ISSN 0915-9916

# Readout

## HORIBA Technical Reports

October 2021
English Edition No. 55

## 2021 Masao Horiba Awards

**Spectroscopic analysis and measurement technology in the life science field**

HORIBA

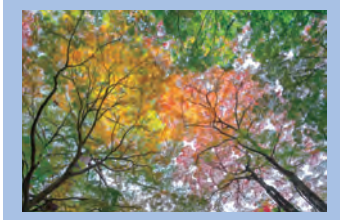
https://www.horiba.com/jp/publications/readout/

Readout
HORIBA Technical Reports
October 2021 No.55

The theme of 2021 Masao Horiba Awards is analytical and measurement technologies for development and production of biopharmaceuticals with high social needs, and vaccines or therapeutic drugs to address today's most significant global health challenge, the COVID-19 pandemic. This research field is directly related to human health and life, and is a field that is attracting global interest and investment.

# 2021 Masao Horiba Awards
# Optical/Spectroscopic Measurement Technologies for Life Science





I visited a forest detached from a famous sightseeing spot searching for the color of true autumn.
When I suddenly looked up, there was another world that seems out of this world with patterns created by red and green leaves.
For a while, I was so absorbed in taking pictures that I didn't notice the passage of time.
*-Photographer MATSUI Hideo-*
*(Member of Nikakai Association of Photographers)*

*Name of this Journal*

This Journal is named "Readout" in the hope that "the products and technology we have created and developed will be read out and so become widely known".

# Foreword

# HORIBA's Contribution to the future of pharmaceutical innovation and human health


NISHIKATA Kentaro

General Manager
Research & Development Division
HORIBA, Ltd.
Ph. D.


Kentaro Nishikata

Since its founding, HORIBA has focused on instrumental analysis, including materials, life science research and medical fields. In the 1980s, HORIBA began a full-scale business activity, via its MEDICAL segment, in the blood analysis field. Looking back on the history of academic fields, in the 1960s when our founder, Masao Horiba, obtained his doctor of medical science, biology in a broad sense, including medicine and agriculture, was positioned as the opposite of the physical sciences. Since then, molecular biology and genetic engineering have developed rapidly, and biological and physical sciences have become closer. Furthermore, when we consider the sense of crisis that such technological developments could bring regarding bioethics, life sciences, as a comprehensive academic discipline, have evolved to carefully consider such social aspects. In recent years, HORIBA has envisioned increasingly higher expectations for the applications of spectroscopic analysis in these life science fields, especially in the field of drug discovery and pharmaceuticals.

Since early 2020, the novel coronavirus continues to rage worldwide. As we all know, we are in a pandemic, not seen since the 1918 influenza, effectively once in 100 years. To work towards combatting such events, we need more than vaccines and therapeutic agents in our global medical toolboxes. Conventional small-molecule medicines, macromolecular medicines that use nucleic acids and antibodies, and creation of cells and extracellular vesicles are necessary for the pursuit of health and wellbeing. Research to develop and deploy such novel medicines are in strong demand. To respond to such changes in the market environment and demands, HORIBA has launched a bio-life science project and is strengthening the development of new technologies with our customers, using HORIBA instruments and technologies.

In addition, in order to deliver the developed drugs to society, one requires a production process that supports each drug modality. In recent years, expectations for

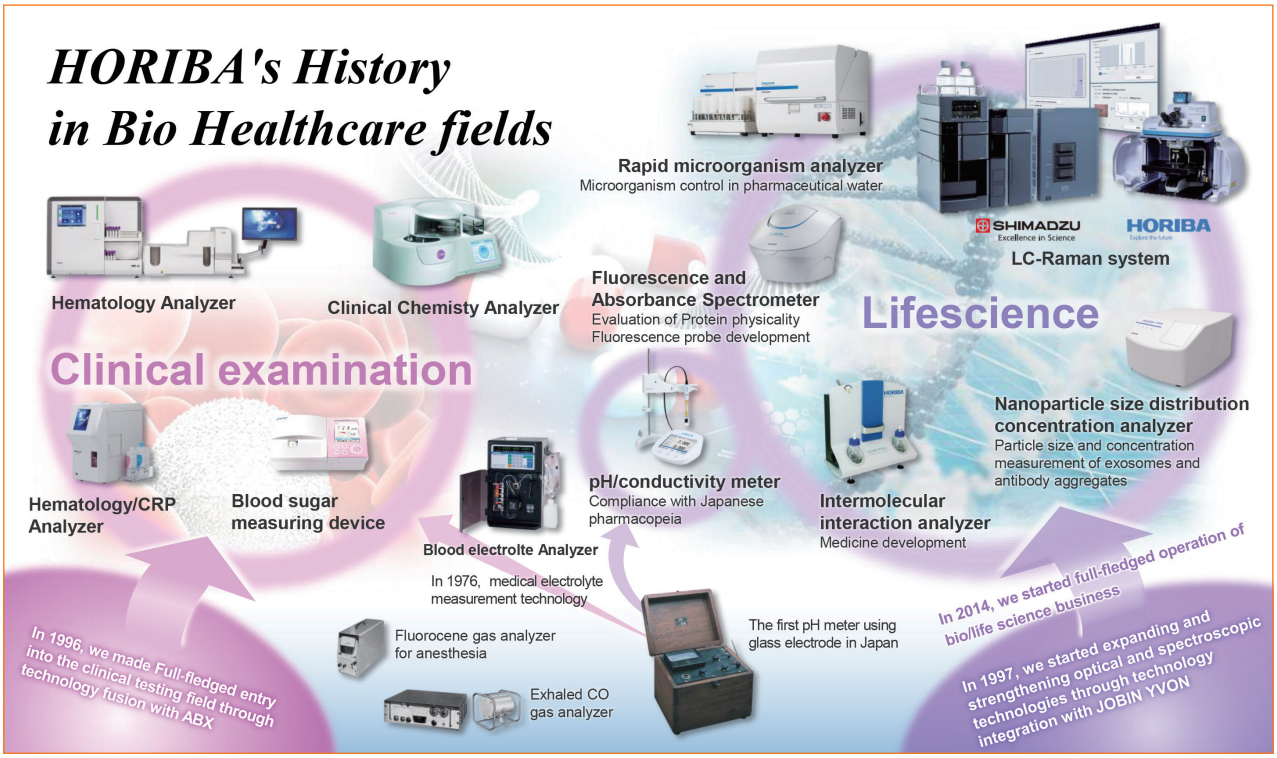
spectroscopic technologies that enable non-invasive, non-destructive, no-contact and rapid analyses have increased. These expectations are generating many demands on HORIBA's fluorescence spectroscopy, Raman spectroscopy, and particle analysis. To leverage the advantages of spectroscopic analytical technologies that enable non-destructive, in-situ analysis, various comncepts and methods for sample handling and sample pre-treatment techniques are also required. HORIBA has launched a new Industrial Solution Project to meet these demands. In this project, with combining Jobin Yvon's 200-years-old spectroscopic technologies with HORIBA's process monitoring technologies, HORIBA has begun providing valuable solutions across various industrial fields.

Furthermore, it is clear that Data Science technologies are important for processing, accumulating, and utilizing a large amount of obtained spectral data and images for process control. These technologies must contribute to R & D efficiency and productivity improvement in the manufacturing process. In response to these demands, including data science as a core technology, HORIBA has launched a new project (IoT and Data Science Project) to improve the reliability, completeness and added value of the data produced by our instruments and systems.

This year, the Masao Horiba Award will celebrate its 18th anniversary. This award has consistently supported the basic research that offer solutions to solve various social issues. Unfortunately, the COVID-19 pandemic forced us to postpone the Award for 2020. It was an unusual situation. For 2021, we have selected the theme for the Masao Horiba award as "Optical/Spectroscopic Measurement Technologies for Life Science", with an eye on analysis and measurement that will contribute to the future of pharmaceutical innovation and human health.

It is our hope and expectation that the winners of the Masao Horiba award will become major contributors to these endeavors.

The theme of the Masao Horiba award is the highlighted feature of this issue of the Readout.




HORIBA has been developing and selling medical analyzers (medical gas analyzers, etc.) since around 1963. In 1996, HORIBA acquired ABX, a French manufacturer specializing in hematology analyzers, and began full-scale entry into the medical (clinical laboratory) business. In 1997, the company acquired the French company JOBIN YVON, which excelled in analytical technology, and has since expanded and strengthened its technology. Based on the combined technologies and products, the company launched its life science business on a full-scale basis in 2014. Recently, in collaboration with Shimadzu Corporation, a manufacturer of analytical instruments, the company has continued to take on challenges in diverse and changing fields, following the legacy of its founder.

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

# Eligible Fields and Award Winners for 2021 Masao Horiba Awards

NOGUCHI Shintaro

The 2021 Masao Horiba Awards focused on optical/spectroscopic analysis and measurement technology in the life science field. Originally scheduled for 2020, the Awards were unusually postponed because of the worldwide COVID-19 pandemic that emerged in early 2020. At the time of writing this review, the worldwide critical situation is still ongoing despite the increasing prevalence of vaccination for the coronavirus on a global scale, especially in developed countries. This widespread virus has brought various changes to our societies and transformed our daily lives and business practices. In that process, people in general throughout the world, not only researchers, have become increasingly concerned about infectious diseases and how they affect our lives, as well as vaccines and pharmaceuticals. Amid such circumstances, we have continued our engagement in the Awards, supported by the sense of significance arising from the selection of life science-related research as being eligible for this year.

In addition to the development and production of novel pharmaceutical modalities (e.g., biopharmaceuticals), which have been in demand since the pre-coronavirus time, the development and production of vaccines and therapeutics for COVID-19, which have been attracting intense attention in society today, were chosen as eligible research areas for this year's Masao Horiba Awards. Directly linked to human health and lives, the latter has become a heavily invested high-profile area of study throughout the world. We hope that the 2021 Awards will serve as an opportunity that will lead to the establishment and expansion of networks for accelerating research and development (R&D) in this area. In the technology aspect, the Award eligibility focused this time on optical/spectroscopic technologies, which are not analysis technologies widely employed in life science, such as separation and mass analyses. Furthermore, considering that R&D and production could make substantive contribution to people's well-being across the globe only when these elements both proceed successfully, we counted real world applicability of research as an important aspect. The Awards' specific intentions and eligible technology areas are as follows.

<2021 Masao Horiba Awards>

Our society seeks to cure disease, and goal that unites the world and the pharmaceutical industry relies on scientific advances in medicine, to drive innovation in medicines. In new collaboration of medical field and drug discoveries, macromolecule drugs derive from nucleic acid and antibody, as well as the pharmaceutical research using calls and extracellular endoplasmic reticulum became active along with the conventional small-molecule drugs.

As research fields diversified, the needs toward analysis methods became diversified, and there is a high expectation toward optical/spectroscopic technologies along with the conventional separation method. In order to disseminate the newly developed drug efficiently to the people, establishment of sophisticated production process responding to the each modality of the drug is

also required. Measurement using optical/spectroscopic technologies is becoming also important for this requirement. In addition, the optical/spectroscopic methods have strong strengths as non-destructive or less damaging method for the live specimen. In order to take full advantage of this strength, development of the sampling and pretreatment method is also important in addition to the research on basic principle and hardware development. It is also obvious that not only the innovation of spectral and imaging but also data science approach to link these data to the actual phenomena is also important. This approach must contribute to R&D efficiency and production process improvement. The Covid-19 pandemic has spread throughout the world, greatly increasing stress in our lives. The race to create a safe and effective vaccine is important not just to prevent the disease itself, but to reduce the additional health issues and suffering that result from systemic stress. Overall, the most effective approach to solve real problems, incorporates an interdisciplinary approach from basic research all the way to implementation.

Based on the background described above, the 2021 Horiba Masao Award solicits research in the field of life science, notably state-of-art optical/spectroscopic measurement technologies which have the potential to transform drug discovery and manufacturing processes. This award asks for research especially applicable to industry and that can be dedicated to production processes.

With this in mind, we have set the following technical fields to be covered.

<Eligible fields for 2021 Masao Horiba Awards>
The award is focused on the optical/spectroscopic analysis and measurement technology which contribute to the advanced drug discovery and pharmaceuticals within the field of life science that increase the efficiency of development and production processes as well as applicable to other industries, specifically the technologies that encase the following aspects.

1: Measurement and automation technologies based on optical/spectroscopic techniques related to the production process of proteins or cells.

The result of the research will improve the efficiency of the production process, or is related to process control using a data science/data management system.

2: Analytical research for bio samples based on spectroscopic techniques in the field of drug discovery and manufacture.

The research takes into account the future industrialization or production process of cells, microorganisms, extracellular particles in the pharmaceutical field.

*We expect the applications relating to the interdisciplinary research and development on the sample preprocess utilizing microfluidic devices as well as the data analysis method applying data science.

As stated above, the eligible areas for this year's Awards included not only research for drug discovery and development (i.e., R&D) and research for higher productivity, but also research that can bridge these two research domains (e.g., research on pretreatment of specimens and on data analysis). As a result, we received many applications from researchers in various fields ranging from pharmaceutical science and biotechnology to information technology. We would like to thank those researchers for taking the time

and effort to submit their applications.

The Screening Committee had a difficult time selecting the Award winners from among the many applicants whose research was valuable, all potentially leading to treatment, diagnosis, and/or drug development; the following decision resulted from their strict evaluation: the Masao Horiba Award went to Dr. Takuya Iida at Osaka Prefectural University, Dr. Sadao Ota at The University of Tokyo, and Dr. Kazuhide Sato at Nagoya University, and the Honorable Mention to Dr. Sanghong Kim at Tokyo University of Agriculture and Technology.

Dr. Iida established a system to condense biomaterials in a microfluidic device (light-induced acceleration system; LAC-SYS) and created a platform for a rapid and highly sensitive optical bioanalysis technology. Dr. Ota, based on the concept that image reconstruction is not necessarily needed in image analysis where it is performed by a machine, established a technique called ghost cytometry, an image-reconstruction-free ultrafast cell identification and sorting method employing direct analysis by artificial intelligence. Dr. Sato developed near-infrared photoimmunotherapy (NIR-PIT), a novel cancer therapy with an extremely low burden on patients. Also, on the basis of the characteristics of the probe used in NIR-PIT, he elucidated the mechanism by which this therapy induces cancer cell death and established a method for therapeutic effect measurement. Dr. Kim, the Honorable Mention winner, developed a novel statistical analysis model utilizing near-infrared absorbance spectral data that enables real-time quality monitoring in pharmaceutical production processes.

In our current situation, where early development and market supply of safe and secure vaccines and pharmaceuticals are awaited so eagerly, highly advanced analysis and monitoring techniques suitable for various pharmaceutical modalities are indispensable. The research by the awardees is certain to contribute to fulfilling such needs for years and decades to come and to provide answers to the aforementioned concerns of people around the world. These researchers' accomplishments are well worthy of the Masao Horiba Awards. It is our hope that the Awards will aid in further advancement in their research activities and thereby realization of the timely availability of vaccines and therapeutics desired in the future.

Last but not least, we would like to extend our gratitude to those who made possible and supported our engagement in the Awards in this difficult time with the coronavirus.

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

NOGUCHI Shintaro

Bio/Life Science Project
Sales Division
HORIBA, Ltd.

# 2021 Masao Horiba Awards Award Details

## About Masao Horiba Awards

The Masao Horiba Award was established in 2003, to support scientists and experts who are devoting themselves to research and development that will generate innovative technology in analysis and measurement. It also aims to strengthen the position of measurement technologies within the scientific and industrial world. The field for the award is decided each year with a focus on the principles and fundamental technologies fostered by HORIBA, and the award highlights unique research and development with the findings and potential that deserves a global recognition.

## Eligible fields

Spectroscopic analysis and measurement technology in the life science field

## Screening Committee

### ● Chairperson

**TAKEUCHI Hirofumi**
Emeritus Professor
Laboratory of Advanced Pharmaceutical Process Engineering, Gifu Pharmaceutical University

### ● Judges

**Jürgen Popp**
Professor, Scientific Director
Spectroscopy/Imaging, Leibniz Institute of Photonic Technology, Jena, Germany

**IBUKI Rinta**
Professor
Research Center for Drug Discovery and Pharmaceutical Development Science, Ritsumeikan University

**TSUMOTO Kouhei**
Professor
Department of Bioengineering, School of Engineering, The University of Tokyo

**MAEKAWA Masato**
Professor
Department of Laboratory Medicine, Hamamatsu University School of Medicine

**UCHIGASHIMA Mikiko**
Deputy Department Manager
Bio/Life Science Project Sales Division HORIBA, Ltd.

**NOGUCHI Shintaro**
Section Leader
BLS Solution Sales Team Bio/Life Science Project Sales Division HORIBA, Ltd.

## Masao Horiba Awards Winners

### 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)

【Research Theme】

**Development of innovative bio-measurement technology by micro-flow** light-induced acceleration

### Dr. Sadao Ota

Associate Professor
Research Center for Advanced Science and Technology, The University of Tokyo

【Research Theme】

Development of ultrafast machine vision-activated cell sorters and its applications

### Dr. SATO Kazuhide

Designated Assistant Professor (selected-YLC program)
Institute for Advanced Research/ School of Medicine, Tokai National Higher Education and Research System, Nagoya University

【Research Theme】

Elucidation of the mechanism of near-infrared light-induced cell death and method establishment for measuring therapeutic effects

## Honorable Mention Winner

### Dr. KIM Sanghong

Associate Professor
Department of Applied Physics and Chemical Engineering,Tokyo University of Agriculture and Technology

【Research Theme】

Real-time monitoring and control of pharmaceutical production processes using spectroscopic data

# 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).

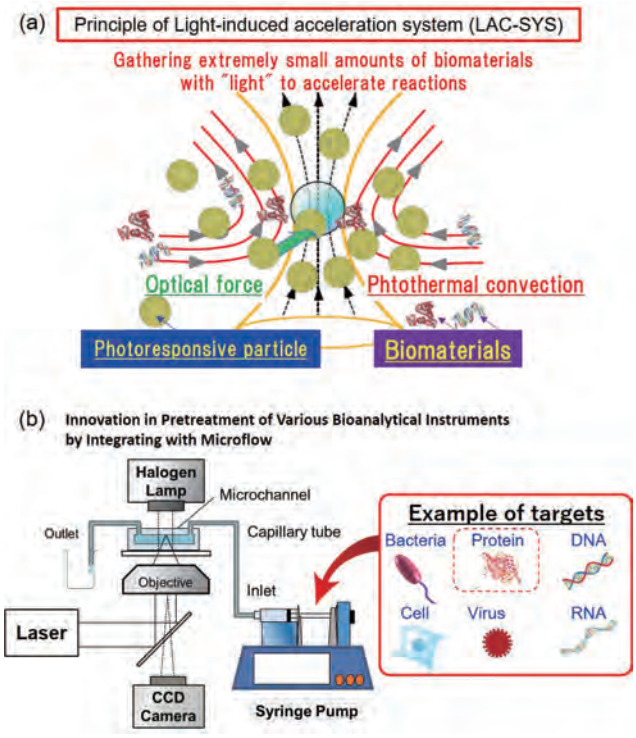



Figure 1 Schematic images of the light-induced acceleration system (LAC-SYS);
(a) Basic principle,
(b) microflow-type LAC-SYS.

## 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)).

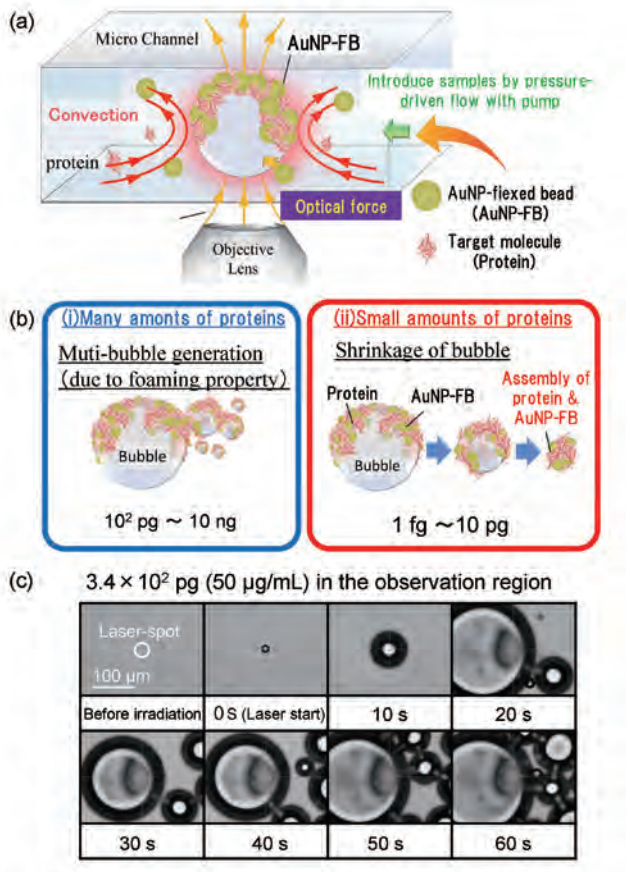



Figure 2 (a) Schematic image of microflow-type LAC-SYS using AuNP-fixed beads as probe particles.
(b) Schematic images of the phenomena depending on the amount of proteins.
(c) Optical transmission image of multi-bubble generation when a large amount of proteins are added.

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

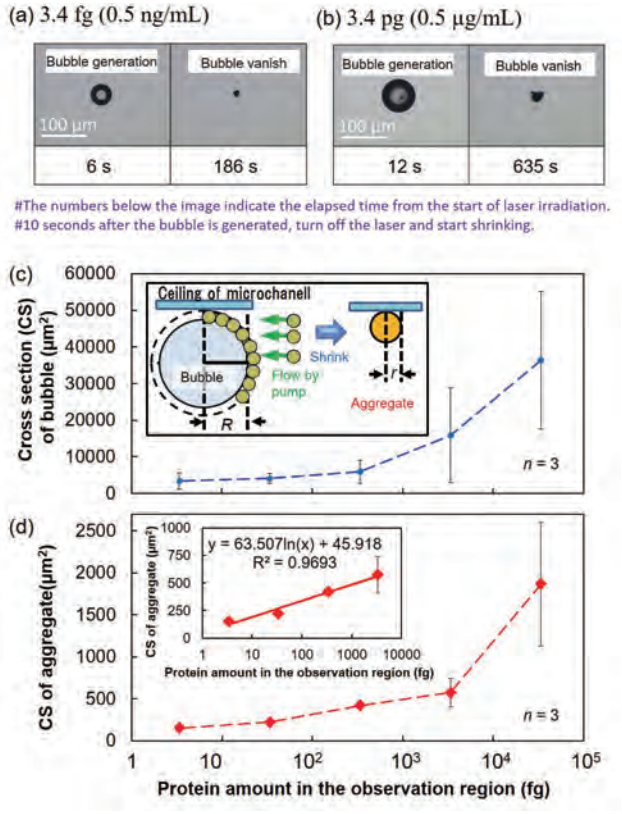



Figure 3 Relationship between the aggregate size and protein amount after the shrinkage of light-induced bubbles (for low concentrations).

