as allergens and pathogenic bacteria. As conventional methods, an enzyme-linked immunosorbent assay (ELISA) using an antigen-antibody reaction is known for protein testing[1], and a polymerase chain reaction (PCR) method is often used for nucleic acid testing such as DNA and RNA[2]. However, the conventional techniques require a high degree of professional pretreatment, and a process such as long-term incubation or complicated cleaning is required, and the detection time often takes several hours or more. There are also problems that a large amount of sample is required for analysis of a dilute target, and that reagents and devices used for fluorescent staining are expensive. In addition, since proteins cannot be amplified like nucleic acids, a breakthrough for the condensation of sample was

required for trace detection.

In order to solve these problems, technologies for non-destructive and non-contact condensation of biological samples for high-throughput analysis and measurement have been desicred[3-5]. It is worth remembering that the technology of optical tweezers, which uses laser light to trap biomaterials contactlessly[6], was awarded the Nobel Prize in Physics in 2018. Although the use of infrared wavelengths in this technology allows for low-damage manipulation, it was limited to precise motion control of a small number of biomolecules and cells. On the other hand, the authors theoretically predicted the possibility of controlling "plasmonic super-radiance" in which a large number of metallic nanoparticles (NPs) are densely arranged by optical trapping under thermal fluctuations in water at room temperature[7]. The plasmonic super-radiance is a quantum-mechanical effect, where the localized surface plasmon (LSP) in each NP strongly interact with each other via the light electromagnetic field for the increase of the radiative relaxation rate (the probability that an excited electron will emit energy as light). Based on this prediction, by focusing on the broadening of the LSP spectrum and the red-shift to the infrared region due to the increase of the radiative relaxation rate in a high-density metallic NPs, the principle of a photothermal biosensor was proposed, where pg-level proteins can be detected by accumulating them in the laser focal area due to the synergistic effect of the light-induced convection generated by the photothermal effect and the optical trapping of gold NP (AuNPs) fixed beads[8]. However, since many proteins and biological substances are sensitive to heat, it is necessary to condense them while controlling the thermal effect.

In this study, we have developed the "Light-induced Acceleration System (LAC-SYS)", which enables high-throughput bioanalysis and measurement by controlling various biochemical reactions involving proteins, double-strand formation of nucleic acids, and condensation of microbes using laser irradiation. Particularly, we have focused on the development of a novel system that integrates microfluidic channels and LAC-SYS to increase the probability of interaction between the laser and NPs or microparticles (MPs) as probes in a narrow space. Furthermore, we also promoted the elucidation of the mechanism of optical condensation without damaging the target biomaterials, and aimed to carry out the entire process from the acquisition of new principles to the development of systems that will innovate spectroscopic analysis and measurement technology in the field of life science (Figure 1).

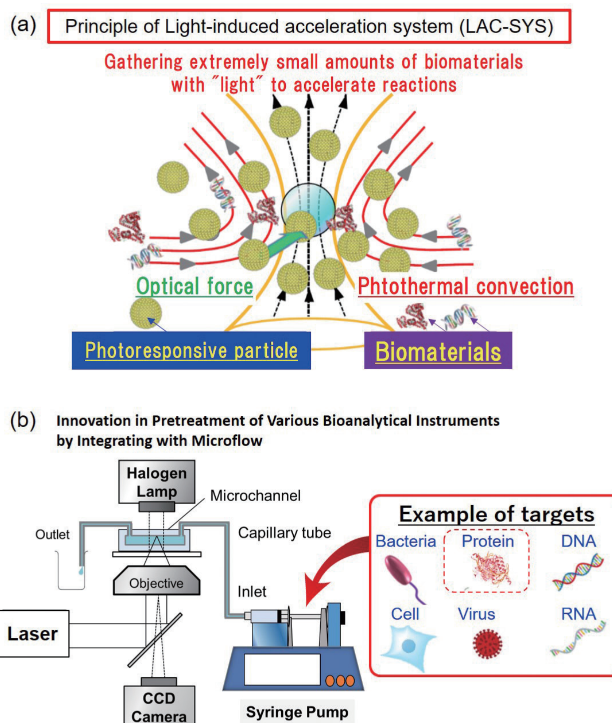

Figure 1 Schematic images of the light-induced acceleration system (LAC-SYS);
(a) Basic principle,
(b) microflow-type LAC-SYS.

Detection of trace amounts of proteins by microflow optical condensation

As a main part of this research, we developed a micro-flow LAC-SYS by constructing a system that can introduce target molecules and small particles as probes, and irradiate them with a laser using pressure-driven flow by a syringe pump in a microchannel as shown in Figure 1(b)[9][10]. For example, in order to elucidate the basic principle in our literature[9], we investigated the phenomenon that occurs when a protein is accumulated on the surface of a bubble (light-induced bubble) by using "optical pressure" derived from dissipative force, which is a kind of light-induced force, to accumulate metal NPs, and convection generated by the photothermal effect was also used as an auxiliary effect (Figure 2(a)). Specifically, an aggregate of AuNPs fixed on the surface of plastic beads (AuNP-fixed beads) were introduced as probe particles into a narrow microchannel of 400 μm in width and 100 μm in height, which is the same scale as blood vessels (for example, the thickness of small veins and arteries is 100-200 μm). They were used as probe to detect the target protein (in this case, albumin derived from egg white), and the research was carried out based on the idea that highly efficient trace detection would be possible by increasing the probability of interaction with laser light. In the search for conditions, we conducted experiments to launch and accumulate

AuNP-fixed beads on the ceiling of microchannel by optical pressure. Theoretical calculations confirmed that high-density metallic NPs on the surface of the beads can generate strong optical pressure even when using a laser in the infrared wavelength range, which is less damaging to living organisms, and we confirmed that AuNPs can efficiently generate optical pressure and light-induced convection in the preliminary experiments before our main research. As a result, AuNP-fixed beads and proteins were transported to the surface of the light-induced bubbles using the optical pressure and the convection generated by the photothermal effect (light-induced convection). As shown in the schematic image of Figure 2(b)(i), in the high concentration region (50 µg/mL to 500 µg/mL), where the amount of protein in the observation region ranged from 340 pg to 3.4 ng, a large number of bubbles were generated and remained stable for a long time (Figure 2(c)).

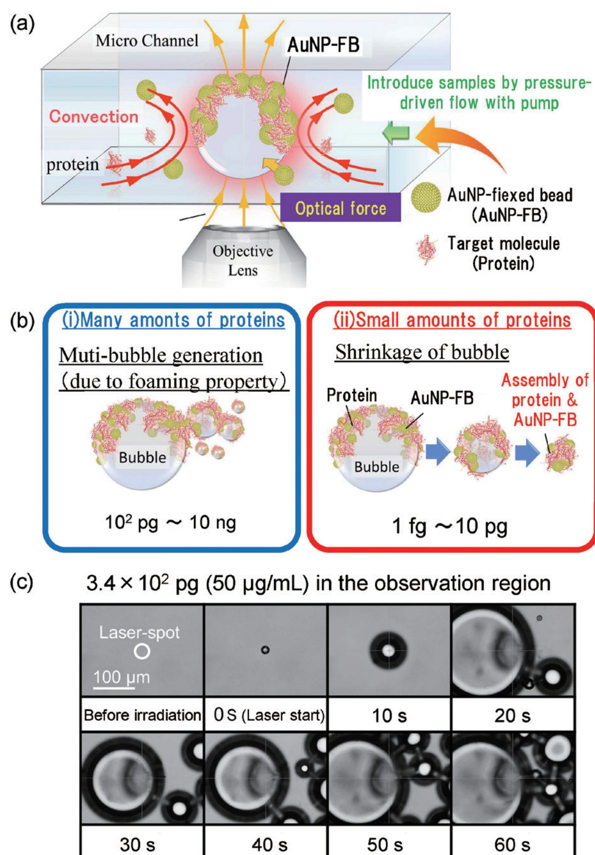

Figure 2 (a) Schematic image of microflow-type LAC-SYS using AuNP-fixed beads as probe particles.
(b) Schematic images of the phenomena depending on the amount of proteins.
(c) Optical transmission image of multi-bubble generation when a large amount of proteins are added.