(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)

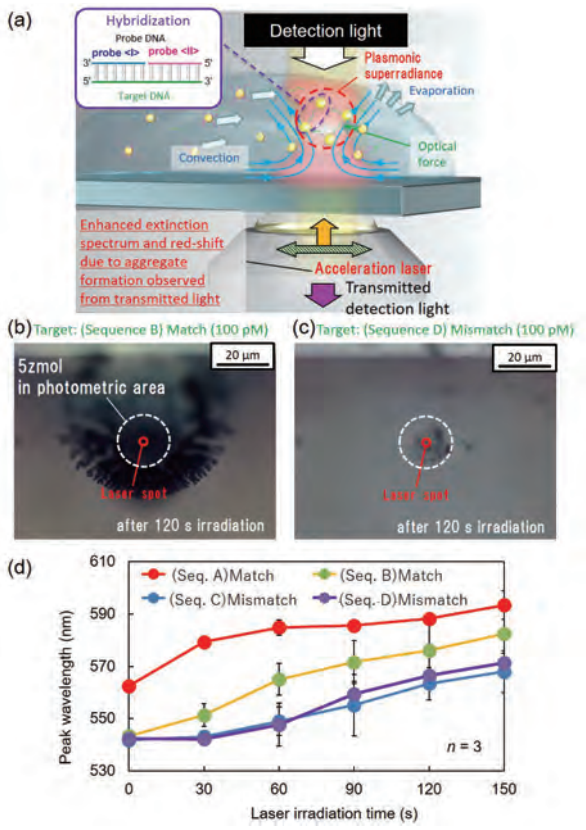



Figure 4 Light-induced acceleration of DNA double strand formation.

(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].

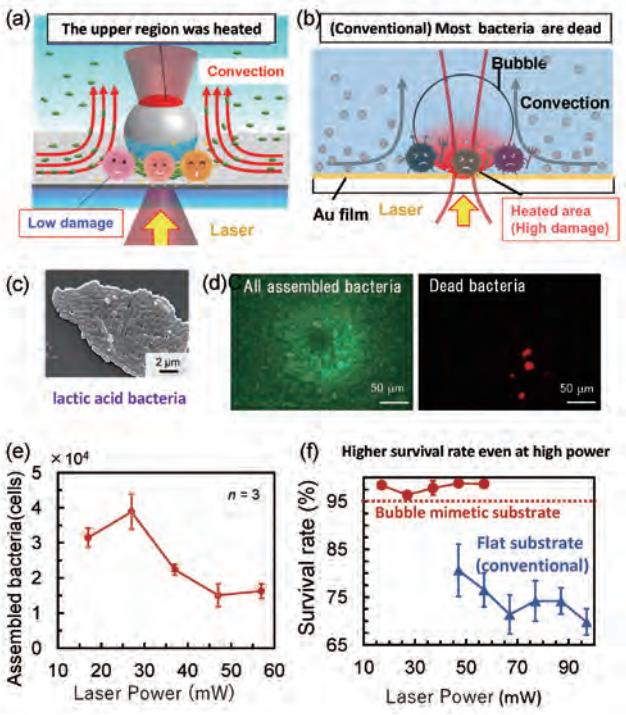



Figure 5 Low-damage light-induced acceleration of microbial condensation by controlling the position of heat sources.

(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)

# Development of Ultrafast Machine Vision-Activated Cell Sorters and Its Applications

OTA Sadao

A cell sorter that performs image information analysis in real-time to separate a large number of cells were desired. However, there is always a tradeoff between the processible information per cell (quality) and the number of cells per time (quantity). Therefore, it has been a great challenge to enable high throughput imaging cell sorter that holds the advantages of optical microscope (high quality) and flow cytometry (high quantity simultaneously). In order to overcome this challenge, we proposed and realized a new approach named "ghost cytometry", originating from a concept that image reconstruction is not always necessary in image analysis when performed by machines, not by humans. This method utilizes the motion of each cell in microchannels to acquire its compressed image signal by a single pixel detector, and directly applies AI-based analysis to the signal, resulting in the development of the ultrafast and accurate image-free "imaging" cell sorter. This technology is expected to be widely applied in various biotechnologies and cell-based clinical methods.

## Introduction

Imaging and analyzing many single cells hold the potential to significantly increase our understanding of heterogeneous systems involved in various complex life systems and diseases. Many key applications in these fields require accurate and high throughput isolation of specific populations of cells according to information contained in the high content images. However, there is always a trade-off between the processible information per cell (quality) and processible number of cells per time (quantity). This trade-off is depicted in Figure 1. For cytometry technologies, this trade-off caused critical challenges that have to be simultaneously solved: (1) ultrafast imaging technology that simultaneously meets the needs of high sensitivity, polychromaticity, high speed and continuous acquisition, and (2) ultrafast information technology that continuously performs both computational image production and analysis which is costly in terms of both time and money. As a result, there has been no high throughput imaging-activated cell sorting technology that holds advantages of both optical microscopy (high quality) and flow cytometry (high

quantity).

In a series of works, we proposed and experimentally demonstrated that directly applying machine learning methods to compressed imaging signals measured using a single pixel detector enables ultrafast, sensitive, and accurate image-free (without image production), imaging-based cell analysis as well as sorting in real time, which we call ghost cytometry[1] (GC, Figure 1). GC has enabled the fast cell sorting based on the cells' image information in both fluorescence and label-free modes, which we name machine-vision based cell sorters (ViCS). Furthermore, using this ViCS, we have developed an ultrafast pooled platform for cell image-based phenotypic screening of genes, which can be scaled up at a large scale.

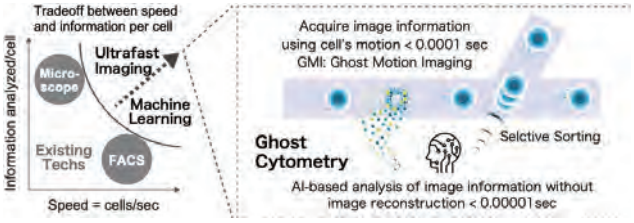



Figure 1 Ghost cytometry simultaneously achieved speed and rich information by "image analysis without image reconstruction"

Moreover, based on the invention, we founded a start-up company ThinkCyte Inc., which develops these ViCS machines through the co-development with renowned companies. Further, we are now striving to realize its practical use in the fields of medical diagnosis, drug discovery, and cell therapy, and a series of joint research with several pharmaceutical companies is underway.

## Key feature of our technology 1: ghost cytometry

Our key concept was ghost cytometry (GC), which is an approach of image analysis without image production. In GC, we applied machine learning methods to compressed imaging signals measured using a single pixel detector for enabling ultrafast, image-free, "image"-based cell analysis as well as sorting in real time (Figure 2). Thanks to skipping the time-consuming image reconstruction process, the time required for image inferring was reduced down to 10 microseconds which is orders of magnitudes shorter than other methods relying on image reconstructions. With GC, we developed a series of ultrafast cell sorters which classifies cell based either fluorescence image information or label-free image information. In the case of the fluorescence mode, fluorescent signals are detected by converting image information into temporal waveforms compressively. In the case label-free modes, refractive index distribution of cells is detected; these label-free morphological modalities obtained are similar label free microscopy images including darkfield and bright field images.

Figure 3 shows an example workflow of GC utilizing a machine learning model to classify the cells by directly analyzing the imaging waveforms. It first starts with preparing a training data set: for each cell, we simultaneously measure the GC imaging waveforms and molecular labels that reflect types, states, phenotypes, and other characteristics of cells. Using this training data set, we then develop a model based on machine learning methods. Finaly this model predicts the labels directly from the GC imaging signals of unknown cells.

In the fluorescence modes, GC can classify localization of fluorescence molecules in the cell morphology, even if their total intensities are the same or if the same kind of cell lines are used. This is particularly useful when users know what image pattern to recognize and what kind of specific molecules are their targets which is often the case in drug screening or biological research. With these pre-knowledge, one can chemically or genetically label the molecule of interest to visualize the fluorescent image pattern of interest with a good signal-to-noise-ratio. Using various optical probes, the GC can classify a variety of fluorescent patterns effectively, including nuclear translocation of molecules, organelle morphologies, and protein-protein interactions. On the other hand, that label-free mode can be useful especially when users don't want to stain cells with chemical or biological labels or damage them before the downstream uses. In addition, this approach is also effective when users are not clear about what the target molecules are or what image patterns should be specifically recognized. This lavel-free GC has demonstrated accurate classification of cells based on their kinds, states, and functions without observing molecular labels.

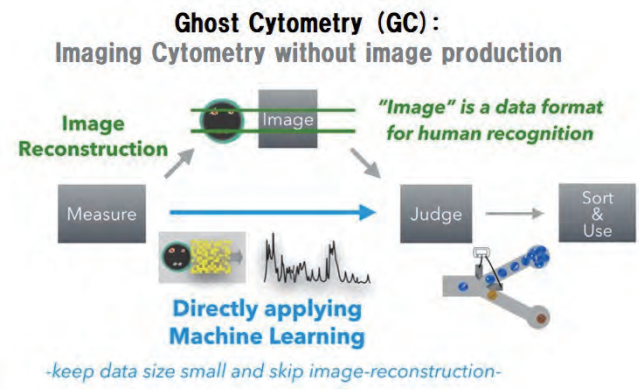



Figure 2 Image-free imaging cytometry driven by machine learning: In conventional workflows of image-based cytometry, imaging technologies were to reconstruct a clear 2D/3D images for human recognition, followed by analysis based on human recognition. Our radical idea of ghost cytometry (GC) is, "apart from human recognition-based imaging cytometry, reconstructing 2D images is not necessary for machine learningbased analysis (AI)". In GC, its compressive imaging technology works to extract cell's imaging information as a temporal waveform of signals measured by a single pixel detector, followed by analysis without image reconstruction using machine learning methods. Thereby, GC enabled the world's first and fastest image-free "imaging" cell sorter by skipping the most time-consuming and computer-intensive image reconstruction in the data-intensive flow cytometry.

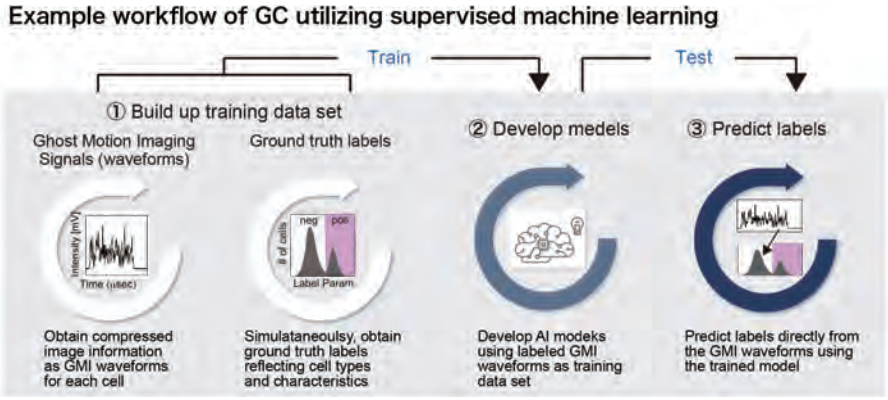



Figure 3 Example workflow of ghost cytometry utilizing supervised machine learning methods

## Key feature of our technology 2: machine vision-activated cell sorting

By combining GC's efficient signal processing implemented in a field programmable gate array with a microfluidic device, we realized ultrafast and accurate cell sorting based on real-time analysis of "imaging" data. Figure 4 shows its first prototype and, since then, this machine vision-activated cell sorter (ViCS) has evolved to be more stable and more flexible one through development by ThinkCyte Inc. together with excellent other companies.

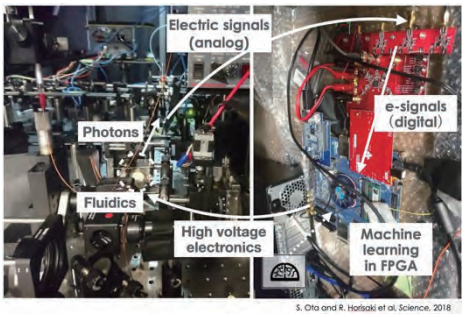



Figure 4 The first prototype of ultrafast machine vision-based cell sorter (ViCS), enabled by combining the optical ghost imaging signal acquisition, the real-time machine learning-based analysis implemented in FPGA, a microfluidic cell sorting technology.

## Application

In the field of drug discovery, we have developed a pooled cell phenotypic screening method using ViCS. In this application, the fluorescence mode ViCS is used when the cell phenotype is specifically known (i.e. the localization of a specific molecule or a specific intracellular organelle), and the label-free mode ViCS is used when no specific explicit imaging feature is available. While high-content screening used to take long time (several months) and high cost, our method can significantly accelerate it at lower cost. We believe we can contribute to societ by greatly accelerating drug discovery research and development.

In the field of cell therapy, regenerative medicine, and cell-based production, the label-free GC and ViCS can evaluate cell types and functions without using stains or antibodies, and to enrich cells of interest. This method can be thus used for automatic monitoring of cell production lines and improving cell qualities by removing unwanted cells or contaminating particles. Similarly, it can be used in the evaluation of cells that produces antibodies and drugs. While remarkably effective cell-based drugs currently seem to put pressure on medical costs, such a system may greatly contribute to its reduction.

## Conclusion

In conclusion, we proposed and realized "ghost cytometry", an approach based on AI-assisted cell analysis of their "image" information without image production. More concretely, by directly applying AI-based analysis to a compressed "image" signals in temporal domains, we skip a time-consuming image reconstruction process and realized the ultrafast and accurate image-free "imaging" cell sorter. We expect this technology to be widely adoped in biological sciences, and industrial applications including biotechnology, drug discovery, and cell therapy and regenerative medicine.

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

## References

[1] Ota, S., Horisaki, R., Kawamura, Y., Ugawa, M., Sato, I., Hashimoto, K., Kamesawa, R., Setoyama, K., Yamaguchi, S., Fujiu, K., Waki, K., & Noji, H. (2018). Ghost cytometry. Science, 360(6394), 1246–1251.

OTA Sadao
Associate Professor
Research Center for Advanced Science and Technology,
The University of Tokyo
Ph.D.

# Elucidation of the Mechanism of Near-Infrared Light-Induced Cell Death and Method Establishment for Measuring Therapeutic Effects

SATO Kazuhide

Near-infrared photoimmunotherapy (NIR-PIT) is a new cancer treatment that involves the application of a near-infrared light probe (IR700) to a specific antibody that binds to antigens on the surface of cancer cells. However, despite its impressive efficacy and innovation, the detailed mechanism of cell death has remained unclear. From the viewpoints of analytical chemistry, condensed matter chemistry, and optics, it has been clarified that NIR-PIT cell death is the world's first photochemical-induced cell death, and the cell death can be measured and quantified by IR700 near-infrared fluorescence spectroscopy at 700 nm, thus establishing the basis for a therapeutic biomarker. Due to the novelty of this cell death, this therapy was recognized as a new cancer modality and was implemented clinically in Japan ahead of the rest of the world.

## Introduction

Near-Infrared photoimmunotherapy (NIR-PIT) is a next-generation cancer-targeting therapy in which antibodies specific to antigens on the surface of cancer cells are attached to probes that react to near-infrared light and are then locally irradiated with near-infrared light (Figure 1) [1]. This photo-targeted therapy is a futuristic, and multidisciplinary treatment method in which probes (chemistry) and antibodies (pharmacology and biology) are conjugated under optimal conditions (conjugation chemistry), and near-infrared light (optics and physics) is added (Figure 1). Irradiation with near-infrared light at 690 nm, the peak absorption of the probe, immediately expands the cancer cells and induces cell death. It is currently undergoing international Phase III trials (LUZERA-301) [2,3] and was approved by PMDA in Japan in September 2020 for the treatment of recurrent and previously treated stage IV head and neck cancer[4]. It is now being recognized as the fifth cancer treatment after surgery, chemotherapy, radiotherapy, and cancer immunotherapy. So far, various application studies of NIR-PIT therapy have been conducted all over the world, and further development of this therapy is expected from here. Although this therapy has attracted attention for its innovation, the detailed mechanism of cell death in NIR-PIT had not been fully elucidated at the time of the introduction of Phase III trials. This was a barrier to its approval and implementation. In this review, we will provide an overview of NIR-PIT, which is attracting attention as a "fifth cancer therapy", its cell death mechanism, and the prediction of its efficacy by near-infrared spectroscopic imaging derived from its elucidation.

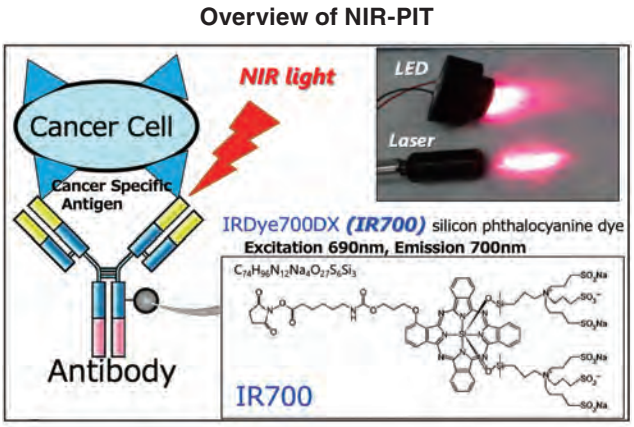



Figure 1 Overview of NIR-PIT
NIR-PIT is a new therapeutic method in which an antibody-IR700 complex, in which IR700DX, a light-absorbing substance, is added to the antibody, binds to cancer-specific antigens that are highly expressed on the surface of target cancer cells, and is then exposed to NIR light.

## About NIR-PIT

There are four known treatment methods for cancer: (1) surgery (surgical and endoscopic resection), (2) chemotherapy (anti-cancer drugs), (3) radiotherapy, and (4) cancer immunotherapy. These existing cancer treatments cause injury not only to cancer cells but also to surrounding normal tissues and organs. In anticancer drug therapy, chemical substances that cause damage to both normal cells and cancer cells are administered, but since the dosage is based on an extremely narrow dosage design that allows normal cells to recover earlier than cancer cells, the dosage is strictly determined, and if an overdose occurs, not only the cancer but also the individual may die. In addition, surgery requires the resection of normal tissues surrounding the cancer as margins, and radiation therapy inevitably causes injury to normal cells within and through the irradiation area. Therefore, it would be ideal if there were a cancer-targeted therapy that could superspecifically damage only cancer cells in the body without damaging normal cells and tissues.

Based on the above idea, Dr. Hisataka Kobayashi and his colleagues at the National Cancer Institute (NCI) in USA announced the development of Near-Infrared Photoimmunotherapy (NIR-PIT) in 2011[1]. NIR-PIT is a photo-targeted therapy that combines near-infrared light and antibody photo-probe adducts, in which IR700, a light-absorbing probe, is covalently attached to antibodies that bind to cancer-specific antigens. NIR-PIT is super-selective because it is doubly targeted by NIR-light and antibodies (Figure 1).

NIR-light is used in TV remote controls, CD players, etc., and is a safe light that has almost no adverse effects when irradiated to the human body. IR700 is a probe that absorbs light in the near-infrared region of 690 nm and emits fluorescence at 700 nm, and was originally developed by LICOR as a dye for microscopic observation in biology. Antibodies are biomaterials that have been actively used in medicine, biology, and engineering in recent years, and are among the existing biomolecules that can bind specifically and stably to molecules on the surface of cell membranes. Among the existing biomolecules, antibodies can bind specifically and stably to cell surface molecules[4]. As described above, NIR-PIT is a multidisciplinary therapy that combines biology, pharmacology, chemistry, optics, engineering, and physics.