On the other hand, in the low-concentration region (0.5 ng/mL to 5 µg/mL), where the amount of protein in the observation region is 3.4 fg to 34 pg, a single bubble disappeared in 3 to 10 minutes when the laser was turned off after 10 seconds of irradiation from bubble generation. In this case, the larger the amount of protein, the larger the aggregates remained (Figure 3(a), (b)). The amount of introduced target protein (in the observation region) is plotted on the horizontal axis, and the cross-sectional area of the bubble observed by optical microscopy is plotted on the vertical axis in Figure 3(c), and the cross-sectional area obtained from the transmission image of the aggregate is plotted on the vertical axis in Figure 3(d). It was found that the larger the amount of protein in the range of 1 fg to 100 pg, the larger the bubble was generated and the larger aggregate remained, which positively correlated with the size of the bubble, indicating the possibility of using this method for quantitative analysis of proteins. In particular, as shown in the inset of Figure 3(d), it shows high linearity and can be used for quantitative evaluation even when the amount of protein is only a few fg to a few hundred fg in the observation region, indicating the possibility of using it for trace analysis of proteins. We have previously reported that proteins as small as 1 pg can be detected by synergistically using light-induced force and light-induced convection in a quiescent fluid[8], and this result shows that we have succeeded in detecting proteins with two to three orders higher sensitivity. Since this is similar to the aggregate formation when bubblegum shrinks, we call this new type of biosensor as a “bubblegum sensor”. Considering that protein adsorption occurs even after laser irradiation is stopped due to pressure-driven flow, we expect that thermal damage can be avoided since the concentration process is based on cooled bubbles. Furthermore, we have succeeded in the quantitative detection of antigens and antibodies on the order of sub-pg/mL concentration by accelerating the antigen-antibody reaction through non-thermal optical condensation using optical pressure as the main driving force[10], and have elucidated the possibility of performing specific detection in just a few minutes with one to two orders higher sensitivity than conventional methods such as ELISA, suggesting the possibility of a breakthrough in protein measurement.

Light-induced acceleration of DNA double strand formation

DNA has a strand-like structure consisting of four types of bases (adenine (A), thymine (T), guanine (G), and cytosine (C)). Base pairs with high complementarity, such as A-T and G-C, selectively bind to each other through a molecular recognition mechanism to form a double strand (hybridization). On the other hand, bases of the same species show low complementarity, called mismatching. AuNPs with a diameter of 30 nm were used as probe particles <I> chemically modified with 12-base, 4.08 nm long single-stranded DNA with a thiol group attached to the end as shown in Figure 4 (a), and the same size AuNPs modified with the same length of single-stranded DNA with the chemical

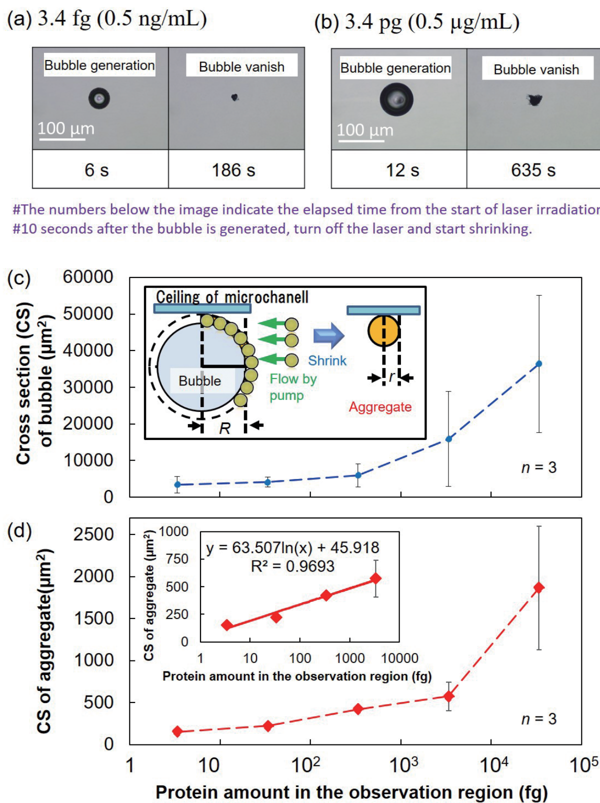

Figure 3 Relationship between the aggregate size and protein amount after the shrinkage of light-induced bubbles (for low concentrations).

(a), (b) Optical transmission images of the aggregates after bubble contraction for each protein concentration. Dependence of (c) the cross section (CS) of the bubble and (d) the area of the aggregate obtained after bubble contraction on the mass of the protein present in the observation region (inset: magnified view and approximate curve in the low concentration region)

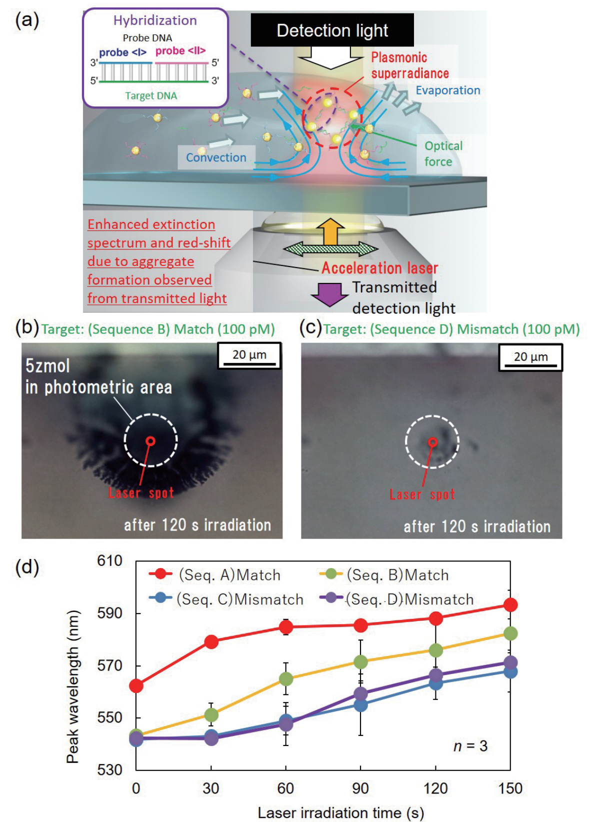

Figure 4 Light-induced acceleration of DNA double strand formation.

(a) Schematic image of the principle. Optical transmission images of light-induced network formation for (b) full-match DNA and (c) mismatch DNA added to DNA on the surface of the probe particle as target DNA. (d) Dependence of the peak wavelength of the absorbance spectra for each sequence on the laser irradiation time.

structure of the end reversed were used as probe particles <II>. Each of them was dispersed in phosphate buffer solution and used. Dispersions of these probe particles <I> and <II> and a dispersion of single-stranded target DNA (24 bases) as a target were dropped onto a glass substrate, mixed, and allowed to diffuse sufficiently, and we try to control hybridization with the single-stranded DNA on the surface of the probe particles by irradiation of an infrared laser focused strongly with a high-magnification objective lens.

When the laser beam is focused near the droplet surface as a non-equilibrium open system, the convection due to the photothermal effect of the AuNPs and the condensation due to the evaporation are expected to occur, and the probe DNA on the surface of AuNP and the target DNA are expected to form a double strand efficiently. Therefore, even with a dilute concentration of 100 pM (about 300 million DNA strands were contained the 5 µL solution) of complementary strand DNA, a macroscopic network-like assembly of about 0.1 mm can be formed after only 2 min of laser irradiation (Figure 4(b)). On the other hand, no large aggregates were obtained when the same concentration of fully mismatched DNA was used (Figure 4(c)), and it was confirmed that the difference in the base sequence also affects the aggregation phenomenon induced by light irradiation. Also, we measured the change in the local extinction spectrum of the white light transmitted through the region surrounded by the dashed lines, and observed a significant broadening and red-shift in a few minutes only when the complementary DNA was added, which is similar tendency to the theoretical prediction in the literature[7]. Even in the same full-match case, the spectral shift was larger for sequences with higher complementarity, such as "sequence A" containing G-C bonds, than for "sequence B" with only A-T bonds (Figure 4(d)). Furthermore, the number of DNA in this photometric region before laser irradiation was estimated to be about 5 zmol estimated from the above concentration (zmol: ~10^2 molecules), indicating that the formation of macroscopic aggregates that can be fully observed by optical microscopy was achieved in only a few minutes by triggering the double-strand formation of a very small amount of DNA. On the other hand, any aggregate could not be observed in a mixture of probe particles and target DNA at the same concentration under an optical microscope simply by letting the mixture stand