## Mechanisms of cell death in NIR-PIT

It has been proposed that oxidative stress is the main mechanism of cell death using photosensitive substances[5]. This pathway is often considered to be one in which the energy difference between photoexcitation and photoemission of a photosensitive substance acts on mitochondria and other organs, resulting in cell injury by oxidative stress agents such as singlet oxygen and free radicals, leading to cell apoptosis. The detailed mechanism of cell death in NIR-PIT has been unknown for a long time, which has been a barrier to its clinical implementation. Since the cell death reaction in NIR-PIT proceeded even after the cell function was stopped at 4°C, and the inhibition of cell death was not sufficiently effective even when oxidative stress inhibitors (free radical scavengers) were added, it was inferred that the oxidative stress was not the main mechanism for NIR-PIT. Therefore, it is inferred that the mechanism of NIR-PIT is not based on oxidative stress as in conventional photodynamic therapy (PDT). [5]

NIR-PIT has a very different therapeutic mechanism compared to conventional cancer cell death. When the antibody-IR700 conjugate bound to the cell surface is irradiated with near-infrared light (690 nm), the cell surface is rapidly injured, the cell morphology changes, and then the cell bursts, leaking the cytoplasmic contents and causing cell death within a few minutes. Thus, the mechanism is necrosis (rapid destruction of cells) rather than apoptosis, which is the conventional slow death of cancer cells. Since near-infrared light irradiation itself is harmless to cells, it can only damage the target cells to which the antibody-IR700 conjugate binds. In fact, when cells with and without antibody-IR700 conjugates were co-cultured and irradiated with NIR light at the same time, cell death was observed only in the cells with antibody-IR700 conjugates, while the neighboring cells without antibody-IR700 conjugates were not injured at all. The antibody-IR700 conjugate can selectively destroy not only cells in vitro but also target cells in vivo in space and time. [6]

**Aggregation by photochemical reaction of IR700 and detection of released products by mass spectrometry**

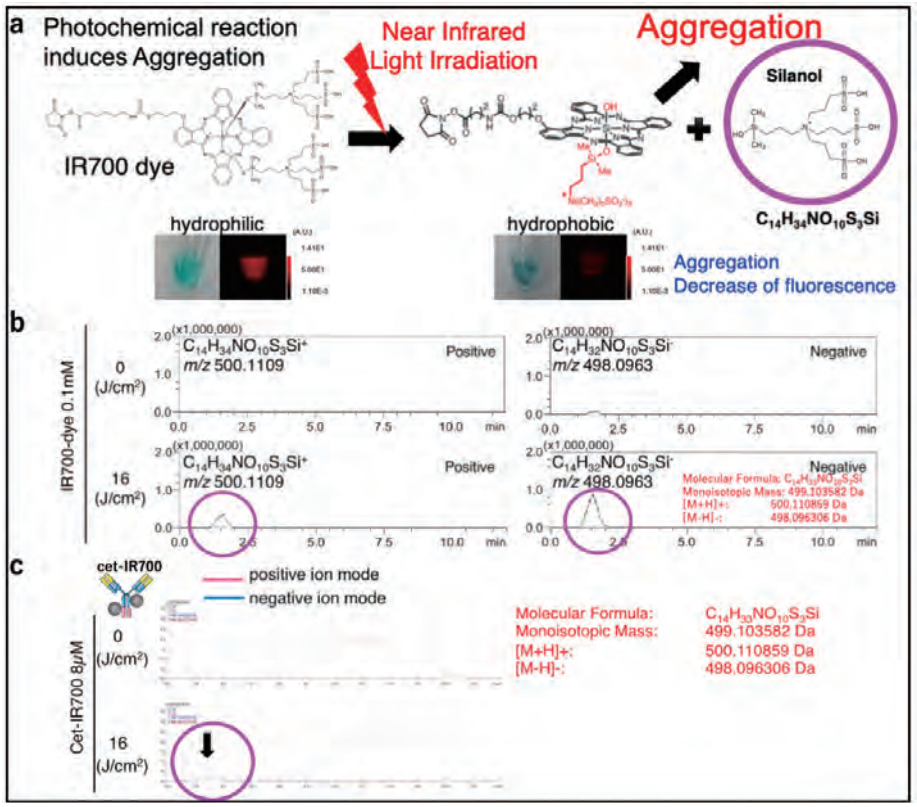



Figure 2 Aggregation by photochemical reaction of IR700 and detection of released products by mass spectrometry
a. The photochemical reaction of IR700 as the starting point of NIR-PIT action: the silanol group attached to the silicon of the silicon phthalocyanine ring of IR700 is released by near-infrared light irradiation, and as a result, the hydrophilic nature of IR700 suddenly becomes hydrophobic and aggregates. As a result of aggregation, the fluorescence is lost.
b. The silanol groups released from the supernatant after irradiation were detected by mass spectrometry (IT-TOF MS), proving the reaction.
c. IT-TOF-MS analysis showed a peak of silanol group only at 16 J/cm2 near-infrared light irradiation. By product scan (MS3) analysis, the structure was determined and proved to be silanol group.

Originally, IR700 dye (IR700 DX) was developed and marketed with the merit that its fluorescence hardly fades compared to other dyes (AlexaFluor680, etc.) during the number of scans in the fluorescence observation environment of microscope slide samples. However, the accumulation of in vitro and in vivo experiments with NIR-PIT clearly showed that the fluorescence of IR700 faded, which was a contradiction. As a hypothesis to resolve this discrepancy, we hypothesized a change in the chemical nature of IR700 due to photochemical reactions. The hypothesis is that the chemical nature (hydrophilic) of IR700 is rapidly changed to hydrophobic by the release of the silanol side chain by near-infrared light. The hydrophobized IR700 forms aggregates in the aqueous solution, and as a result of the aggregation, the fluorescence is lost from the solution in a series of photochemical reactions (Figure 2a)[7]. It was thought that the aforementioned discrepancy could be explained by the difference between the inclusion state of the slide samples using non-water-soluble inclusion material and the water-rich biological environment.

Based on these hypotheses, in order to prove this mechanism, we attempted to measure the silanol groups (molecular weight 500) above and below the silicon phthalocyanine ring of IR700, which are thought to be released upon aggregation, by mass spectrometry. IT-TOF-MS was able to detect the peak of the molecular weight only in the case of light irradiation (Figure 2b). In addition, it has been found that this liberation reaction is enhanced in the absence of oxygen and in an environment rich in electron donors, while the presence of electron absorbers inhibits the reaction. This indicates that this reaction is independent of oxygen and free radicals, and has the advantage that the reaction proceeds more easily in tumors under hypoxic conditions.

Next, in order to prove that the above reaction also occurred in the antibody-IR700 adduct, cetuximab-IR700 (cet-IR700) was irradiated in the near-infrared to prove that silanol could be measured and detected by mass spectrometry. In addition, the solution of cet-IR700 was confirmed by the product scan method to be of IR700 origin, not antibody origin, with the structure of the silanol group (Figure 2c).

SDS-PAGE analysis was performed to study the effect of photochemical reaction of IR700 on antibody IR700 adduct and antibody IR700 adduct-antigen protein complex. First, we found that NIR light irradiation (peak 690 nm NIR light laser irradiation) caused the antibody (cetuximab) band to smear on SDS-PAGE, resulting in the loss of IR700 fluorescence. The photochemical reaction of IR700 resulted in the protein denaturation, i.e., aggregation of the added antibody proteins (Figure 3a). As shown in the figure, the denaturation increased in a near-infrared light-dependent manner, and the aggregation was almost complete at 64 J/cm$^2$. It is also clear that the near-infrared fluorescence is lost due to aggregation. In order to investigate the effect

**Examination of protein aggregation and loss of fluorescence of cet-IR700 by near-infrared irradiation and changes in the antibody-IR700 adduct-antigen protein complex using SDS-PAGE**

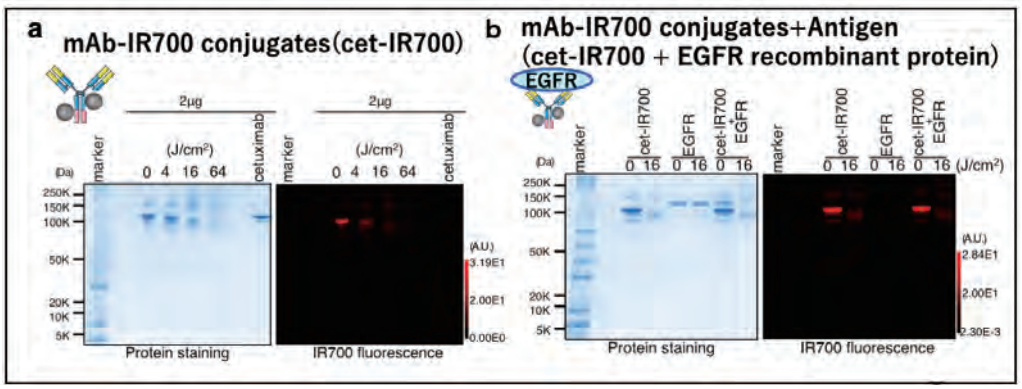



Figure 3 Examination of protein aggregation and loss of fluorescence of cet-IR700 by near-infrared irradiation andchanges in the antibody-IR700 adduct-antigen protein complex using SDS-PAGE
a. The band of cet-IR700 became a smear in a light-dependent manner and lost its fluorescence, indicating that it was aggregated.
b. When cet-IR700 was bound to an antigen protein (EGFR) and irradiated with near-infrared light, the band of the antigen protein also became smeared, indicating that not only cet-IR700 but also its binding partner antigen was aggregated. With aggregation, fluorescence was also lost.

of the photochemical change of cet-IR700 on the binding partner antigen protein, we conducted a near-infrared irradiation experiment with cet-IR700 bound to EGFR protein (antibody-IR700 adduct-antigen protein complex) (peak 690 nm near-infrared laser irradiation measured at 16 J/cm$^2$). SDS-PAGE showed that not only cetuximab but also EGFR protein, the antigen, was aggregated (Figure 3b). In addition, the loss of NIR fluorescence of IR700 was observed. From the above, it was confirmed that the complex of antibody IR700 adduct and antigen was aggregated by light irradiation. This indicates that the photochemical aggregation of IR700 causes the antibody to aggregate and also causes the antigen protein, the binding partner of the antibody, to aggregate.

Although we were able to prove the photochemical reaction by mass spectrometry above, it is necessary to capture the change visually. This mechanism can be clarified by imaging the changes in a single antibody molecule and quantitatively measuring the photochemical reaction. Therefore, we attempted to perform nano-imaging in liquid by atomic force microscopy (AFM). Panitumumab-IR700 (pan-IR700) was adhered to a mica substrate and the top of the substrate was filled with 10 mM phosphate buffer containing 50 mM $MgCl_2$ for nano-imaging in liquid. Macroscopic views of 1.0 μm and microscopic images of

**Observation and Quantification of Changes in panitumumab-IR700 (pan-IR700) under Near-Infrared Light Irradiation by Nano-Imaging in Liquid Using FM-AFM**

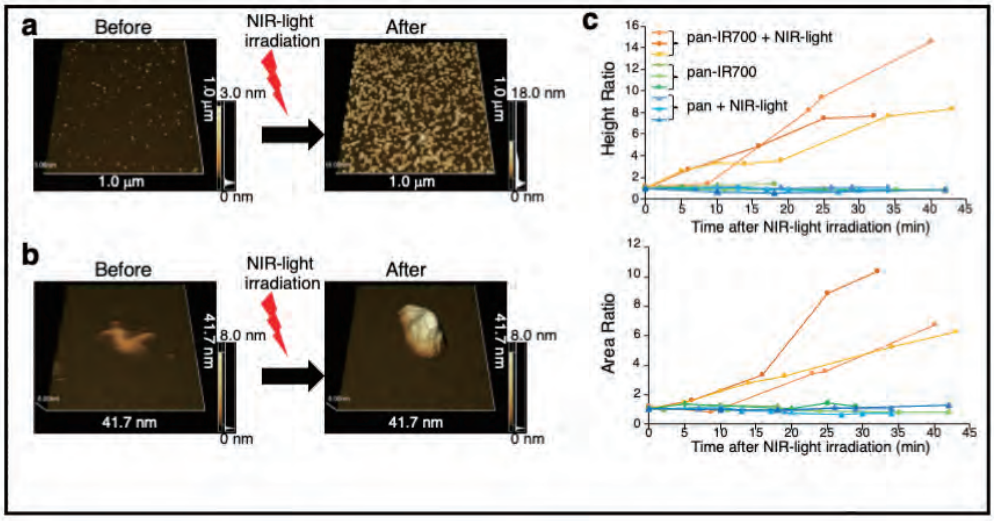



Figure 4 Observation and Quantification of Changes in panitumumab-IR700 (pan-IR700) under Near-Infrared Light Irradiation by Nano-Imaging in Liquid Using FM-AFM The changes of pan-IR700 before and after near-infrared light irradiation were investigated by nano - imaging in liquid using FM-AFM.
a. The pan-IR700 molecules depicted on the mica substrate aggregated and became larger after light irradiation, and adhered to the mica substrate.
b. pan-IR700 molecules depicted on the mica substrate have a Y-shape, but they aggregate and completely change their structure after light irradiation.
c. Results of change quantification by liquid nano-imaging using FM-AFM; the height and area significantly increased only in the group where Pan-IR700 was irradiated with NIR-light.

**Relationship of IR700 fluorescence and in vitro cell death, in vivo anti-tumor effects, respectively**

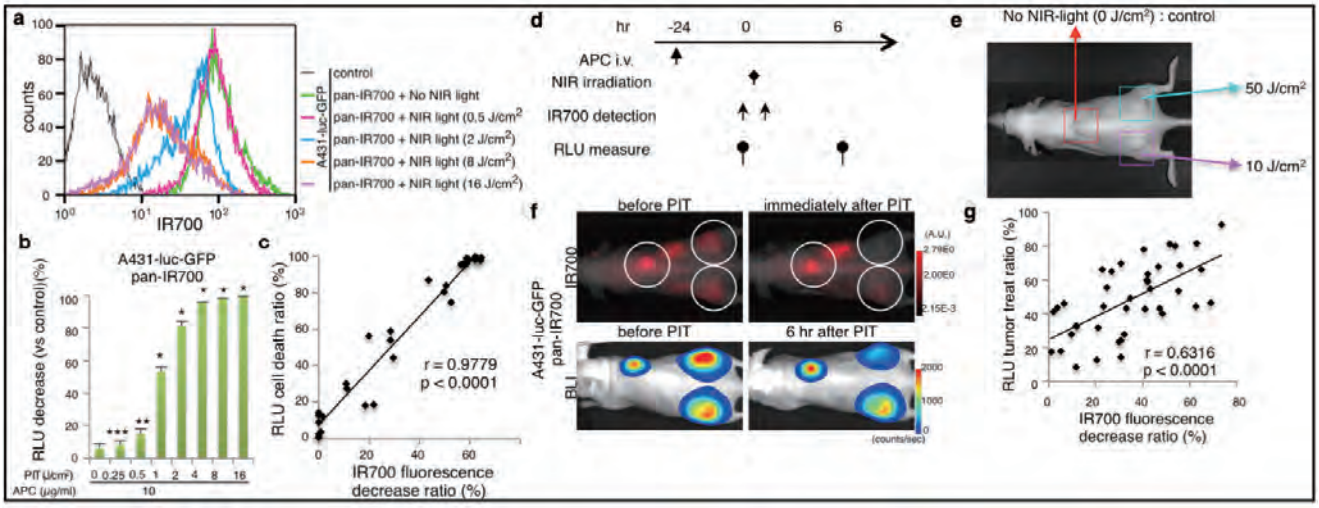



Figure 5 Relationship of IR700 fluorescence and in vitro cell death, in vivo anti-tumor effects, respectively
a. IR700-fluorescence in vitro was detected before and after NIR-PIT with flow cytometry.
b. Cytotoxicity in vitro was detected before and after NIR-PIT with luciferase activities.
c. Positive correlations between decrease of IR700 fluorescence and cytotoxicity is detected (r = 0.9779).
d. in vivo NIR-PIT regimen is showed.
e. Mouse tumor inoculation outline and light dose.
f. Representative image of treated mice.
g. Positive correlations between decrease of IR700 fluorescence and anti-tumor effects is detected (r =0.6316).

41.7 nm were taken. In the macroscopic image, a single molecule of pan-IR700 attached to the mica substrate was agglomerated and expanded as an oligomer by near-infrared light irradiation (measured value: 16 J/cm$^2$). In the micro-image, a single molecule of pan-IR700 in the shape of a Y-shape underwent structural changes upon light irradiation, and expanded, deformed, and aggregated as an oligomer (Figure 4a, b). As a control, pan-IR700 alone (no light irradiation) and antibody (pan) plus light irradiation (16 J/cm$^2$) were tested in the same way for quantification. As a result of quantification of the height and Area values over time, only the combination of pan-IR700 and light irradiation (16 J/cm$^2$) was found to be dominant, with the height and Area values expanding over time (Figure 4c). Therefore, nano-imaging confirmed that the antibody-IR700 agglomerates upon near-infrared light irradiation.

In order to analyze the relationship between photochemical reactions and cell death in vitro, we analyzed the relationship between the loss of IR700 fluorescence on the cell membrane before and after light irradiation and cell death. It is known that near-infrared fluorescence is lost in a dose-dependent manner, and measuring the near-infrared fluorescence of IR700 in IR700 antibody adducts was thought to reflect the degree of protein aggregation. Changes in near-infrared fluorescence were quantified by flow cytometry (Figure 5a), and cell death was quantified by luciferase activity (Figure 5b), and the respective changes were analyzed as a scatter plot. The results showed a positive correlation between the decrease in IR700 fluorescence and the increase in cell death (r = 0.9779) (Figure 5c). This result suggests that cell death can be measured from the amount of decrease in near-infrared fluorescence in target cells bound with IR700 antibody adducts, proving that IR700 photochemistry is strongly associated with cell death in NIR-PIT.

To analyze the relationship between the photochemical reaction and the in vivo anti-tumor effect, we analyzed the relationship between the loss of IR700 fluorescence in tumors before and after light irradiation and the anti-tumor effect (Figure 5d). As shown in Figure 5e, three tumors were created in the same individual and irradiated with 0, 50, and 100 J/cm$^2$ of near-infrared light, respectively, and the amount of change was measured. The change in IR700 fluorescence was quantified by the 700 nm channel of the PEARL imager, and the anti-tumor effect was quantified by the luciferase activity, which was analyzed in relation to the scatter plot (Figure 5f). The results showed a positive correlation between the decrease in IR700 fluorescence and the increase in antitumor effect (r = 0.6316) (Figure 5g). This result suggests that the anti-tumor effect can be measured from the amount of decrease in IR700 fluorescence (700 nm) across the target tumor bound with IR700 antibody adducts, proving that the photochemical reaction of IR700 is strongly related to the anti-tumor effect of NIR-PIT. Since this decrease in IR700 fluorescence may predict therapeutic efficacy, optimization of real-time imaging of IR700 fluorescence is underway.

## Conclusion

The above results suggest that the initiation point of cell death in NIR-PIT is the hydrophobization of IR700 by

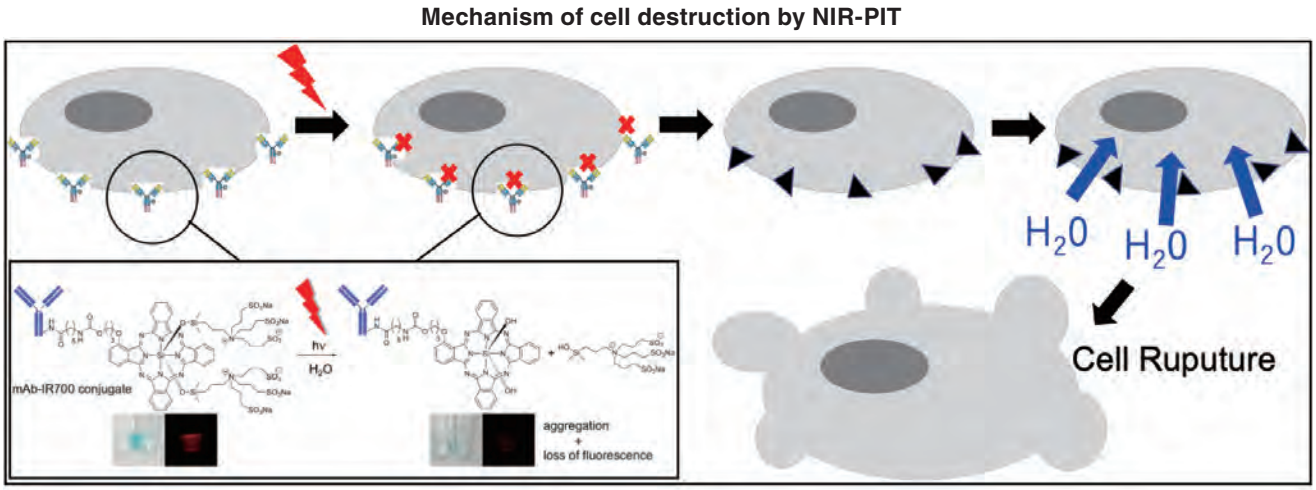



Figure 6 Mechanism of cell destruction by NIR-PIT
The antibody-IR700 complex binds to cancer-specific antigens on the cell surface. When the cells are irradiated with near-infrared light, the silanol group is released from IR700 and rapid hydrophobization occurs, resulting in the formation of aggregates including antibody and antigen proteins. This causes physical damage to the cell membrane. The osmotic disparity causes water to flow into the cell from outside the cell, causing the cell to rupture. Thus, the cell contents are disseminated to the surrounding area as necrosis to produce active cell destruction.

near-infrared light irradiation and the subsequent aggregation of antibody and antigen proteins[7]. In this study, the aggregation of antibody-IR700 adducts by near-infrared light irradiation was revealed by nano-imaging, and the results were convincing enough to propose a new concept of cell death in NIR-PIT. Integrating these analytical chemical data, cell culture, and animal studies, the authors propose the following mechanism of action of NIR-PIT (Figure 6). The antibody-IR700 complex, which binds to cancer-specific antigens on cell membranes, suddenly aggregates upon NIR irradiation and acts as a physical cell membrane barrier by denaturing the cell membrane antigen to which it binds. This mechanism was considered to be a novel photocell death that is completely different from previous concepts. The clarification of this mechanism proved the superiority of NIR-PIT as a new anti-cancer modality, and led to its limited approval in Japan as a "fifth cancer treatment" ahead of other countries. In addition, as a further application, it is thought that it is possible to predict the effect of treatment in real time by measuring and evaluating near-infrared fluorescence before and after treatment as described in this paper, and devices are being developed that apply intraoperative fluorescence imaging devices. We look forward to the development of future applications.

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

## References

[1] Mitsunaga M.; Ogawa M.; Kosaka N.; Rosenblum L. T.; Choyke P. L.; Kobayashi H. Cancer Cell-Selective in Vivo near Infrared Photoimmunotherapy Targeting Specific Membrane Molecules. Nat. Med. 2011, 17 (12), 1685–1691.

[2] NCT03769506, https://clinicaltrials.gov/ct2/show/NCT03769506

[3] https://rakuten-med.com/jp/news/press-releases/2020/09/25/7637/

[4] Buss NA, Henderson SJ, McFarlane M, Shenton JM, de Haan L : Monoclonal antibody therapeutics : history and future. Curr Opin Pharmacol 12 : 615-622, 2012

[5] Agostinis P, Berg K, Cengel KA, Foster TH, Girotti AW, Gollnick SO, Hahn SM, Hamblin MR, Juzeniene A, Kessel D, Korbelik M, Moan J, Mroz P, Nowis D, Piette J, Wilson BC, Golab J. Photodynamic therapy of cancer: an update. CA Cancer J Clin. 2011 Jul-Aug;61(4):250-81.

[6] Sato K, Nakajima T, Choyke PL, Kobayashi H.Selective cell elimination in vitro and in vivo from tissues and tumors using antibodies conjugated with a near infrared phthalocyanine RSC Adv. 2015 Mar 3;5(32):25105-25114.

[7] Sato K, Ando K, Okuyama S, Moriguchi S, Ogura T, Totoki S, Hanaoka H, Nagaya T, Kokawa R, Takakura H, Nishimura M, Hasegawa Y, Choyke PL, Ogawa M, Kobayashi H. Photoinduced Ligand Release from a Silicon Phthalocyanine Dye Conjugated with Monoclonal Antibodies: A Mechanism of Cancer Cell Cytotoxicity after Near-Infrared Photoimmunotherapy. ACS Cent Sci. 2018 Nov 28;4(11):1559-1569.

SATO Kazuhide

Designated Assistant Professor
Institute for Advanced Research, Respiratory Medicine / S-YLC, Nagoya University.

# Real-Time Monitoring and Control of Pharmaceutical Production Processes Using Spectroscopic Data

Dr. Sanghong Kim

To realize the continuous production in the pharmaceutical industry, it is essential to monitor the production process in real time, but it is often difficult. In addition, conventional methods for predicting pharmaceutical quality from near-infrared spectra have drawbacks, such as a decrease in prediction accuracy over time. In this study, a stable and accurate method for predicting the quality of pharmaceuticals from near-infrared spectra was developed using data science techniques. As a result, a new analysis method was established that can contribute to realize real-time quality management and control, and to improve the efficiency of the pharmaceutical production processes.

## Introduction

In the pharmaceutical production process, strict quality control based on Good Manufacturing Practice (GMP) is required. In recent years, there has been a shift from batch processes to continuous processes and the introduction of Quality by Design (QbD) and Process Analytical Technology (PAT) [1]-[4] to improve the efficiency of pharmaceutical production processes. In order to achieve the above goals, it is necessary to monitor and control the quality of pharmaceuticals in real time. However, it is often difficult to measure the quality of a drug directly in real time. In order to solve this problem, this research developed and put into practical use a method for constructing a statistical model to estimate drug quality from variables that can be measured relatively easily in real time, such as near-infrared spectra.

## Main

### Process Nonlinearity, Model Maintenance, Input Variable Selection

There are many studies on methods for predicting drug quality from near-infrared spectra, but most of them use the classical method of partial least squares (PLS) [5]-[8]. However, PLS is known to have some problems such as inability to cope with nonlinearity and high maintenance load because the estimation accuracy decreases with time. In this research, a new method called locally-weighted PLS to improve PLS was developed. Locally-weighted PLS can cope with nonlinearity by selectively using historical data that are similar to those around the conditions for which quality prediction is required. In addition, since a new model is automatically constructed for each quality prediction, the estimation accuracy can be maintained for a long time. Figure 1 shows the results of PLS and locally-weighted PLS prediction of the concentration of the active ingredients in the powder after mixing for a mixing process. Locally-weighted PLS reduced the root mean square error of prediction (RMSEP) from 1.84 to 1.13. This allows for more strict quality control and more efficient drug production processes.

In addition to the development of locally-weighted PLS, a method to select the variables that are useful for estimation was developed. It is said that various physicochemical

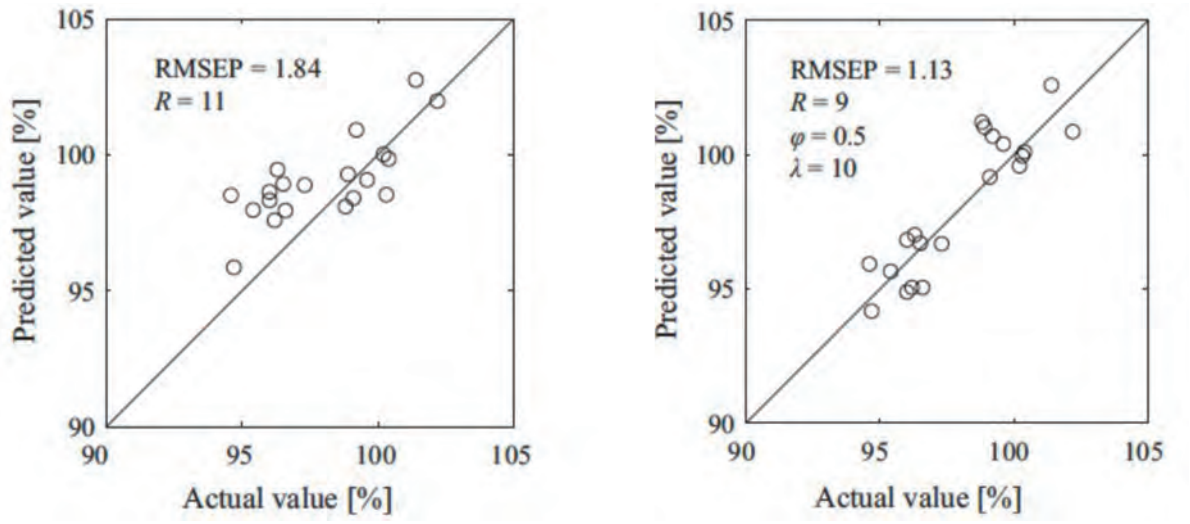



Figure 1 Prediction results by PLS (left) and locally-weighted PLS (right)

factors coexist in near-infrared spectra, and it is not always possible to make good predictions by selecting functional groups and their corresponding wavelength regions, or by selecting wavelength regions by referring to the spectra of active ingredients and other ingredients as pure substances. The wavelength regions selected by the developed method are shown in Figure 2 as the blue region. The target granule is composed of API (Active Pharmaceutical Ingredient) and five other components. The peaks of API and other components do not exist in the selected region, which may seem useless for estimation. However, the RMSEP was reduced to 1.13 from 1.84 by using absorbance at those wavelengths compared to use the absorbance at wavelengths selected by conventional method: variable importance on the projection (VIP) [12].

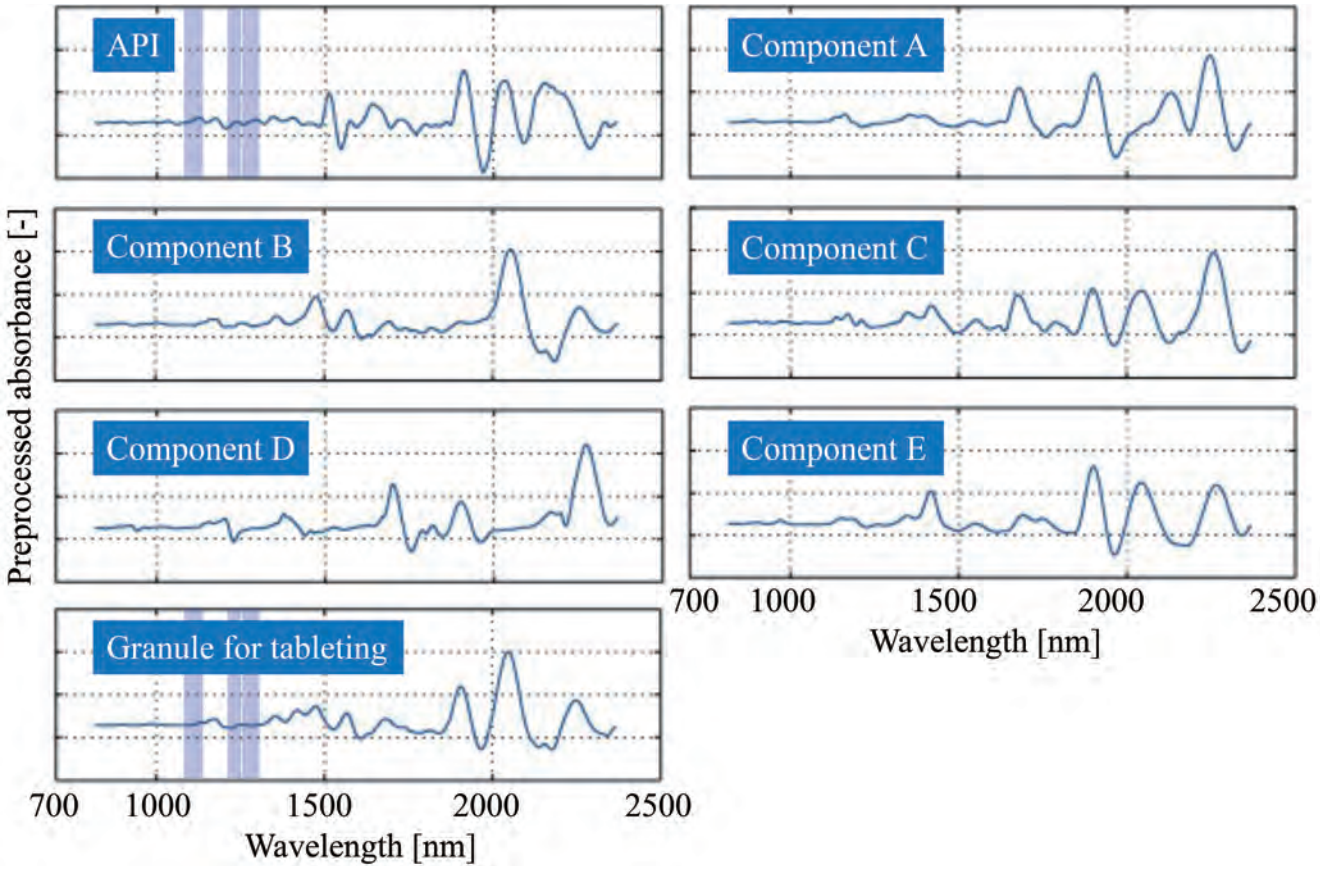



Figure 2 Wavelength selection result of the proposed method and preprocessed absorbance of each component of a drug

The detailed information can be found in the paper [9].

### Input Variable Scaling

When using statistical methods to build a quality prediction model, it is necessary to pre-process the input variables. Although input variable scaling, a data preprocessing method in which the values of each input variable are multiplied by the scaling factor of the input variable, can have significant effect on the estimation performance of soft sensors, research on input variable scaling has not been actively conducted. Hence, this research focuses on input variable scaling. In past research, autoscaling was commonly used [11-13]. In addition, Pareto scaling, level scaling, Poisson scaling, range scaling, and VAST scaling [14] have been considered.

In this research, a new method of preprocessing near-infrared spectra was developed, and it was found that it can improve the prediction accuracy of the impurity concentration inside the device. In the case of spectral data, it is difficult to evaluate the importance of each variable because hundreds to thousands of variables need to be handled simultaneously.

An example of the application of the proposed method to the prediction of magnesium stearate concentration is shown in Figure 3. Figure 3 shows the near-infrared spectra of magnesium stearate and methanol solutions of magnesium stearate with different concentrations. Unlike Section 2-1, it was known in advance that the blue region in the figure is important for concentration prediction because it is a solution system. The evaluation results are shown in Figure 4. From Figure 4, it can be seen that the proposed method is able to correctly determine the important regions for concentration prediction. Accordingly, the prediction error was reduced by 45%.

The detailed information can be found in the paper [15].

### Model and Parameter Selection

Research on statistical modeling methods has been actively conducted, however, the usefulness of the methods has usually been evaluated by using a single dataset in most of the research, and the robustness of the methods have not

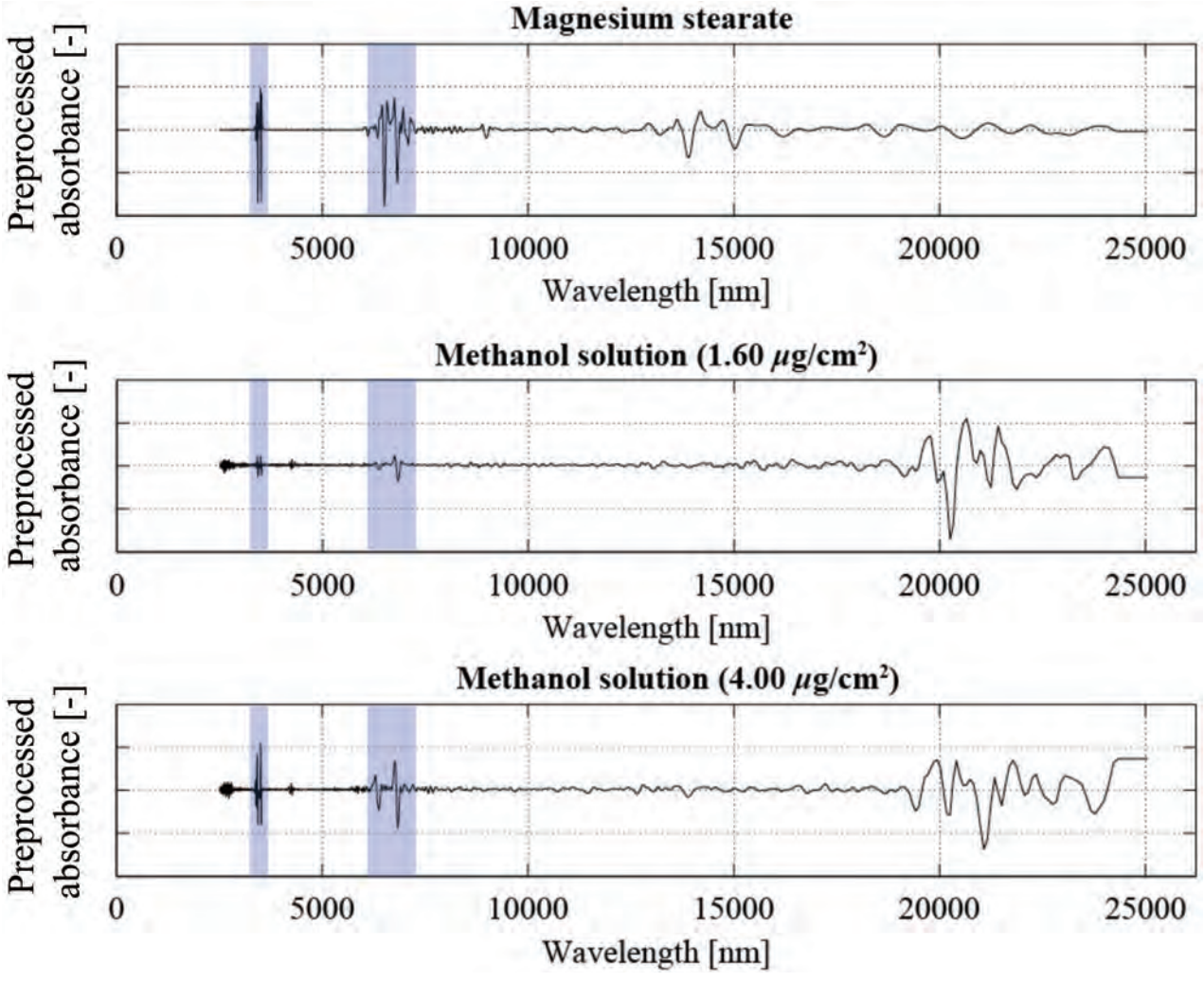



Figure 3 Preprocessed absorbance of magnesium stearate and its methanol solution

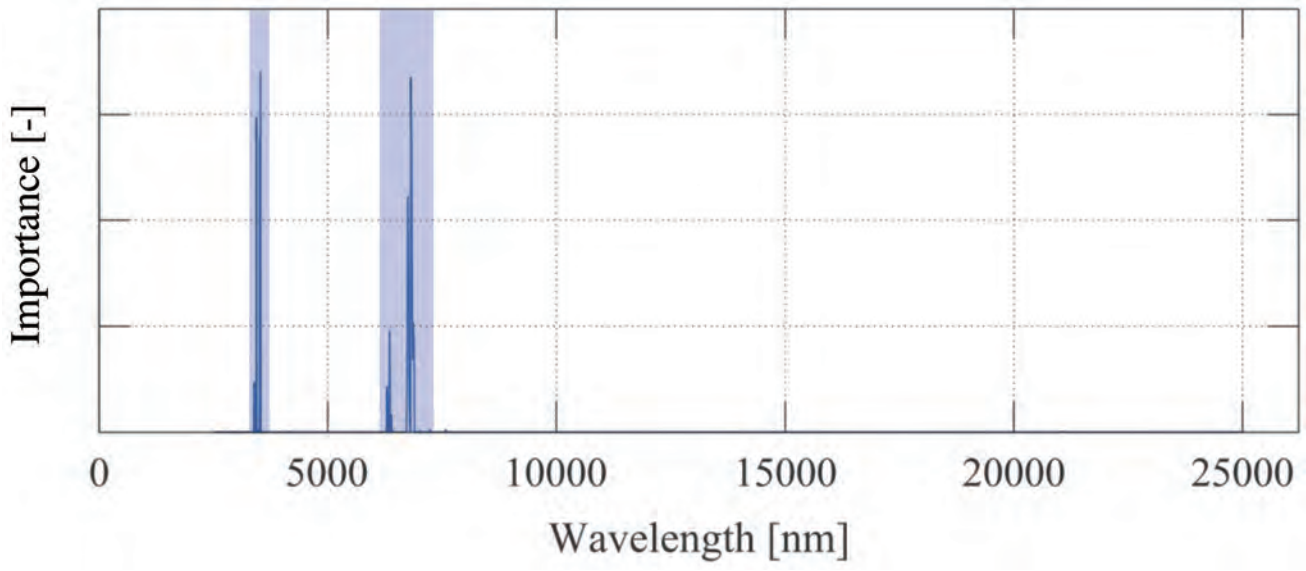



Figure 4 Importance evaluation result of the proposed method

been evaluated. To propose a highly reliable method, it is necessary to evaluate whether the developed soft-sensor can stably estimate the difficult-to-measure variables for various processes and a wide range of operating conditions. In this research, partial least squares (PLS), locally weighted PLS, support vector regression (SVR), and artificial neural network (ANN), which are widely used for soft-sensor design, are compared using twelve real-world datasets. The result of comprehensive comparative study in 6 datasets is show in Figure 5. In Figure 5, $p$ denotes the ratio of the number of samples used to model construction to the number of all the samples in each dataset. The result showed that locally weighted PLS outperformed the other methods. At the same time, the drawback of locally weighted PLS has also been made clear. locally weighted PLS is a modified version of PLS which is a linear regression method, and locally weighted PLS can deal with nonlinearity. However, while the conventional parameter tuning method can adapt to the nonlinearity of the target process, it has also a risk of excessively adapting to variations in measured values due to the influence of noise. Thus, a new parameter tuning method is proposed to improve the accuracy while guaranteeing the minimum prediction accuracy, and showed that the prediction accuracy of active ingredient and impurity concentrations can be improved.

The detailed information can be found in the paper [16].

## Conclusion

In this research, real-time monitoring methods of continuous pharmaceutical processes were investigated. Some of the developed technologies have already been put to practical use and have achieved social contribution. In addition to the content of the former section, the research on measurement and automation using data science and process control technology for many processes in the pharmaceutical production process, including the granulation process, drying process, and tableting process have been conducted. This research is expected to make a significant contribution to society, as it is planned to be put to practical use by our joint research partners.

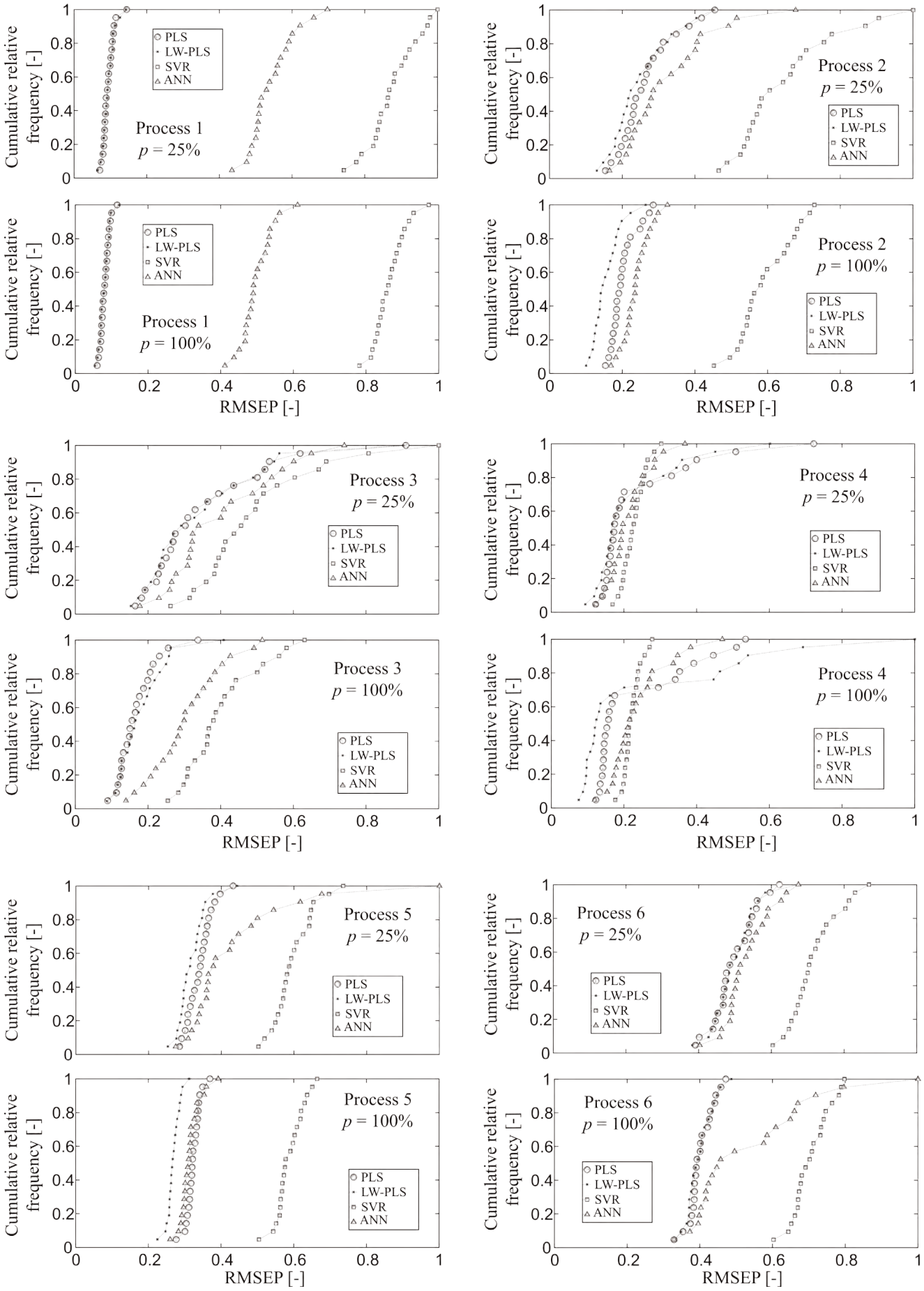



Figure 5 Result of comprehensive comparative study

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

## References


[1] FDA 2004. Pharmaceutical cGMPs for the 21st century—a risk-based approach final report.