for 3 hours, but macroscopic aggregates could be formed very quickly by accelerating the reaction under optical condensation. This is an important achievement that shows that specific detection of trace DNA is possible through this process.

Principle of damage-free light-induced assembly of microbes by bubble-mimetic substrate

We have measured the number of bacteria in liquid based on the optical condensation of microbes such as bacteria using "light-induced convection" and "bubble" generated when a substrate coated with a metallic nanofilm exhibiting a finite absorption rate in the wavelength range of the irradiated light is locally heated by laser irradiation[12], and we have also performed the optical condensation of useful bacteria with the high viability using a multi-pore substrate[13]. In these studies, there were some problems such as the reduction of the survival rate when the laser power was increased. However, we also clarified the principle of "damage-free light-induced assembly" by coating the upper part of the solid-liquid interface with a metallic nanofilm to mimic a bubble and minimize the thermal effect on the trapping site[14].

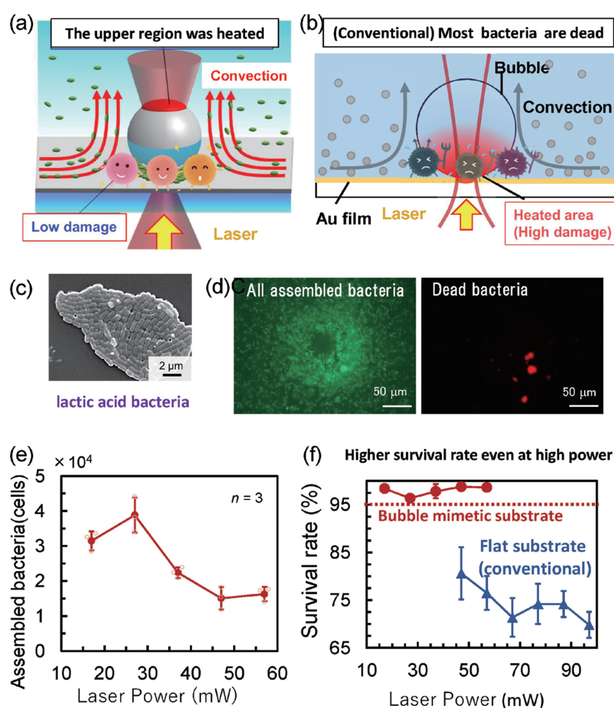

Figure 5 Low-damage light-induced acceleration of microbial condensation by controlling the position of heat sources.
(a) Schematic of optical condensation using an imitation bubble,
(b) Schematic of optical condensation using a conventional flat photothermal substrate.
(c) Electron micrograph of an example of microbe.
(d) Fluorescence images of optically-condensed bacteria.
(e) Laser power dependence of the number of aggregated bacteria.
(f) Laser power dependence of the survival rate of assembled bacteria.

As shown in Figure 5(a), polystyrene beads with a diameter of 100 µm were chemically fixed onto a glass bottom dish as an imitation bubble, and a thin platinum (Pt) film was coated on top of the bead by ion sputtering to create a "bubble-mimetic substrate (BMS)". In particular, *Lactobacillus casei (L. casei)*, a type of lactic acid bacteria, was used as a target, and green fluorescent dye (SYTO 9), which stains all the bacteria including live ones, and red fluorescent dye (PI), which stains only dead ones, were used to determine the viability. The optical system used for the experiment was an inverted microscope as shown in Figure 1(b), where a laser was defocused from below onto a Pt thin film coated on the top of a 100 µm diameter bead, and optical condensation was performed in the power range of about 15-60 mW without syringe pump.