[2] ICH 2005. ICH harmonised tripartite guideline—pharmaceutical development Q8 (R2).

[3] ICH 2005. ICH harmonised tripartite guideline—quality risk management Q9.

[4] ICH 2008. ICH harmonised tripartite guideline—pharmaceutical quality system Q10.

[5] Moes, J.J., Ruijken, M.M., Gout, E., Frijlink, H.W., Ugwoke, M.I., 2008. Application of process analytical technology in tablet process development using NIR spectroscopy: blend uniformity, content uniformity and coating thickness measurements. Int. J. Pharm. 357, 108–118.

[6] Berthiaux, H., Mosorov, V., Tomczak, L., Gatumel, C., Demeyre, J., 2006. Principal component analysis for characterising homogeneity in powder mixing using image processing techniques. Chem. Eng. Process. 45, 397–403.

[7] Virtanen, S., Antikainen, O., Yliruusi, J., 2007. Uniformity of poorly miscible powders determined by near infrared spectroscopy. Int. J. Pharm. 345, 108–115.

[8] Wu, H., Tawakkul, M., White, M., Khan, M., 2009. Quality-by-design (QbD): an integrated multivariate approach for the component quantification in powder blends. Int. J. Pharm. 372, 39–48.

[9] Eriksson, L., Johansson, E., Kettaneh-Wold, N., Wold, S., 2001. Multi- and Megavariate Data Analysis. Principles and Applications. Umetrics Academy.

[10] Kim, S., Kano, M., Nakagawa, H., Hasebe S., 2011. Estimation of active pharmaceutical ingredients content using locally weighted partial least squares and statistical wavelength selection, Int. J. Pharm., 421, 269–274

[11] Engel J, Gerretzen J, Szyma´nka E, Jansen JJ, Downey G, Blanchet L, et al. 2013. Breaking with trends in pre-processing? Trends Anal. Chem. 50, 96–106.

[12] van den Berg RA, Hoefsloot HCJ, Westerhuis JA, Smilde AK, van der Werf MJ., 2006. Centering, scaling, and transformations improving the biological information content of metabolomics data. BMC Genomics, 7, 142.

[13] Todeschini R, Consonni V, Maiocchi A., 1999/ The k correlation index theory development and its application in chemometrics. Chemom Intell Lab Syst 46, 13–29.

[14] Keun HC, Ebbels TMD, Antti H, Bollard ME, Beckonert O, Holmes E, et al., 2003. Improved analysis of multivariate data by variable stability scaling application to NMR-based metabolic profiling. Anal Chim Acta, 490 265–76.

[15] Kim, S., Kano, M., Nakagawa, H., Hasebe S., 2015. Input variable scaling for statistical modeling, Comput. & Chem. Eng., 74, 4, 59–65

[16] Matsuyama, Y., Kim, S., Hasebe S., 2021 Robust parameter tuning method of LW-PLS and verification of its effectiveness by twelve industrial processes", Comput. & Chem. Eng. 146 107224



**Dr. Sanghong Kim**

Associate Professor
Department of Applied Physics and Chemical Engineering,
Tokyo University of Agriculture and Technology.

# The Role of Particle Design Studies in Developing Pharmaceutical Dosage Forms

**TAKEUCHI Hirofumi**

Emeritus Professor
Laboratory of Advanced Pharmaceutical Process Engineering
Gifu Pharmaceutical University

In order to realize patient centric therapy, studies on drug administration is progressed focusing on its route and type of dosage forms. In addition to guaranteeing efficacy and safety in drug administration, there are development of new dosage forms that take into consideration ease of administration, and development of DDS for the purpose of more effective drug delivery. In either study, particle design research in developing the dosage forms is important. Recent trends in dosage form design development and the particle design researchs to support them will be introduced.

## Introduction

Human drugs, administered in divided doses as required, are designed in optimal dosage forms to ensure efficacy and safety; especially well-known forms include tablets, capsules, injections, ophthalmic solutions, and adhesive patches. As you can see from this listing, diverse administration routes are available and the optimal route is selected for individual drugs. Some drugs have multiple routes. The decision on the administration route is based on various rationales and considerations. Among them, recently gaining much attention is patient-centric therapy (PCT).

Considering PCT in terms of formulation and administration route, the oral form is deemed to be minimally burdensome and therefore preferrable for the patient. Oral administration is also seen as desirable from a medication adherence aspect, i.e., assurance that the drug administration is executed as prescribed. Eight oral dosage forms are currently set forth in the Japanese Pharmacopeia (JP), 17th Edition (JP17): tablets, capsules, granules, powders, liquids and solutions for oral administration, syrups, jellies for oral administration, and films for oral administration.

The tablets section includes the subsection of orally disintegrating tablets (ODTs) as a formal dosage form. The films section was newly introduced in the JP17, together with the orally disintegrating films (ODFs) subsection. The JP started to provide the definitions and specifications of the formulations sorted and aligned by the route of administration in the 16th edition, published roughly a decade ago, in concordance with the time when a number of pharmaceutical products were developed giving consideration to the ease of administration for the patient, such as ODTs, with PCT becoming a prevalently accepted notion.

Another important issue in pharmaceutical preparation investigations is the effective delivery of the drug to the target site, i.e., research and development of the drug delivery system (DDS). The early DDS studies included investigations of controlled release oral drugs and change from injectable to transdermal dosage forms; these studies led to early realization into products. As a DDS for drugs administered into the circulation, encapsulation in a microparticle carrier to achieve efficient delivery to the site of action has been studied vigorously and has led to the development of

anticancer drug products, among others. Such carriers include liposomes (Lips) consisting of phospholipids, lipid emulsions, and albumins. While ideal targeting drugs that are 100% delivered to their target sites (e.g., tumor tissue) are yet to be realized, the use of microparticle carriers has improved the targeting efficiency.

Particle design studies have been a tradition at the pharmaceutical laboratory at Gifu Pharmaceutical University, where I belonged. On the basis of these studies, my colleagues and I have pursued pharmaceutical designing with the goal of "patient-centric formulation design"[1] since the early 2000s. Here I would like to share with you some findings mainly from our work to show the benefits of particle design studies in accomplishing target formulation designs.

## Easy to swallow solid formulations and particle design studies

### Orally disintegrating tablets

The first product of the aforementioned ODTs on the market was developed abroad under the trade name Zydis. This product is prepared from the aqueous solution or suspension of the drug filled in the blister pockets, which is then subjected to freeze drying.[2] Zydis is known as a typical ODT of a porous structure that disintegrates very rapidly when coming into contact with water. Several domestic ODT products prepared by the same method are also available. Advances in ODT development have enabled manufacturing of ODTs having similar hardness as conventional oral tablets and even using similar tableting machines (compression molders) as those for conventional tablets, bringing about many ODT products into the clinical setting.

Tablets are formulated using various excipients, such as diluents/fillers, disintegrants, binders, and lubricants. The process for determining the kind and amount of each excipient to be used is called a formulation study. In the case of ODTs, a regular formulation study alone may not suffice to clear their property requirements: disintegration within 30 seconds in the oral cavity plus the hardness of conventional tablets. Mannitol and erythritol, for example, are desirable as a filler because of their superior solubility, but they are poor in compression and compaction properties; investigations to improve their compactibility are under way. Some ODT products are made using mannitol products suited for direct tableting; such a filler is processed for granulation using the spray-dry granulation method, as is lactose. Also, to ensure the drug's disintegration property, a comprehensive formulation study is needed for other excipients as well as the filler.

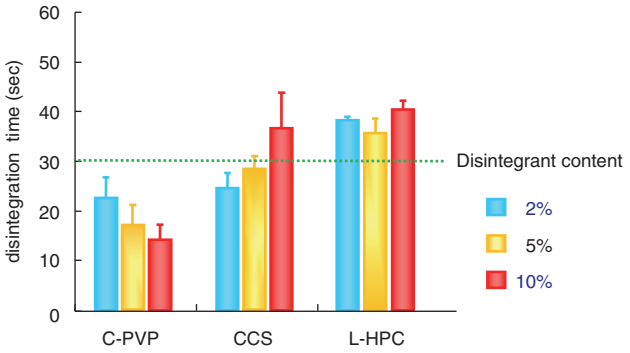



Figure 1 Disintegration time of erythritol tablet containing composite particle prepared by spray-drying with silica and disintegrant
Formulation of tablets: Erythritol + 10% composite particle + 2-10% disintegrant + 0.2% lubricant
Disintegrant: C-PVP: cross povidone, CCS: croscarmellose sodium, L-HPC: low substituted hydroxy propyl cellulose

Allow me to show you some of our studies in which we worked on particle designs for ODT development. With respect to diluents/fillers, we used erythritol, which is hardly compactible, and porous silica to formulate composite particles employing the spray-dry method and compressed this formulation into tablets, which we showed were equivalent in hardness to conventional tablets.[3] The addition of small quantities of porous silica particles was empirically known to slightly improve the tablet hardness, but the presence of silica in the formulation alone was not enough to explain the resulting drastic improvement in compactibility. Powder X-ray diffraction showed that the sugar alcohol remained in a crystalline state, ruling out disintegration of sugar alcohol crystals as a cause of the improved compactibility. Subsequent thermal and other analyses of different sugar alcohol to silica ratios revealed the contribution of sugar alcohol placed in a high-energy state.[4] Identical peaks displayed in the powder X-ray diffraction pattern showed the absence of so-called crystal polymorphism while a study on particles dispersed in a solid medium indicated the contribution of porous silica to stabilization of the high-energy state.[5] Figure. 1 presents model formulations containing composite particles that achieved our target disintegration property (disintegration within 30 seconds) by the addition of proper amounts of disintegrants.

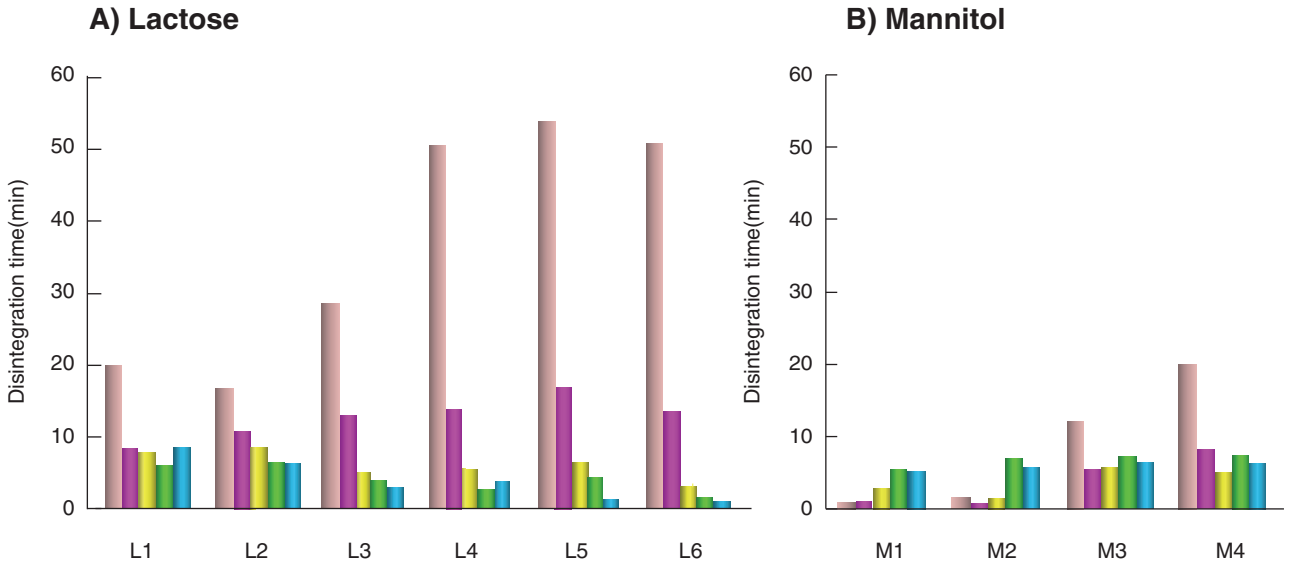



Figure 2 Disintegration time of tablets formulating magnesium stearate (Mg-st) or different type of sugar alcohol (SE) as a lubricant
Formulation of tablet: Mannitol or lactose + magnesium stearate (1%) or sugar alcohol
Mg-st S-370F S-770 S-1170 S-1570
L1: Dilactose S, L2: Super Tab 21AN, L3: Fast-Flo Lactose, L4: Tablettose 100, L5: Flow lac 100, L6:Super Tab 11SD
M1: Parteck M200, M2: Fine mannitol, M3: PEARITOL 200SD, M4: Mannitol (crystal)

Table1 Premixed or co-processed excipients with mannitol for direct tableting of ODT

| Trade name | Formulation |
|---|---|
| F-MELT | Mannitol, xylitol, microcrystalline cellulose, cross povidone, Metasilicate alumic acid magnesium or Anhydrous calcium hydrogen phosphate |
| SmartEx | Mannitol, L-HPC, PVA |
| Ludiflash | Mannitol, Collidone CL-SF, Collicoat SR30D |
| Pearitol FLASH | Mannitol, corn starch |
| Granfiller D | Mannitol, carmelose, cross-povidone |

L-HPC: Low-substituted hydroxy propyl cellulose
PVA: poly vinyl alcohol

Improving compactibility with the use of a binder is a common strategy in tablet designing. In the case of ODTs, however, it was confirmed that upon disintegration the binder in the tablet dissolved, causing delayed hydration and longer disintegration time than intended. In the striving process for assuring compactibility with the addition of minimum quantities of the binder to avoid disintegration delay, we found that this aim was achievable by the use of micronized hydroxypropyl cellulose (HPC), a widely used binder. Further, taking HPC grades into consideration, we presented some model ODT formulations using HPC-SSL fine powders.[5] With an established manufacturing process, HPC-SSL fine powders are commercially available, and at present they are utilized for broader purposes, such as hardness adjustment of tablets. Regarding lubricants, magnesium stearate (Mg-st) is the most freaquently used in tablet formulation has now become far more common than before, whereas other substances are also used in some medications and health food products. Sucrose fatty acid esters (SEs) are one of them. We assessed the effects of some SE products that were much less hydrophobic than Mg-st. Figure. 2 summarizes the disintegrating time of tablets formulated using lactose, a typical filler for tablets, with the addition of different types of SEs (S-370F, S-770, S-1170, or S-1570; in the ascending order of hydrophilic property). As expected, a higher hydrophilic property of the lubricant was associated with shorter disintegration time (Figure. 2).[6] Conversely, in a similar study on tablets formulated with different types of mannitol, a typical filler for ODTs, we found shorter disintegration time for certain combinations of mannitol and Mg-st or SEs of higher hydrophobic properties (Figure. 2). This reversal phenomenon may be explained as follows: in instances of a very fast disintegration, the presence of a hydrophobic portion within the tablet may contribute to rapid hydration by reducing the amount of water consumed for mannitol dissolution.

Drug additive manufacturers, going beyond the development of ODT fillers for direct compaction, are engaged in the development of products having higher functionalities, such as improved disintegrable profiles, based on optimal mixtures of additives (premixed products) or formulations with composite particles (composite formulations). Table 1 lists some of such products along with their formulations. Their manufacturers are not limited to companies in Japan,

where ODT development is active, but multiple overseas manufacturers are also present. Increased attention to and further advances in ODTs are anticipated.

### Orally disintegrating films

Orally disintegrating films (ODFs), similar in characteristics to ODTs, are expected to be prescribed for extended use because of their dosage form. As stated before, ODFs have been included in the JP. The film developed abroad by Prestige Medical under the trade name Chloraseptic Sore Throat Relief Strips was the first ODF approved as a medicinal product in 2004, which was followed by many other products of the same kind. In Japan, Voglibose OD film was launched in 2006 as the first medicinal ODF on the domestic market. Over-the-counter ODF development has been ongoing since then as in the U.S. and European markets.

ODFs are advantageous over ODTs in ease of dosage adjustment as well as ease of administration, and hence high expectations are held as a new pediatric drug. ODFs for children have already been approved in the U.S. and Europe. In Japan although such products are yet to be made available, ODFs are drawing greater attention for their dosage form suited for use in children. Clinical dose levels for pediatric patients are often based on their weight and age group. For better dosage adjustability, it has been proposed abroad to develop long tape-like ODFs and administer them in the length determined based on the calculated required dose. Wening et al.[7] presented a method to graduate the film itself and different types of devices to sectionalize the film. Niese et al.,[8] taking the example of warfarin, which requires frequent dose adjustment, reported on their development of a device for film dose adjustment (e.g., a tape dispenser) and formulations suitable for use with the device (e.g., those ensuring flexible dosing). The usefulness of the film as a dosage form suited to personalized formulation, not limited to pediatric patients, was also reported. Visser et al.[9] evaluated the feasibility of small-scale ODF preparation by the solvent casting method using different model active ingredients added to the film forming agent hydroxypropyl methylcellulose (HPMC) and concluded that such customized ODF production would be possible. We also assessed preparation of ODFs of high fluconazole content with sufficient tensile strength and practically short-enough disintegration time for in-hospital ODF preparation purposes.[10]

The components used to form films, which are equivalent to excipients for tablets, are called film forming agents, which include HPMC, hydroxypropyl cellulose (HPC), polyvinyl alcohol (PVA), and pullulan. Among them, HPMC is most commonly used, probably because, for one, accumulated data are available on film formability and other properties as a tablet film coating agent. We conducted various evaluations, aiming to clearly characterize varying film forming agents to contribute to future development of ODF as well as ODT products. One of our studies was on the development of ODFs using HPMC; we tested films formulated with wet-ground micronized low substituted HPC, the application development of which was ongoing at that time, and reported that those films achieved drastically shortened disintegration time.[11] We also found that ODFs of high mechanical strength were preparable using HPC without the addition of a plasticizer, which is required for other film formers,[12] and that the disintegration time was controllable by the inclusion of suitable hydrophilic microparticles in the formulation.[13] For novel dosage forms, it is especially important to establish assessment methods. We evaluated a technique that employed a taste sensing system to detect the bitter taste of sample ODFs[14] and developed and assessed a disintegration test system for ODFs capable of automatically detecting the ending of disintegration time.[15]

## Particle designing for drug delivery

### Lips as drug carrier systems

Phospholipids, which comprise the cell membrane, are reported to form closed vesicles in water. These vesicular structures, called Lips, consist of bilayers. They were expected to serve as highly biocompatible microparticle drug carrier systems in addition to their role as an experimental biomembrane model. Actually, Lips are used as carriers for the anticancer drug doxorubicin products that are available in injectable form (e.g., Doxil®). Research to enable gastrointestinal absorption of insulin utilizing Lips was also initiated early on, but this objective has not been realized yet clinically.

Drug-loading particles are usually of submicron size. Lips prepared by the most common method, thin-film

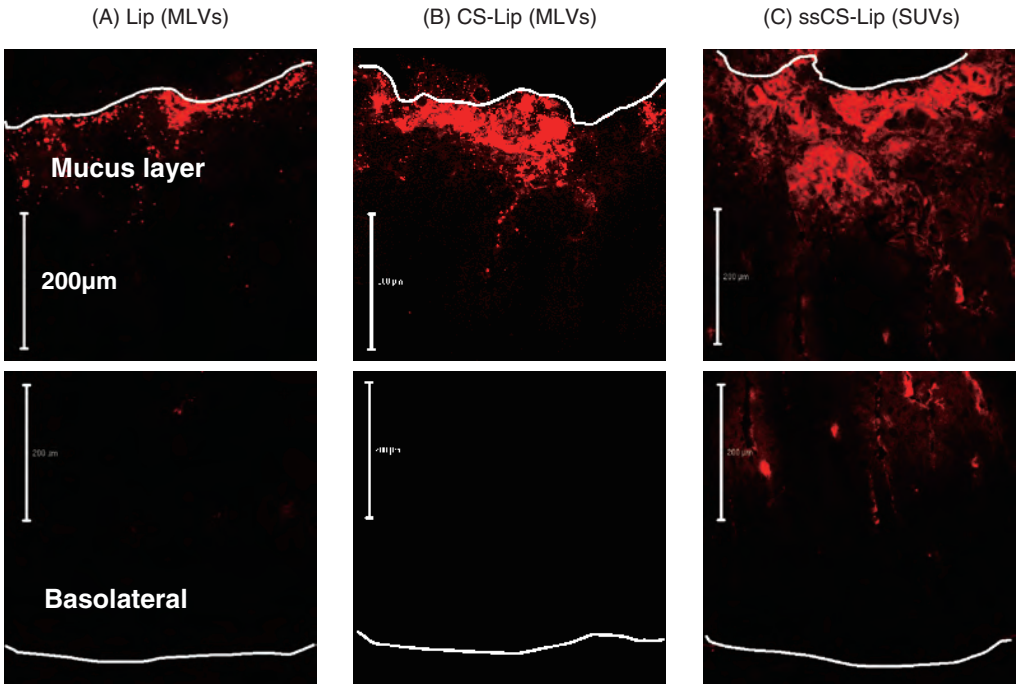



Figure 3 Confocal laser scanning micrographs of slice samples of upper part of the intestinal tract at 2hr after intragastrical administration of (A) Lip (MLVs), (B) CS-Lip, (C) ssCS-Lip in rats
Lip: liposome, CS-Lip: chitosan coated liposome, ssCS-Lip: submicron sized CS-Lip
The mean particle size of liposomes: (A) 7.56 μm (B) 3.58 μm (C) 0.28 μm. Lipid formulation of liposomes: DSPC: DCP: Chol.=8: 2: 1. Red parts means presence of corresponding liposomes.

hydration, can be several micrometers, but the size can be adjusted with relative ease to approximately 100 to 200 nm. It is also possible to render Lip particles either positively or negatively charged by adding small quantities of a charged substance together with phospholipids. The hydrophobic properties of Lips can be controlled by altering their surface membrane fluidity with the addition of a proper amount of cholesterol in drug preparation. Since the surfaces of Lip particles can be modified with substances such as macromolecules or surfactants, it was deemed feasible to achieve particle designs suited to different administration routes.