Figure 5(d) shows the main results of the light-induced assembly of fluorescently stained *L. casei*. We found that about 40,000 bacteria could be aggregated after 5 minutes of irradiation with 27 mW laser, and a high aggregation rate of the order of 10^4-10^5 bacteria was achieved after only a few minutes of laser irradiation (Figure 5(e)). Furthermore, we found that the survival rate of higher than 95%, which is almost the entire population, can be maintained while using convection due to the photothermal effect (Figure 5(f)). In order to elucidate the mechanism of the highly efficient light-induced assembly and the high survival rate, we evaluated the convection velocity by simulations combining theories of electrodynamics and thermo-fluid dynamics, and estimated the number of bacteria entering the region around the imitation bubble to be about 50,000, which is consistent with the order of the number of bacteria trapped in the experiment. Furthermore, it was found that the bubble-mimetic substrate causes less damage even with high laser power because the heat source and trapping site are spatially separated. On the other hand, as shown in Figure 5(b), the conventional method of generating a bubble on the surface of a flat substrate[12] inevitably causes damage to the microbes due to the high temperature near the laser irradiation point, and the survival rate decreased with higher power (Figure 5 (f)).

Conclusion

Based on the accelerating assembly process of biological nanomaterials and probe particles introduced into a micro-flow system using "optical condensation" by the electromagnetics and thermohydrodynamics actions of light, we have clarified that it is possible to achieve a dramatically high-throughput in terms of measurement time, sensitivity, and sample volume for the first time in the world. In particular, we clarified the highly efficient photothermal effect of densely assembled metallic NPs arising from quantum effect and utilized it for "optical condensation

of biological samples," and clarified a new principle that enables the detection of proteins on the order of fg (10^{-15} gram) in just a few tens of seconds to a few minutes by condensing them with laser irradiation. Remarkably, we have obtained initial results on "light-induced acceleration" of antigen-antibody reaction by extending this principle[10], and have found that it can speed up protein detection by 2-3 orders of magnitude faster (from 5 hours to 3 minutes), increase sensitivity by 1-2 orders of magnitude higher (from pg-order to fg-order), and drastically reduce sample volume (from several hundred µL to several hundred nL) by eliminating the multi-step process that has been the bottleneck in conventional ELISA and a significant reduction in sample volume (from several hundred µL to several hundred nL). Moreover, we succeeded in light-induced acceleration of DNA double strand formation on the order of zmol (hundreds of DNA molecules), which could be applied to rapid and highly sensitive genetic testing. In addition, they succeeded in developing a substrate that can be used to measure the number of bacteria in just a few minutes, which takes several days by the conventional culture method.

These results have a high potential to open up an important core technology for the construction of a next-generation platform for spectroscopic analysis and measurement technology for biological science, which boasts overwhelming speed and sensitivity. As shown in Figure 6, our developed micro-flow LAC-SYS can contribute to the creation of a platform for spectroscopic analysis and measurement technology in the life science field, which will lead to ultra-smart applications in drug discovery, medical testing, food testing, and environmental measurement, as well as to scientific and technological innovations that transcend the fields of physics, chemistry, and biology regarding the optical control of various biological functions.

Figure 6 Future image of innovation in medical testing and drug discovery process by micro-flow type LAC-SYS.

The basic structure of the system is based on a simple optical system, which makes it easy to be compact and portable, and it is expected to be developed into a " future spectroscopic analysis and measurement technology" useful for smart drug discovery, self-care medicine and telemedicine, and on-site food inspection. Furthermore, our technology will contribute to the development of low-cost and simple devices for evaluating the local effects of rare and expensive new drugs and fluorescent probes in cancer treatment and screening, and to environmental measurements such as the measurement of biological genes (DNA, RNA) and harmful particles in the environment, including advanced measurement technology for biological cells and microbes.