## Oral administration of peptides using mucoadhesive Lips

Studies of DDSs utilizing Lips have been active since around the 1970s, soon after phospholipids were reported to form Lips. I started working full-fledgedly on Lip formulations in around 1990, when mucoadhesion-based control of tablet and granule retention time was a high-profile topic in formulation studies. Our interest in Lip particle surface modification and adhesion of small particles to intestinal mucosa led us to prepare chitosan (CS)-coated Lips (CS-Lips; Lips with CS-modified surfaces; CS is known to be mucoadhesive) and evaluate the effects thereof.[16] Mucoadhesive Lips were successfully designed as planned. Lips encapsulating insulin were orally administered to rats, and to analyze their pharmacological effects, changes in the blood glucose level were monitored versus noncoated Lips loading insulin. The administration of insulin encapsulating CS-Lips resulted in a significant reduction in the blood glucose level, and this effect lasted for 12 hours or more. These results confirmed in vivo manifestation of the mucoadhesive effect observed in vitro.[17]

Given the above results, we assessed the usefulness of mucoadhesive Lips and optimization of their functions from various viewpoints. The finding that drew our attention most from a particle design perspective was the wide variability noted in Lip retention time depending on Lip size.[18] Direct observation was the only method available to check the particle behavior in the intestine. Thus, Lips loading a lipid-soluble fluorescent substance were intragastrically administered to rats, and horizontal sections of their intestinal tracts were observed post-administration with confocal laser scanning microscopy. All the micrographs in Figure. 3 were taken 2 hours post administration. You can see that the amount of Lips (shown in red) differs greatly depending on the particle size of the Lips administered. This trend was augmented by CS coating of the Lips. While it is generally perceived that Peyer's patches' function is

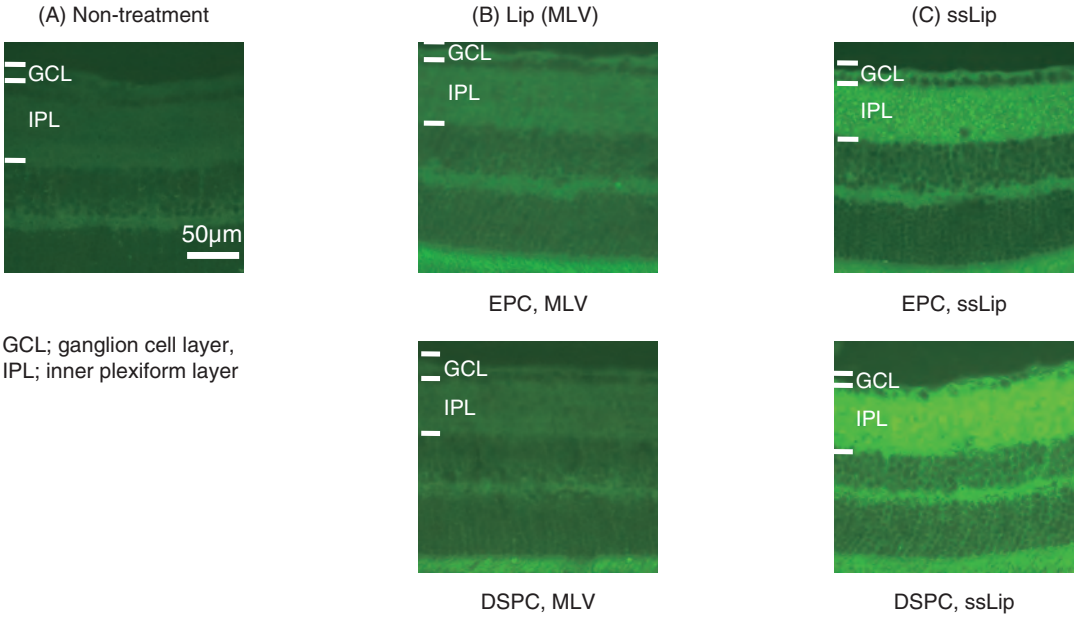



Figure 4 Delivery of coumarin-6 (fluorescence marker for liposomes) with liposome to posterior part of eye observed with fluorescence microscopy 30 min after instillation to the eye in mice
Lip: liposome, ssLip: submicron sized Lip, EPC: egg phosphatidyl choline, DSPC: di-stearoyl phosphatidyl choline
EPC liposomes are relatively softer compared with DSPC liposomes.

involved in the intestinal absorption of particulate substances, such a tendency was not noted for Lip absorption. Some Lip particles were seen penetrating into the intestinal epithelial mucosa.[19] Studies up to recent times have detected an existing model macromolecular compound (fluorescein isothiocyanate dextran) in the circulation in experiment systems similar to ours. This finding has convinced us that CS-Lips are a useful carrier system to increase the absorption of macromolecules from the intestine. In addition, the simultaneous delivery of a permeation enhancer with the macromolecular drug as entrapped in Lips was shown to augment both the pharmacological effect and absorption levels of the entrapped macromolecular drug, which indicates that part of the drug is released and absorbed in the vicinity of the mucosa.[20]

## Retention of Lips administered via lung

Pulmonary administration via inhalation has been considered to be an efficient route for better absorption of macromolecular drugs such as peptides. The presence of fewer degrading enzymes and thinner epithelial cells make the lung a more desirable site for drug absorption than the intestine. Inhaled insulin powders to treat type I diabetes with frequent dosing were actually commercialized, but regrettably, this dosage regimen was not widely accepted as an alternative to an injection and is no longer available on the market.

Pulmonary administration of Lips is intended for controlled drug release and, as in the intestine, prolonged drug retention. The respiratory system being a closed system within the lung, I took interest in what effects would result from mucoadhesive properties of Lips in the alveolus, where macrophages are known to ingest foreign materials. To clarify the behavior of surface-modified Lips after pulmonary administration, the amounts of Lips on the tissue and in the bronchoalveolar lavage fluid (BALF) were evaluated at designated time points post-administration. CS-Lips were primarily found adhered to the tissue, whereas Lips with PVA (a comb-shaped hydrophilic polymer) formed on the surface (PVA with a hydrophobic anchor [PVA-R]-coated Lips; PVA-R-Lips) were detected in abundance in the BALF. PVA-R-Lips loading a model peptide drug were associated with a longer-lasting pharmacological effect of the peptide than were CS-Lips, which led to the conclusion that prolonged drug retention in alveoli was more effective than delivery to the pulmonary tissue as a function of the DDS.[21]

Regarding PVA-R-Lips, we have demonstrated in experimental animals that intravenously administered PVA-R-Lips are well retained in the circulation, like pegylated Lips, and hence show higher levels of delivery to the target tumor tissue. These aspects are

evaluable by blood sampling and isolation and quantification of the DDS. To assess long-term retention in the tissue, a less invasive methodology is more appropriate. The IVIS Imaging System is an effective instrument for such assessment for DDS microparticles. This system was used to monitor PVA-R-Lip retention time in the lung, using indocyanine green as a suitable marker, versus surface-unmodified Lips; although results varied between animals monitored, the retention time was significantly greater for PVA-R-Lips than that for unmodified Lips.[22] The PVA layer over the PVA-R-Lip surface is deemed to effectively decrease the uptake by alveolar macrophages, as observed in the liver, where intravenously administered PVA-R-Lips reduced the elimination by hepatic macrophages.

### Delivery of Lip eye drops to the posterior eye segment

Eye drops are the most common dosage form for ophthalmic pharmacotherapy. They are formulations that are primarily used on the anterior part of the eye, such as the conjunctiva. Situated in the posterior part is the retina, which is a crucial tissue for eyesight. No eye drops are currently available for retinal damage. Recently in Japan, as super-graying of society progresses, incidence rates of serious posterior eye diseases such as diabetic retinopathy and age-related macular degeneration are on the rise. These diseases are particularly problematic in that loss of vision may result if they are left untreated. Neovascularization secondary to edema that develops in the vicinity of the macula is a symptom that requires treatment. Fortunately, potent medication is now available to inhibit this occurrence at high rates. However, its dosage form is currently limited to invasive intravitreal injection. Medication in the form of an ophthalmic solution, if made available, would greatly contribute to advances in pharmacotherapy.

We assessed intraocular behaviors of Lips in mice given Lip suspension containing a fluorescent marker (coumarin-6) by observing their retinae for Lip delivery characteristics. Nano-sized Lip particles were found to reach the posterior segment of the eye (Figure. 4). Factors affecting the delivery efficiency were shown to include particle size, that is, the smaller the particles, the higher the delivery efficiency, and Lip particle rigidity.[23] Since there were no established methods to quantify nano-sized particle rigidity, we proposed to express it numerically using the ratio (Rd) calculated from the mean particle size (d50) determined with dynamic light scattering and the particle size (perpendicular height) obtained with atomic force microscopy. As expected, these two values were almost the same for highly rigid polymer particles like polystyrene, yielding Rd of nearly 1.0. Lip particles exhibited rigidity that reflected the value roughly predictable from the type of component phospholipid.[24] The effects of use of Lips in the form of an ophthalmic solution were assessed in model animals treated with either antioxidant- or anti-inflammatory drug-loaded Lips. They were shown to reduce light-induced retinal damage[25] and choroidal neovascularization,[26] among others.

## Closing remarks

In preparing a patient-centric formulation, the design of its constituent particles carries a solid weight, as shown above. Variable particles are available, ranging from powder particles of several tens of micrometers in size for solid formulations to submicron-sized Lip particles for the DDS. Whatever particles are selected, the target formulation design can only be achieved when the preparation of the selected particles and assessment thereof both go well. Each industry normally has its own product assessment methods. It would be crucial for researchers to identify the essence of assessment required and seek cooperation of professionals in other fields across industries where necessary. Promotion of information exchange in that regard is one of the objectives of the Division of Particulate Design and Preparations of the Society of Powder Technology, Japan, where I have long belonged for research activity purposes. It is my wish to continue with the development of formulations that contribute to PCT through promotion of interdisciplinary matching.

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

## References


[1] Takeuchi H. Patient centric solid formulation design. In: Strategy and Novel Technology on Pharmaceutical Preparations [in Japanese]. Takeuchi H. ed. CMC Publishing Co., Ltd. (2007) 3–7.

[2] Seager H., Drug-delivery products and the Zydis fast-dissolving dosage form., J. Pharm. Pharmacol., 50 (1998) 375–382

[3] Takeuchi H., Fuji Sylysia Chemical Ltd., Jpn. Patent No. 5026675 (2012)

[4] Tanimura S, Tahara K, Takeuchi H. Spray-dried composite particles of erythritol and porous silica for orally disintegrating tablets prepared by direct tableting. Powder Technol. 286 (2015) 444–450.

[5] Tanimura S, Kawazoe H, Yamamoto H, Takeuchi H. Direct compaction of poorly compactable pharmaceutical powders with spray-dried HPC-L [in Japanese]. Powder Technol. 43 (2006) 648–652.

[6] Hayashi T, Takeuchi Y, Tahara K, Uchida M, Takeuchi H. Studies on the control mechanism and on disintegrating property of lactose tablet with sucrose fatty acid esters as a lubricant [in Japanese]. Powder Technol. 49 (2012) 750–757.

[7] Wening, K., J. Breitkreutz J., Oral drug delivery in personalizedmedicine: Unmet needs and novel approaches. Int. J. Pharm. 404 (2011) 1–9.

[8] Niese, S. Quodbach J., Development of a dosing device for individualized dosing of orodispersible warfarin films. Int. J. Pharm., 561(2019) 314–323

[9] Visser J. C., Woerdenbag H. J. Crediet S., Gerrits E., Lesschen M. A., Hinrichs W. L.J., Breitkreutz J., Frijlink H. W., Orodispersible films in individualized pharmacotherapy: The development of a formulation for pharmacy preparations. Int. J. Pharm., 478 (2015) 155–163.

[10] Matsumoto M., Arai K., Fujita A. Tsuji M., Hayakawa S., Sawakami K., Sawakami K, Shida S., Nohara Y., Sumiyaa K., Murata K., Takeuchi Y., Takeuchi H., Murata R., Characterization of Orally Disintegrating Films Containing Fluconazole, Journal of Drug Interaction Research, 43 (2019) 55–59.

[11] Takeuchi H., Yamakawa R., Nishimatsu T., Takeuchi Y., Hayakawa K., Maruyama N., J. Design of rapidly disintegrating drug delivery films for oral doses with hydroxypropyl methylcellulose. Drug Deliv. Sci. Technol., 23 (2013) 471–475.

[12] Takeuchi Y., Umemura K., Tahara K., Takeuchi H., Formulation design of hydroxypropyl cellulose films for use as orally disintegrating dosage forms. J. Drug Deliv. Sci. Technol., 46 (2018) 93–100

[13] Takeuchi Y., Nishimatsu T., Tahara K., Takeuchi H., Novel use of insoluble particles as disintegration enhancers for orally disintegrating films. J. Drug. Delivery Sci. Technol., 54, (2019) 101310.

[14] Takeuchi Y, Usui R, Ikezaki H, Tahara K, Takeuchi H. An advanced technique using an electronic taste-sensing system to evaluate the bitterness of orally disintegrating films and the evaluation of model films. Int J Pharm., 531 (2017) 179–190.

[15] Takeuchi Y., Kawamoto M, Tahara K, Takeuchi H. Design of a new disintegration test system for the evaluation of orally disintegrating films. Int J Pharm., 553 (2018) 281–289

[16] Takeuchi H, Yamamoto H, Niwa T, Hino T, Kawashima Y. Mucoadhesion of Polymer-Coated Liposomes to Rat Intestine in Vitro. Chem. Pharm. Bull., 42 (1994) 1954–1956.

[17] Takeuchi H., Yamamoto H., Niwa T., Hino T., Kawashima Y., Enteral Absorption of Insulin in Rats from Mucoadhesive Chitosan-Coated Liposomes. Pharm. Res., 13, (1996) 896–901

[18] Takeuchi H, Yamamoto H, Kawashima Y. Mucoadhesive nanoparticulate systems for peptide drug delivery. Adv. Drug Del. Rev., 47 (2001) 39–54.

[19] Takeuchi H, Matsui Y, Sugihara H, Yamamoto H, Kawashima Y. Effectiveness of submicron-sized, chitosan-coated liposomes in oral administration of peptide drugs. Int. J, Pharm., 303 (2005) 160–170.

[20] Makhlof A, Werle M, Tozuka Y, Takeuchi H. A mucoadhesive nanoparticulate system for the simultaneous delivery of macromolecules and permeation enhancers to the intestinal mucosa. J. Cont. Rel., 149 (2011) 81–88

[21] Murata M, Nakano K, Tahara K, Tozuka Y, Takeuchi H. Pulmonary delivery of elcatonin using surface-modified liposomes to improve systemic absorption: Polyvinyl alcohol with a hydrophobic anchor and chitosan oligosaccharide as effective surface modifiers. Eur. J. Pharm.Biopharm., 80 (2012) 340–346.

[22] Murata M, Tahara K, Takeuchi H. Real-time in vivo imaging of surface-modified liposomes to evaluate their behavior after pulmonary administration. Eur J Pharm Biopharm. 86 (2014)115–119.

[23] Hironaka K, Inokuchi Y, Tozuka Y, Shimazawa M, Hara H, Takeuchi H. Design and evaluation of a liposomal delivery system targeting the posterior segment of the eye. J Contr. Rel., 136 (2009) 247–253.

[24] Nakano K, Tozuka Y, Yamamoto H, Kawashima Y, Takeuchi H. A novel method for measuring rigidity of submicronsize liposomes with atomic force microscopy. Int. J. Pharm., 355 (2008) 203–209.

[25] Hironaka K, Inokuchi Y, Fujisawa T, Shimazaki H, Akane M, Tozuka Y, Tsuruma K, Shimazawa M, Hara H, Takeuchi H. Edaravone-loaded liposomes for retinal protection against oxidative stress-induced retinal damage. Eur. J. Pharm. Biopharm., 79 (2011) 119–125.

[26] Shimazawa M, Inoue Y, Masuda T, Onodera R, Tahara K, Shimizu Y, Mibe Y, Tsuruma K, Takeuchi H, Hara H. Topical diclofenac-loaded liposomes ameliorate laser-induced choroidal neovascularization in mice and non-human primates. Curr. Neurovasc Res., 14 (2017) 46–52.

# Translational Raman Spectroscopic Approaches for Clinical Routine

Prof. Dr. Jürgen POPP

Leibniz Institute of Photonic Technology,
Member of the Leibniz Research Alliance - Leibniz Health Technologies,
Albert-Einstein-Str. 9, D-07745 Jena, Germany

Institute of Physical Chemistry & Abbe Center of Photonics
Friedrich-Schiller University Jena,
Helmholtzweg 4, D-07743 Jena, Germany

The article reviews our latest results on innovative technological and data analysis concepts for bringing Raman spectroscopy closer to clinical use in terms of diagnosing and targeted therapy of infectious diseases and cancer. The first part will report on clinical Raman solutions for rapid diagnosis of infectious diseases - in terms of rapidly identifying the infection causing pathogen its antibiotic resistance pattern and ideally also its immune response - being decisive parameters for a targeted antibiotic administration, which is crucial for the survival of patients. The second part will focus on nonlinear multimodal Raman imaging for a fast and safe precise intraoperative tumor margin control, because reliable tumor margin recognition during an intervention is the key to effective tumor treatment.

Keywords
Raman spectroscopy, Artificial intelligence, Chemometrics, Microbial analysis, Infectious diseases, Cancer, Spectral histopathology

## Introduction

Understanding the causes of diseases, recognizing them earlier and treating them more specifically - hopes that are associated with modern biomedicine - requires the determination of diagnostic, prognostic and predictive factors including their comprehensive evaluation in just a few steps or ideally in a single step. In this context, the sharp rise in cancer due to an ageing society and the rapid spread of life-threatening infectious diseases (due to in part unknown pathogens) and antibiotic-resistant germs, which is partly due to an increasing worldwide mobility but also to the ill-considered administration of broad-spectrum antibiotics, should be mentioned in particular. An effective and early diagnosis and personalized therapy of cancer and infections requires new methods of differential diagnosis and represents an outstanding task of medicine. In principle, the following applies to all diseases: the earlier treatment begins, the better the chances of cure. There is therefore a great need for new diagnostic methods for a targeted early diagnosis of diseases in order to be able to use a targeted therapy as early as possible.

Raman spectroscopy plays a key role in the implementation

of these ambitious goals. The application of Raman methods to address biomedical research has grown rapidly over the past ten years and has advanced into a new era due to advances in instrumentation and most importantly due to an enhanced cross-disciplinary dialogue between spectroscopists and clinicians, which e.g. is fostered in Europe by the COST action Raman4clinics (https://www.raman4clinics.eu/).[1, 2]

This contribution will highlight our recent advances in translating Raman approaches towards routine clinical applications with focus on infectious diseases and cancer. Addressing this question requires new Raman instrumentation, which can be applied out of specialized labs in a clinical environment (e.g. operation theatre, bedside or in a doctor's practice).

## Point-of-care Raman spectroscopy for a rapid infectious disease diagnosis and treatment

Let's assume the following situation: a patient is at the doctor's office and after a short anamnesis the doctor decides that it is a bacterial infection and prescribes a certain antibiotic. Now one can ask the question, how does the doctor know that it is a bacterial infection, or in case is a bacterial infection, that the causing germs are not resistant to the prescribed antibiotic. While this approach was appropriate a few years ago, nowadays in the course of increasing antibiotic resistance, it would be very beneficial, that prior to prescribing an antibiotic or before taking an antibiotic, a precise diagnosis in terms of rapidly identifying the pathogen and characterizing its resistance profile takes place on which then a targeted therapy follows. In the following the great potential of optical technologies with focus on Raman spectroscopy to successfully address these unmet needs will be shown. At the same time, the hurdles that have to be overcome in order to move from an academic scientific process to a validated and verified product will be outlined.

First the question, what exactly is needed for an optimal and personalized treatment of infectious diseases will be addressed. Here, it is important to know whether the symptoms a patient - the so-called host response - is exhibiting are symptoms of an infectious disease or of another inflammatory disease. In case it is an infection, the next step is to find out what kind of pathogen is causing the infection, i.e. are the infection causing pathogens viruses, bacteria, or fungi? This knowledge is crucial for initiating an effective therapy. In the case of a bacterial infection, it is also necessary to know whether the bacterial pathogen is resistant to certain antibiotics or not.

### The host response:

In order to find out if the patient suffers from an infection or not it is important to consider the role of immune cells during an infection. When infectious agents enter the body, they interact with immune cells, i.e. among other with white blood cells including neutrophils, monocytes, eosinophils, T cells, B cells or the basophils. The interaction of pathogenic microorganisms, i.e. viruses, bacteria or fungi, with these immune cells leads to a certain cell response, causing molecular changes within the immune cells. Raman spectroscopy offers a very simple approach (requiring only a laser, a microscope, some filters and a spectrometer together with a sensitive camera) to monitor these molecular changes via the Raman spectral fingerprint. Raman spectroscopy requires very simple sample preparation steps. The isolation of white blood cells from whole blood can be achieved very easily via lysis of the red blood cells. After isolation, the white blood cells are placed under the microscope and irradiated with the Raman excitation laser. A notch filter separates the elastically scattered from the inelastically scattered Raman light, which is spectrally dispersed by a monochromator and detected by a camera (e.g. CCD camera). This simple analysis process, which has been established within the EU project Hemospec (https://cordis.europa.eu/project/id/611682), requires just 60 minutes from sampling to the final result. However, in order to achieve this white blood cell analysis two challenges had to be overcome: (1) since the Raman spectra of the different cell types or the activation of the different cells differ only minimally, a simple spectral analysis by eye is not possible and artificial intelligence methods are required to analyze the cellular Raman spectra (see below). (2) a large number of cells need to be examined and commercially available Raman setups are not able to measure several 1000 cells in a very short time. Therefore, a high-throughput Raman (HTR) setup has been developed within Hemospec and put to use in the clinic.[3, 4]

The investigation of the host response started with *in vitro* experiments, where isolated white blood cells here neutrophils from whole blood were spiked with various infectious agents such as bacteria and fungi. The individual cellular Raman signatures were analyzed by a PC-LDA analysis subsequent to a sophisticated data pretreatment.[5] In detail, the Raman spectral data of infected neutrophils have been compared with those of uninfected neutrophils and with an accuracy of 90% the infected could be distinguished from the non-infected neutrophils.[5] The examination of the infected neutrophils revealed that it is possible to distinguish a fungal infection from a bacterial infection with an accuracy of 92%. Finally, neutrophils associated with Gram-positive bacteria such as *Staphylococcus aureus* could be separated from

Gram-negative *E. coli* with an accuracy of 84%, i.e. Raman spectroscopy also allows to distinguish Gram-positive bacteria from Gram-negative bacteria. This *in-vitro* microbial infection diagnostics study[5] (see Figure 1) demonstrates that pathogen-specific activation can be detected with Raman data and opens up new possibilities for clinical diagnostic applications in terms of timely personalized therapy. For this, however, it is necessary to transfer the concept of the *in-vitro* stimulated cells shown in Reference [5] to patient samples.

In a recently published clinical study[4] we were able to show that it is possible to distinguish between inflammation, infection and sepsis by analyzing the Raman spectroscopic fingerprints of leukocytes from the blood of hospitalised patients from an emergency department. Using the aforementioned HTR approach[3], we were able to clearly distinguish patients with viral infection from patients with bacterial infection. This clinical study paves the way for translating this Raman spectroscopic host response approach as a first steps towards a targeted therapy, as it allows to quickly choose the appropriate therapeutic approach based on the host response.[5]

## Pathogen diagnostics and antibiotic resistance:

The currently approved microbiological methods to identify the infection-causing pathogens require the cultivation of bacteria and are therefore time-consuming, which means that a targeted antibiotic therapy is usually performed too late. Therefore, broad-spectrum antibiotics are often used empirically before the appropriate antibiotics matched to the infection-causing pathogen are found. However, this approach leads to more and more pathogens becoming resistant to antibiotics. To treat an infection successfully, the physician needs the information about the type of the pathogen and its resistance to antibiotics - as quickly as possible. As mentioned above, standard microbiological methods usually require more than 24 hours due to cultivation, which is often too late.

To overcome this unmet medical need we have developed the so-called RAMANBIOASSAY™ - an approach that allows the identification of the pathogens as well as the determination of the minimum inhibitory concentration (MIC) in a very short time. The unique feature of this RAMANBIOASSAY™ approach is the dramatically reduced diagnostic time: in only 2 to 3.5 hours and with a very small number of microbial pathogens (a few hundred bacteria are sufficient), the pathogens can be clearly identified and their resistance properties can be determined without a prior cultivation step. Based on this information a physician can adapt the therapeutic antibiotic treatment specifically to the pathogen in question.

The basis of the RAMANBIOASSAY™ approach is the combination of chip-based Raman microspectroscopy with classical imaging. The smart use of laser light in a Raman setup in combination with a light microscope enables a label-free, non-destructive and culture-independent optical and spectroscopic characterization and identification of bacteria down to the level of single cells. Each molecule generates an individual signature in the Raman spectrum, creating a specific molecular fingerprint for each bacterium. Statistical evaluation algorithms enable automated classification and identification of bacteria and resistance to antibiotics. Furthermore, the additional integration of chip-based enrichment methods into miniaturized structures covers the entire process chain

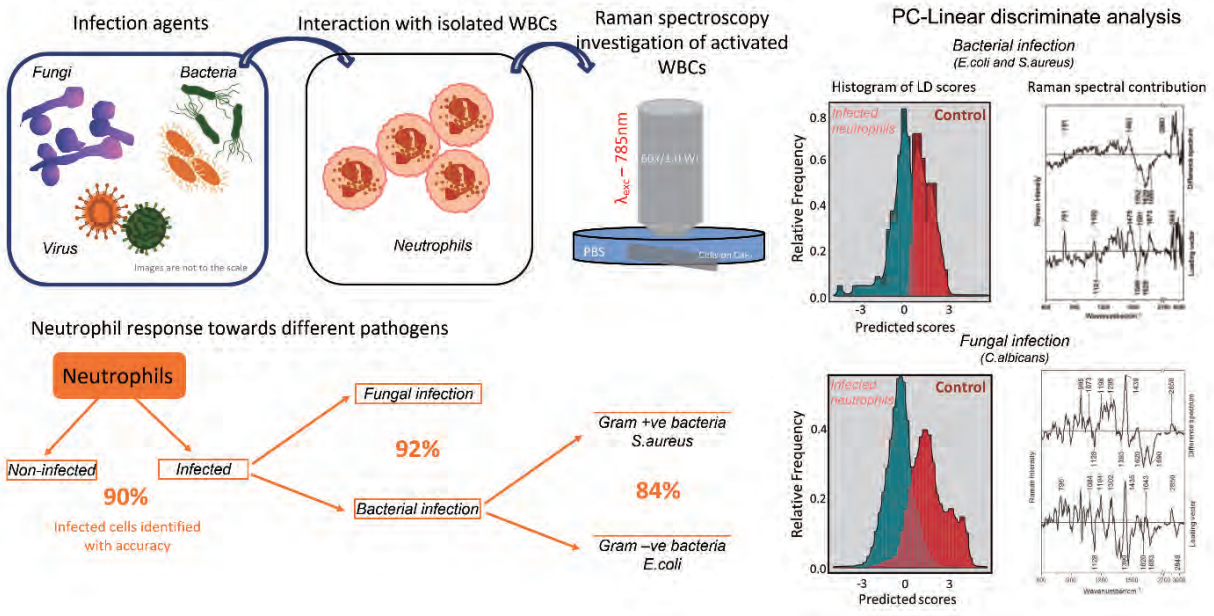



Figure 1 Summary of *in-vitro* Raman study to unravel the host response.[5] For details see text.

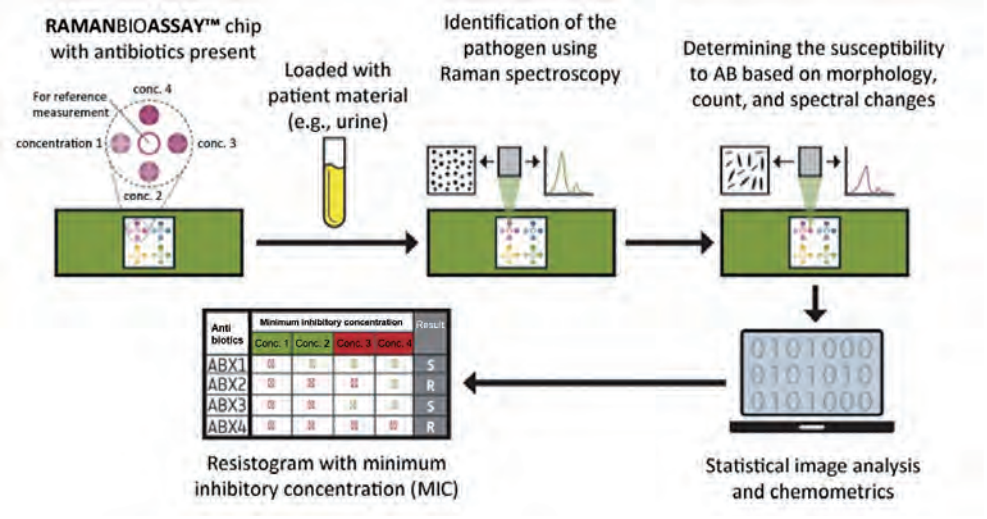



Figure 2 Schematic of the individual steps of the RAMANBIOASSAYTM: The core is a chip onto which the bacteria from a patient sample are applied. Up to four different antibiotics are already present in four concentrations each. This means that the chip is ready for immediate use.

from sampling to final results (see Figure 2).

The Raman spectroscopic identification of the pathogen can be completed after 35 minutes. At the same time, the morphological changes of the pathogens in a short-term culture (on the RAMANBIOASSAY™ chip) together with the Raman spectra are used to determine to which antibiotic agent resistance exists and what the MIC is. This information is extracted from the morphological images and the Raman spectra using computer-assisted statistical image data analysis and chemometrics. By doing so the complete resistogram is thus already available after 2 to 3.5 hours and the physician can react much earlier than currently and does not have to resort to the application of a broad-spectrum antibiotics.

The potential of the RAMANBIOASSAY™ approach was demonstrated using the example of urinary tract infections with an identification of the pathogens, their resistance to antibiotics and their MIC from patient material.[6-11]

The uniqueness of the RAMANBIOASSAY™ approach lies in its independence from culture and the scalability of the approach. The independence from culture allows the analysis time to be reduced to 2 to approximately 3.5 hours after sample collection. The scalability enables universal use on the one hand in clinical microbiology in combination with pipetting robot compatible Raman chip platforms, which allows a high degree of automation in the clinical microbiology workflow, and on the other hand in the form of a closed cartridge-based approach with a miniaturized Raman reader for direct use in a physician's office or in a hospital without its own microbiology unit. Figure 3 shows the current status using the RAMANBIOASSAY™ chip together with the BioParticle Explorer (a manual Raman spectroscopy system together with a microscope for morphological analysis) as well as future developments of the RAMANBIOASSAY™ for clinical microbiology or use in a physician's office.

Equally important as the development of clinically usable Raman devices (see Figure 3) is the development of tailored Raman spectroscopic analysis routines. As mentioned above the analysis of bacterial Raman spectra or changes within induced by e.g. antibiotics treatment cannot be analyzed by naked eye and require sophisticated artificial intelligence based spectral analysis routines.

In general, the success of Raman spectroscopy for medical diagnosis and therapy (and also other applications like e.g. in life sciences, process analytics, pharmacy or environmental analysis) is inherently connected with the development of customized Raman data evaluation algorithms for translating Raman measurement data (spectral data sets, image data, etc.) into qualitatively and quantitatively usable information for end users. In this context we have developed a universally applicable Raman data analysis software called RAMANMETRIX

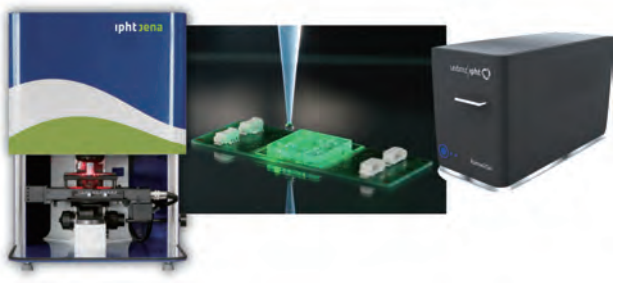


Figure 3 Photograph of RAMANBIOASSAY™ chip (middle) together with the BioParticle Explorer(left) and future developments towards a cartridge-based system with miniaturized reader for doctor's office and clinics without clinical microbiology (right).

(see: https://docs.ramanmetrix.eu/). This software allows for a one-click data analysis of Raman spectroscopic data in a robust and reliable way.

## Non-linear spectroscopic multimodal instrumentation for intraoperative tumor identification

The diagnostic gold standard in most surgeries is the extraction of biopsies and their histopathological examination to confirm the tumor and the tumor borders. The histopathological examination is carried out by means of rapid sections of non-contrasted tissue sections during the operation and definitively also on tissue sections of fixed material. Depending on the number of removed rapid sections, the rapid section diagnostic takes about 20-30 minutes. Then the surgeon is typically informed by telephone about the result and can decide on his/her further approach. If, for example, a sample in the border region of the tumor still shows tumor tissue, the surgeon will resection here and, if necessary, provide a new border section for rapid section diagnostics until the rapid sections indicate complete tumor resection.

This procedure is time-consuming, investigator-dependent, and dependent on the size, number and quality of the removed tissue samples, which are, of course, limited. Furthermore, since the quality of intraoperatively prepared frozen sections is not comparable to that of embedded tissue, the results of frozen section diagnostics often differs from those of an examination on embedded sections, which is why subsequent diagnostic confirmation using embedded sections is still necessary. For example, in head and neck surgery, depending on tumor size and location, the incidence of residual tumor (R1), i.e., subsequent finding of tumor cells in the incision margin, is approximately 7.5 - 10%. For such cases, the patient has to undergo a new operation and often also postoperative radiotherapy, which is an enormous burden for the patient. Therefore, even within the primary surgery, where possible, more tissue is removed than would actually be necessary to reach the tumor limit. However, the more tissue that is removed, the greater the impact on the patient's healing process. Thus, new methods and approaches are urgently needed for a fast and reliable intraoperative diagnosis.

Over the last few years, we have been investigating a multimodal nonlinear imaging approach that has the potential to reliably assess tissue and the success of surgery or endoscopy directly in the operating theatre or endoscopy room. The approach combines three different nonlinear imaging techniques namely two-photon excited autofluorescence (TPEF), second harmonic generation (SHG) and coherent anti-Stokes Raman scattering (CARS) displaying vibrations in the CH-stretch wavenumber region. The combination of these three modalities allows to determine the morphological and chemical composition (morphochemistry) of unfixed tissue sections in a label-free manner.[12] In order to translate the morphochemical information encoded in the multimodal spectroscopic images into medical relevant information photonic data science, i.e. machine and deep learning approaches are necessary.[13-16]

In the following we will briefly highlight the potential of multimodal nonlinear imaging in combination with innovative image analysis routines as a powerful tool for computer based spectral histopathology allowing for an automatic prediction of tissue types / disease and thus offering great potential to fulfill the aforementioned unmet medical needs in terms of reducing the time in an operative theatre due to instant feedback and smaller workload due to automatization.

In a recent study we investigated head and neck squamous cell carcinomas using the multimodal combination of CARS, TPEF and SHG. The analysis of the images by a machine learning classification model features a 90% accuracy compared to gold standard diagnosis of a blinded pathologist.[14, 15, 17, 18]

Furthermore, we could show that the utilization of deep learning approaches also allows for a pseudo-staining of multimodal images. The deep learning generated pseudo H&E images nicely represent the real H&E images and show that the combination of the three label-free non-linear imaging modalities CARS, TPEF and SHG yields information that can be translated into computational pseudo hematoxylin and eosin (HE) images.[13,16] For a clinical application compact and easy to use devices are needed. Thus, we have transferred the presented CARS/SHG/TPEF approach into a compact microscope suitable for clinical use in terms of a rapid *ex-vivo* tissue analysis.[12]

In order to further extend the applicability of this multimodal microscopy approach for *in vivo* tissue screening, various endoscopic probe concepts were also realized.[19-23] The core of all these setups are robust and alignment-free fiber laser concepts. In the following two concepts of endoscopic probes for multimodal nonlinear imaging, which have been researched or are still currently under investigation will be briefly summarized. The first is a rigid needle endoscope for neurosurgical applications.[21] This probe provides very good imaging in a compact design, there are no moving or electric parts in the probe head, but it is not flexible (which is fine for neurosurgery and some other fields of application, e.g., head and neck).

For applications requiring flexible endoscopes, a unique endoscopy concept using a double-core double-clad fiber and focus-combining micro-optical concept allowing for a background free, low-loss, high peak power laser delivery, and an efficient signal collection in backward direction has been successfully realized.[23]

## Conclusion

In conclusion this short review highlights the great potential of Raman spectroscopy in combination with innovative photonic data science concepts for clinical diagnosis and therapy.

In the first part of this contribution, we report on the culture-free isolation and identification of pathogens, their host-response and their antibiotic resistance by using a combination of Raman spectroscopy, chip-based sampling strategies as well as chemometric spectroscopic data analysis methods. The main advantages of this rather simple approach compared to conventional microbiological analysis methods is that the analysis result is available in just a few hours. This represents a major step forward, as antibiotic therapy can be started promptly and specifically tailored to the pathogen. It will be shown how this approach was transferred into an automated clinically applicable system the RAMANBIOASSAY™

The second part of this presentation reports on multimodal non-linear imaging solutions in the field of pathological cancer diagnostics. Here, the combination of CARS with SHG and TPEF imaging in combination with adapted image analysis methods represents a powerful *ex-vivo* and *in-vivo* approach for a labelfree clinical intraoperative tissue diagnostic for tumor margin detection in terms of computer based spectral histopathology.

## *Acknowledgement*

*Financial support of the EU, the "Thüringer Ministerium für Wirtschaft, Wissenschaft und Digitale Gesellschaft", the "Thüringer Aufbaubank", the Federal Ministry of Education and Research, Germany (BMBF), the German Science Foundation, the Fonds der Chemischen Industrie and the Carl-Zeiss Foundation are greatly acknowledged.*

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

## References


[1] C. Krafft, I. W. Schie, T. Meyer, M. Schmitt, and J. Popp, "Developments in spontaneous and coherent Raman scattering microscopic imaging for biomedical applications", Chem. Soc. Rev. 2016, 45, 1819-1849 and references cite therein.

[2] C. Krafft, M. Schmitt, I. Schie, D. Cialla-May, C. Matthäus, T. Bocklitz, and J. Popp, "Label-free molecular imaging of biological cells and tissues by linear and non-linear Raman spectroscopic approaches", Angew. Chem. Int. Ed. 2017, 56, 4392-4430 and references cite therein.

[3] A. S. Mondol, N. Töpfer, J. Rüger, U. Neugebauer, J. Popp and I. W. Schie, "New perspectives for viability studies with high-content analysis Raman spectroscopy (HCA-RS)", Sci. Rep. 2019, 9, 12653.

[4] A. Ramoji, D. Thomas-Rüddel, O. Ryabchykov, M. Bauer, N. Arend, E. J Giamarellos-Bourboulis, J. Eugen-Olsen, M. Kiehntopf, T. Bocklitz, J. Popp, F. Bloos, U. Neugebauer, "Leukocyte Activation Profile Assessed by Raman Spectroscopy Helps Diagnosing Infection and Sepsis", Crit. Care Explor. 2021, 3(5), e0394.

[5] N. Arend, A. Pittner, A. Ramoji, A. S. Mondol, M. Dahms, J. Rüger, O. Kurzai, I. W. Schie, M. Bauer, J. Popp and U. Neugebauer, "Detection and Differentiation of Bacterial and Fungal Infection of Neutrophils from Peripheral Blood Using Raman Spectroscopy", Anal. Chem. 2020, 92, 10560-10568.

[6] U.-C. Schröder, F. Bokeloh, U. Glaser, J. Popp, U. Neugebauer, M. O'Sullivan, J. Ducrée, K. Wolf and W. Pfister, "Rapid, culture-independent, optical diagnostics of centrifugally captured bacteria from urine samples", Biomicrofluidics 2015, 9, 044118.

[7] U. C. Schröder, C. Beleites, C. Assmann, U. Glaser, U. Hubner, W. Pfister, W. Fritzsche, J. Popp and U. Neugebauer, "Detection of vancomycin resistances in enterococci within 3 (1/2) hours", Sci. Rep. 2015, 5, 8217.

[8] U.-C. Schröder, J. Kirchhoff, U. Hübner, G. Mayer, U. Glaser, T. Henkel, W. Pfister, W. Fritzsche, J. Popp and U. Neugebauer, "On-Chip spectroscopic assessment of microbial susceptibility to antibiotics within 3.5 hours", J. Biophotonics 2017, 10, 1547-1557.

[9] J. Kirchhoff, U. Glaser, J. A. Bohnert, M. W. Pletz, J. Popp and U. Neugebauer, "Simple ciprofloxacin resistance test and determination of minimal inhibitory concentration (MIC) within two hours using Raman spectroscopy", Anal. Chem. 2018, 90, 1811-1818.

[10] S. Kloß, B. Kampe, S. Sachse, P. Rösch, E. Straube, W. Pfister, M. Kiehntopf and J. Popp, "Culture independent Raman spectroscopic identification of urinary tract infection pathogens - A proof of principle study", Anal. Chem. 2013, 85, 697-708.

[11] S. Kloß, B. Lorenz, P. Rösch, S. Dees, I. Labugger and J. Popp, "A Raman spectroscopy- compatible isolation procedure of single bacteria cells from sputum samples", Anal. Bioanal. Chem., 2015, 407, 8333-8341.

[12] T. Meyer, M. Baumgartl, T. Gottschall, T. Pascher, A. Wuttig, C. Matthaeus, B. F. M. Romeike, B. R. Brehm, J. Limpert, A. Tuennermann, O. Guntinas-Lichius, B. Dietzek, M. Schmitt and J. Popp, "A compact microscope setup for multimodal nonlinear imaging in clinics and its application to disease diagnostics", Analyst 2013, 138, 4048-4057.

[13] T. Bocklitz, F.S. Salah, N. Vogler, S. Heuke, O. Chernavskaia, C. Schmidt, M. Waldner, F.R. Greten, R. Bräuer, M. Schmitt, A. Stallmach I. Petersen, J. Popp, "Pseudo-HE images derived from CARS/TPEF/SHG multimodal imaging in combination with Raman-spectroscopy as a pathological screening tool", BMC Cancer, 2016, 16, 534/1-1.

[14] T. Meyer, D. Akimov, O. Guntinas-Lichius, M. Schmitt, B. Dietzek, F. v. Eggeling and J. Popp, "Multimodal nonlinear microscopic investigations on head and neck squamous cell carcinoma: Toward intraoperative imaging", Head Neck 2012, 35, E280-287.

[15] E. Rodner, T. Bocklitz, F. von Eggeling, G. Ernst, O. Chernavskaia, J. Popp, J. Denzler and O. Guntinas-Lichius, "Fully convolutional networks in multimodal nonlinear microscopy images for automated detection of head and neck carcinoma: Pilot study", Head Neck 2019, 41, 116-121.

[16] P. Pradhan, T. Meyer, M. Vieth, A. Stallmach, M. Waldner, M. Schmitt, J. Popp, T. Bocklitz, „Semantic Segmentation of Non-linear Multimodal Images for Disease Grading of Inflammatory Bowel Disease: A SegNet-based Application", ICPRAM 2019: 396-405; DOI:10.5220/0007314003960405.

[17] T. Meyer, D. Akimov, O. Guntinas-Lichius, M. Schmitt, B. Dietzek, F. v. Eggeling and J. Popp, "Multimodal nonlinear microscopic investigations on head and neck squamous cell carcinoma: Toward intraoperative imaging", Head Neck 2012, 35, E280-287.

[18] S. Heuke, O. Chernavskaia, T. Bocklitz, F. B. Legesse, T. Meyer, D. Akimov, O. Dirsch, G. Ernst, F. von Eggeling, I. Petersen, O. Guntinas-Lichius, M. Schmitt and J. Popp, "Multimodal nonlinear microscopy of head and neck carcinoma - toward surgery assisting frozen section analysis", Head Neck 2016, 38, 1545-1552.