Acknowledgement

The author would like to express his sincere thanks to Prof. Shiho Tokonami, the Deputy Director of the RILACS of Osaka Prefecture University, for her support through joint research, and to Dr. Mamoru Tamura, Dr. Yushi Nishimura, Dr. Yasuyuki Yamamoto, Ms. Mayu Ueda, Mr. Kota Hayashi, and other alumni and graduate students for their cooperation in carrying out the research. Also, the author would like to express his deep gratitude to Prof. Hajime Ishihara and Prof. Syoji Ito of Graduate School of Engineering Science in Osaka University, and Prof. Ikuhiko Nakase, the Assistant Director of RILACS, for their fruitful discussions. This work was partially supported by JST-Mirai Program (No. JPMJMI18GA, No. JPMJMI21G1), Grant-in-Aid for Scientific Research (A) (No. 17H00856, No. 21H04964), Grant-in-Aid for Scientific Research (B) (No. 18H03522), Grant-in-Aid for Scientific Research on Innovative Areas (No. 16H06507) from JSPS KAKENHI, AMED (No. JP20he0622017), and the Key Project of Osaka Prefecture University.

* Editorial note: This content is based on HORIBA's investigation at the year of issue unless otherwise stated.

References

[1] E. Engvall, P. Perlmann, "Enzyme-linked immunosorbent assay (ELISA) quantitative assay of immunoglobulin G", Immunochemistry, 8 , 871-874 (1971).

[2] R. K. Saiki, S. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, H. A. Erlich, N. Arnheim, "Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia", Science, 230, 1350-1354 (1985).

[3] E. A., Raiber, R. Hardisty, P. van Delft, et al. "Mapping and elucidating the function of modified bases in DNA", Nat. Rev. Chem. 1, 0069 (2017).

[4] A. Altelaar, J. Munoz, A. Heck, "Next-generation proteomics: towards an integrative view of proteome dynamics", Nat. Rev. Genet. 14, 35 (2013).

[5] Y. Bian, R. Zheng, F. P. Bayer, et al. "Robust, reproducible and quantitative analysis of thousands of proteomes by micro-flow LC–MS/MS". Nat. Commun. 11, 157 (2020).

[6] A. Ashkin, J. M. Dziedzic, J. E. Bjorkholm, S. Chu, "Observation of a single-beam gradient force optical trap", Opt. Lett. 11, 288 (1986).

[7] T. Iida, "Control of Plasmonic Superradiance in Metallic Nanoparticle Assembly by Light-Induced Force and Fluctuations", J. Phys. Chem. Lett., 3, 332 (2012).

[8] Y. Nishimura, K. Nishida, Y. Yamamoto, S. Ito, S. Tokonami, T. Iida, "Control of Submillimeter Phase Transition by Collective Photothermal Effect", J. Phys. Chem. C, 118, 18799 (2014).

[9] M. Ueda, Y. Nishimura, M. Tamura, S. Ito, S. Tokonami, T. Iida, "Microflow-mediated optical assembly of nanoparticles with femtogram protein via shrinkage of light-induced bubbles", APL Photon. 4, 010802 (2019).

[10] T. Iida, S. Tokonami, I. Nakase, "Detection method for substance to be detected and detection system for substance to be detected", PCT/JP2020/032758 (2020).

[11] T. Iida, Y. Nishimura, M. Tamura, K. Nishida, S. Ito, S. Tokonami, "Submillimetre Network Formation by Light-induced Hybridization of Zeptomole-level DNA", Sci. Rep. 6, 37768 (2016).

[12] Y. Yamamoto, E. Shimizu, Y. Nishimura, T. Iida, S. Tokonami, "Development of a rapid bacterial counting method based on photothermal assembling", Opt. Mater. Exp., 6, 1280 (2016).

[13] S. Tokonami, S. Kurita, R. Yoshikawa, K. Sakurai, T. Suehiro, Y. Yamamoto, M. Tamura, O. Karthaus, T. Iida, "Light-induced assembly of living bacteria with honeycomb substrate". Sci. Adv. 6, eaaz5757 (2020).

[14] K. Hayashi, Y. Yamamoto, M. Tamura, S. Tokonami, T. Iida, "Damage-free Light-induced Assembly of Intestinal Bacteria with a Bubble-mimetic Substrate", Commun. Biol., 4, 385 (2021).

Dr. IIDA Takuya

Professor, (Concurrent) Director of RILACS
Department of Physical Science,
Graduate School of Science,
Osaka Prefecture University.
(Concurrent) Research Institute for Light-induced Acceleration System (RILACS)