[19] A. Lukic, S. Dochow, O. Chernavskaia, I. Latka, C. Matthäus, A. Schwuchow, M. Schmitt and J. Popp, "Fiber probe for nonlinear imaging applications", J. Biophotonics., 2016, 9, 138-143.

[20] A. Lukic, S. Dochow, H. Bae, G. Matz, I. Latka, B. Messerschmidt, M. Schmitt and J. Popp, "Endoscopic fiber probe for nonlinear spectroscopic imaging", Optica 2017, 4, 496-501.

[21] P. Zirak, G. Matz, B. Messerschmidt, T. Meyer, M. Schmitt, J. Popp, O. Uckermann, R. Galli, M. Kirsch, M. J. Winterhalder and A. Zumbusch, "Invited Article: A rigid coherent anti-Stokes Raman scattering endoscope with high resolution and a large field of view", Apl. Photonics 2018, 3, 092409.

[22] J. Tragardh, T. Pikalek, M. Sery, T. Meyer, J. Popp and T. Cizmar, "Label-free CARS microscopy through a multimode fiber endoscope", Opt. Express 2019, 27, 30055-30066.

[23] B. Messerschmidt, E. Pshenay-Severin, G. Matz, K. Reichwald, J. Bierlich, J. Kobelke, H. Bae, T. Meyer-Zedler, M. Schmitt, J. Popp, „CARS endomicroscopic imaging probe enabled by a double-core double-clad fiber and related focus-combining micro-optical concept", Proceedings Volume 11636, Optical Biopsy XIX: Toward Real-Time Spectroscopic Imaging and Diagnosis; 116360A (2021) https://doi.org/10.1117/12.2578538.

# Improving Speed and Safety of Biopharmaceutical Manufacturing with HORIBA Analytical Solutions

## Innovative spectroscopic (A-TEEM and Raman) and optical techniques (ViewSizer) offer faster, accurate and less costly alternatives to chromatographic quantitative analysis in biotechnology

Linda Kidder YARLOTT
Adam M. GILMORE
Sean TRAVERS

The transition of biotechnologies from research project to pharmaceutical products requires analytical solutions that ensure safety and efficacy. In this paper we describe several innovative HORIBA products and highlight how these novel approaches rapidly deliver key information to accelerate processes in biopharmaceutical manufacturing. The HORIBA product portfolio can transform a variety of biopharmaceutical QC processes including the rapid characterization of cell culture media and viral vectors, vaccine manufacturing and analysis of components needed for cell and gene therapies.

Key words
Fluorescence Spectroscopy
EEM Fluorescence Spectroscopy
A-TEEM Fluorescence Spectroscopy

## Introduction

The publication of the human genome in February of 2001 was a tremendous achievement, and is just one of the multiple pieces of fundamental knowledge that has fueled the rapid pace of discovery and innovation in biotechnology. The tangible benefits to human health of this and other advances, like CRISPR-Cas9 gene editing, are just now being felt in the mainstream, as there is natural delay between discovery and commercialization. In many instances this transition to commercialization of transformative innovations is slowed by the lack of complementary analytical technologies to confirm safety and efficacy. The innovation of novel analytical tools (or repurposing of existing ones) to enable biopharmaceutical manufacturing may not receive the same recognition as the initial discoveries, but commercialization absolutely depends on it. In recognition of the need for innovations in analytical tools, governments around the world have created public-private partnerships to solve this problem.

NIIMBL (National Institute for Innovation in Manufacturing

Biopharmaceuticals)[1] in the US, for example, is focused on the development of "novel real-time analytical technologies for integrated continuous processing for process integration and intensification." Their goals are to enable "flexible and adaptive manufacturing, de-risk innovation, lower manufacturing costs and accelerate development and approval." NIIMBL want to speed commercialization of novel biopharmaceutical product categories, such as Cell and Gene Therapy, and also want to streamline processes for existing products that lack efficient analytical tools such as: monoclonal antibodies (mAbs), vaccines, antibody-drug conjugates (ADCs), bispecifics, and virus-like particles (VLPs). Another government funded consortia is the Cell and Gene Therapy Catapult (CGT Catapult) in the UK,[2] aimed at accelerating the development of process analytical technologies (PAT) for cell and gene therapy manufacturing. HORIBA has joined CGT Catapult with the purpose of assessing the A-TEEM fluorescence technology as a way of reducing batch failures and manufacturing costs.[3]

## Traditional Analytical Approaches and Their Limitations

Chromatography is the workhorse in biotechnology, configurable with a wide range of detectors, stationary and mobile phase options, it is used to solve many analytical challenges. It is used for quantitative analysis, purification, classification, in R&D and for QA/QC. As omnipresent and useful as it is, separations techniques have drawbacks - chromatography is almost exclusively a lab-based technique. Although there have been attempts to make process-ready chromatography platforms, the challenges presented by these physical separations approaches are difficult to overcome robustly. Separations also tend to be quite expensive on a per-measurement basis, requiring solvents or gases for the mobile phase, columns and standards, and waste disposal fees for expended solvents. It is also slow compared to optical techniques - a fast chromatographic method typically takes tens of minutes to run compared to seconds-to-minutes for spectroscopy. Additionally, there are fundamental limitations in what chromatographic methods can detect, as they are limited to the chemistry exposed on the outside of molecules, with no visibility to what may be buried inside. Ultimately separations techniques confirm **expected** components, therefore, unexpected components often go unnoticed, being "invisible" to the developed chromatographic method.

Molecular spectroscopy approaches like Fluorescence, Raman, near-infrared (NIR) and Fourier-transform infrared spectroscopy (FT-IR), on the other hand, are green, rapid, robust, and non-destructive technologies that can sit near-, at-, or even in-line. These techniques are sensitive to molecular environment in total, not just the molecules exposed on the exterior of a molecule, and spectroscopic libraries can be used to identify "unexpected" components. These attributes have contributed to molecular spectroscopy approaches, largely Raman and NIR, being incorporated as standard Process Analytical tools (PAT).

There is however a continuum of capability across these optical techniques, and some struggle to detect low concentration components, or to easily distinguish very similar components. Vaccines, ADCs, bispecifics, Adeno-Associated Virus (AAV) characterization (serotype differentiation, full vs. empty capsid) are all samples that require the highest level of specificity and sensitivity, so that even Raman cannot always reliably provide the detailed characterizations needed. Multidimensional excitation-emission matrix (EEMs) fluorescence should be the technique of choice based on its sensitivity and specificity. However, standard EEM implementations have struggled with repeatability and reproducibility. HORIBA has solved this with a patented 2-in-1 approach to multidimensional fluorescence spectroscopy called Absorbance, Transmittance and Excitation Emission Matrix (A-TEEM).[4] Table 1 compares A-TEEM with other optical spectroscopy techniques.

Summarizing, we find that in support of biopharmaceutical manufacturing, standard separations approaches,

Table 1 Comparison of Molecular Spectroscopy Analytical Techniques for Biopharmaceutical Analysis

| Technique | Sensitivity | Selectivity | Comments | LOD (PPM) |
|---|---|---|---|---|
| A-TEEM (2-in-1) Fluorescence | ↑ | ↑ | UV/Vis & Fluorescence, Quantitative across broad concentration range | <0.001 |
| 2D/EEM Fluorescence | ↑ | ↑ | Poor analytical quantification, molecular fingerprint is concentration dependent | <0.1 |
| Raman | ○ | ○ | Struggles with low concentrations | 25-150 |
| FTIR | ○ | ○ | Water interferes with molecular fingerprint | 100-2000 |
| UV/VIS | ○ | ↓ | Low selectivity<br>Low information content | 0.3 |
| NIR | ↓ | ↓ | Struggles with low concentrations<br>Low selectivity | 0.1-1 |

although effective, are slow and mostly confined to the laboratory, and unable to characterize unexpected sample components, or differentiate samples based on non-surface characteristics. Vibrational spectroscopic techniques such as Raman, NIR, and FT-IR, are rapid and effective for many samples, able to operate on the manufacturing line, and can characterize samples on overall molecular environments, not just surface variations; but have constraints for low concentrations or highly similar samples. Fluorescence A-TEEM shares the speed and efficiency of Raman, adds the high specificity and sensitivity of fluorescence EEMs, and finally implements reproducibility and repeatability that transform it into a true analytical tool. We'll go into a bit more detail about A-TEEM implementation and applications in the next section.

### Fluorescence spectroscopy progression - from EEM to A-TEEM™

Fluorescence spectroscopy is an exceptionally sensitive tool. In some sense this sensitivity presents challenges for its adoption as a routine analytical tool, as clearly assigning a spectral change to a corresponding sample change can be quite complicated. There have been several advances though, that have enabled even complex fluorescence profiles to be converted into actionable information. Multivariate analysis and fluorescence Excitation Emission Matrix (EEM) spectroscopy taken together have advanced the use of fluorescence spectroscopy for routine use. An EEM is a 3D scan, resulting in a contour plot of excitation wavelength vs. emission wavelength vs. fluorescence intensity. EEMs are used for a variety of applications and, coupled with chemometrics, have shown success in monitoring batch endpoint and quality of final product in a bio-fermentation process.[5]

Fluorescence EEM spectroscopy unites the sensitivity of chromatography with the attributes of spectroscopic implementation, such as speed, robustness, low cost of measurement, and is effective for low concentration samples, and in looking for very slight environmental or molecular structural changes.

### The Limitation of Standard EEM Spectroscopy

The molecular identification capabilities of a standard EEM spectroscopy has some fundamental limitations. The inner filter effect (IFE) distorts the measured fluorescence spectrum of a molecule due to re-absorption that occurs at higher concentrations. The standard correction protocol uses a secondary measurement from a different absorbance spectrometer to adjust the measured fluorescence signal. But as the measurement does not occur simultaneously and with the same exact volumes, this approach doesn't provide a perfect correction. The imperfect match between data and correction means that an EEM for a single component won't necessarily be reproducible, and this severely limits the ability of a traditional EEM for robust component identification as well as quantification.

### A-TEEM™ Spectroscopy Overcomes These Limitations

To overcome this limitation, HORIBA Scientific developed a new technology, called A-TEEM™ spectroscopy,[4] giving a spectrofluorometer the ability to simultaneously acquire Absorbance, Transmittance and a fluorescence Excitation Emission Matrix (A-TEEM) of a particular sample. Because the absorption spectra used for IFE correction are collected at the same time on the same sample as the fluorescence measurement, the correction is reproducible, and the A-TEEM data can be used for accurate sample ID as well as highly sensitive and specific component quantification.

A-TEEM provides ***true and accurate representations of fluorescent molecules over a broad concentration range*** (typically up to ~2 absorbance units). Additionally, absorbance and color information for all molecules, including non-fluorescent ones, is acquired and can be used in multivariate methods for comprehensive multi-component molecular identification. Interestingly, color can be a very sensitive indicator of protein product quality, and this information is acquired as part of the measurement. The A-TEEM method is a robust and extremely sensitive analytical technique, comprising the best attributes of both molecular spectroscopy (speed, low per measurement cost, lab-to-line placement) and chromatography (sensitivity and selectivity). A-TEEM therefore has the potential to replace traditional instruments like HPLC, GC, LCMS, and GCMS as a simpler, and faster analytical tool, with significantly lower per-sample measurement costs. A-TEEM is also a green approach to quantification compared to chromatography, as the need for a mobile phase goes away. Based on simultaneous use of fluorescence and absorbance spectroscopy, A-TEEM is validatable using the standard approaches for these two techniques.[6] A-TEEM molecular fingerprints are extremely information-rich, and multivariate analysis techniques are used to simplify the data and answer specific questions, providing direct and unambiguous results.

The A-TEEM fluorescence method provides a complete and traceable optical fingerprint of liquid samples, overcoming limitations that previously hampered adoption of fluorescence EEMs. This technique is well suited to the characterization of vaccines and other biopharmaceutical samples, with not only the sensitivity and specificity comparable to chromatographic methods, but also the speed, cost savings, and reproducibility of vibrational spectro-

scopic approaches.

## Cell Culture Media - Where Bioproducts Begin

The fundamental process in creating biopharmaceutical products is the growth of cells in bioreactors, fed by cell culture media, a specially formulated broth which contains a balance of nutrients to optimize cell productivity. The cost of the media itself is a small fraction of the value of the final product, therefore assessing its quality prior to the start of a bio-fermentation is a cost-effective way to ensure end-product quality and quantity. Standard approaches to perform this critical QC step are chromatography and mass spectrometry, making these measurements time consuming, expensive, and designed only to confirm what is expected, not to detect the unexpected. There is a growing interest in the use of molecular spectroscopic approaches for media QC, and both Raman and EEMs have been tested.[7,8] As is often the case, the combination of these two spectroscopic approaches may be the best way to cover the breadth of components that need to be characterized for this application.

As expected, A-TEEM provides a robust approach to assess the quality of cell culture media prior to the addition of cells.[9] We investigated eight different commercially available cell culture media samples, with the goal to differentiate between them, and also to study the sensitivity of the technique to detect environmental degradation. A more in-depth analysis of specific cell media components is a topic for future studies, and not addressed in this overview. Five samples from each of the eight types of media were analyzed, each sample was measured in triplicate resulting in 120 measurements, and each measurement took ~60 seconds. All cell culture media was stored at 4°C before testing and allowed to equilibrate to ambient laboratory temperature prior to analysis. The PCA showed clear differentiation between the different categories of media. To explore the specificity of the technique, a separate analysis including data

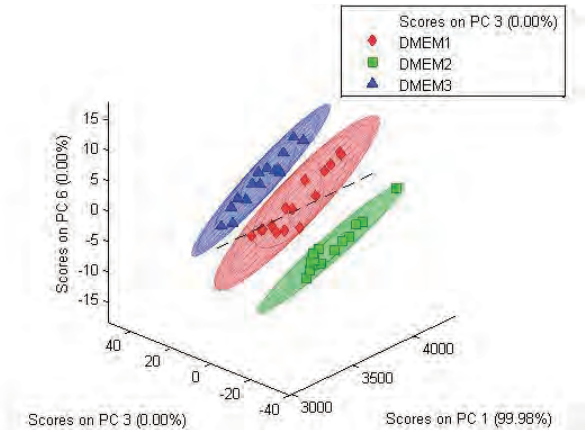



Figure 1 3D principal component scores plot showing ability of A-TEEM to differentiate within a cell culture category. DMEM samples of varying composition (types 1-3) were analyzed.

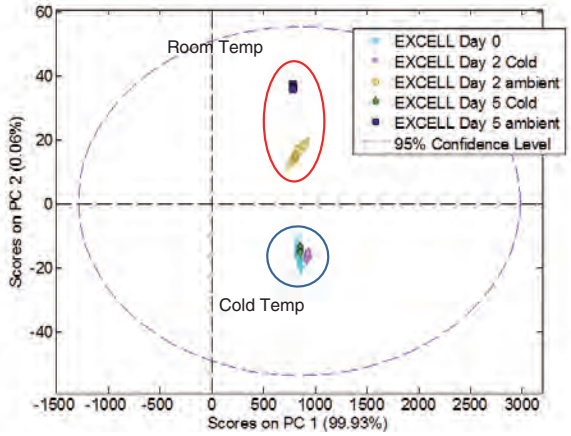



Figure 2 A-TEEM characterization of fresh from degraded cell culture media samples

from only a single category of media was performed. Figure 1 shows three different DMEM samples with slight compositional differences were easily distinguishable.

The final test for A-TEEM characterization of cell culture media was to compare fresh samples with those degraded through exposure to light, and elevated (room) temperature. A-TEEM was able to track the degree of degradation over a period of 5 days, as shown in Figure 2. A-TEEM provides a rapid approach to differentiating eight commercial cell media formulations, classifying three similar formulations, and tracking degradation of a cell media sample against adverse storage conditions.

## Viral Vectors Characterization

Researchers have turned to nature to understand how to deliver genetic material into human cells, and viruses provide a perfect mechanism. Several specific viruses, Adeno-Associate Virus (AAV) and lentivirus, for example, have been found to have appropriate characteristics, allowing scientists to manipulate them for the purpose of delivering genetically engineered payloads into human cells. These so-called viral vectors are the work horses of cell and gene therapy, and are making inroads into other applications, such as vaccines. In developing and deploying viral vectors, there are multiple parameters that are important to characterize for a complete picture of product quality. Measurements need to discriminate: 1) whole, broken, empty, aggregates, and infectious or non-infectious viruses; 2) empty from full capsids; and 3) vector serotypes. We'll present results from HORIBA's multi-laser Nanoparticle Tracking Analysis (NTA) approach using the ViewSizer 3000™ technology showing how it can be used to determine infectious titer, and then briefly touch on as-yet unpublished results from the A-TEEM method on its ability discriminate empty from full capsids and discriminate between vector serotypes.

### Infectious Titer Determination with ViewSizer 3000

The ViewSizer 3000 is a nano-particle tracking (NTA) platform that incorporates three lasers to collect the most accurate distribution and concentration data across a wide range of sample sizes. The point of the three lasers is to compensate for a variety of known problems when looking at a polydisperse sample: large particles scatter too much light and will saturate the detector from one laser, whereas small particles will only weakly scatter, and will be hard to detect. With three different lasers with independent power control, particles size can be determined across a very broad range, with scattering signals optimized to provide the most accurate distribution and concentration results.

The Viral Plaque Assay is the most robust approach to determine Infectious Titer, and it is highly manual and very time consuming. Viral preparations are serially diluted and allowed to grow on the planar surface of cell culture monolayer. Over a period of time (hours to weeks) the viral cells proliferate, infecting the confluent cells and destroying them. Regions of dead cells appear as empty patches on the culture surface, and these regions or "plaques" are then manually counted to determine the number of Plaque Forming Units (PFU) in a given dilution. Multiplying the PFUs by the dilution factor determines the final infectious titer of the parent stock, expressed in PFU/mL.

To determine the utility of the ViewSizer 3000 approach, the following protocol was followed: samples were transferred to the ViewSizer 3000 cuvette, fitted with a magnetic stir bar, and twenty-five video segments were collected, with five seconds of stirring in between each to ensure completely independent sets of particles in each video. The measurements were recorded with the following parameters: frame rate: 30 frames/second; exposure: 15 ms; gain: 30; blue laser power: 210 mW; green laser power: 12 mW; and red laser power: 8 mW; temperature control: active, 22°C. Each run took between 15-20 minutes. Multiple users independently analyzed the samples using the same measurement and acquisition parameters described above. The data were then processed and plotted using the same settings. Particle sizes ranged from 50 nm to 1000 nm, with a significant enrichment of particles around 200 nm, as shown in Figure 3. The total viral particle concentration within the sample was 1.6 x 107 particles/mL. The distribution tailing towards 1000 nm suggests the existence of host cell debris or other background particles contained within the cell culture growth media. The image extracted from the video recordings further validated the presence of larger viral aggregates.

Infectious titer data from multiple assay replicates performed by independent analysts were plotted as a function of measured particle concentration from the ViewSizer 3000 and fitted with a result shown in Figure 4. The result indicates an excellent $R^2$ value of greater than 0.9. When the results are analyzed using only data from one of the three lasers available, correlation dropped to an $R^2$ value of 0.6 because of its inability to properly count the aggregates. By using three lasers, all particles were counted and the improved correlation is presumably because infectious aggregates contribute to the titer and were quantified along with single virus particles. This result indicates that over the course of multiple experiments, the ViewSizer 3000 accurately and reproducibly determined the viral titer within a heterogeneous sample.

This demonstrates the ViewSizer 3000's ability to assess not only intact viral particles and aggregates, but also potential cell debris and background noise generated during upstream processing. Since downstream processing depends strongly on the removal of these impurities to minimize further aggregation, quantifying infectious viruses reliably depends on understanding both the purity of the upstream sample and the downstream analytical precision. The ability to detect these aggregates and cell

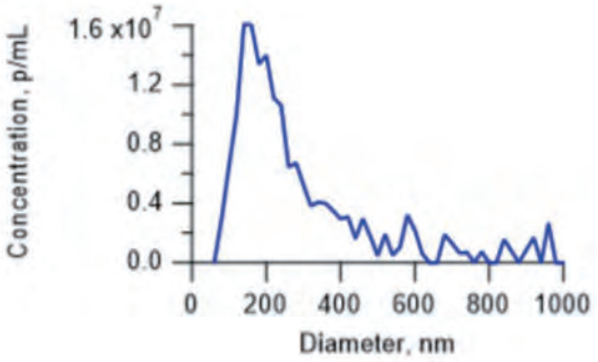



Figure 3 Measurement result of a human viral vector sample. Note the distribution tailing towards 1000 nm suggests the presence of host cell debris and aggregates.

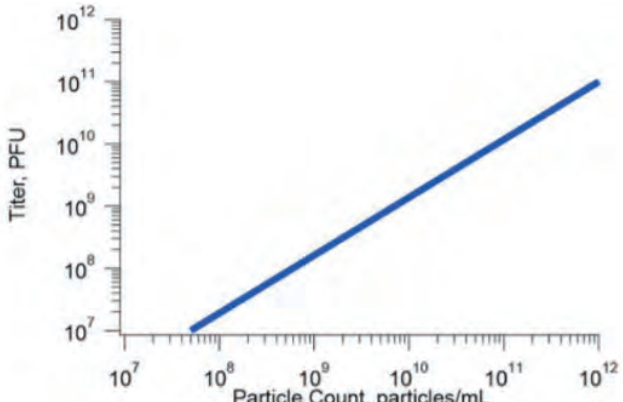



Figure 4 $R^2$ value of > 0.9 was achieved between titer and data collected from the ViewSizer 3000.

debris is also critical when examining the stability of the final product over time. As we saw with the roll out of Coronavirus vaccines, the stability of biologic preparations within a freezer is a crucial factor to understand. With Viewsizer we are able to compare fresh preparations of a sample to ones that have been in the freezer for a number of weeks. By monitoring the increasing number of aggregates/cell debris that are present in the stored samples versus time, it is possible to gain an understanding of how long a sample is stable in a freezer. Utilizing the multiple lasers of the system is a crucial factor in these studies, as the particles of interest are usually. around 80-120 nm, while aggregates and debris can appear all the way up to a micron; suggesting that the ability to measure across that entire size range is an indispensable tool in understanding the stability of these biologic preparations.

In addition to the applications described above, there are several unpublished studies that point towards other very interesting applications of A-TEEM, including the differentiation of AAV serotypes (AAV2 from AAV9), and quantification of empty vs full capsid ratio.

## Vaccine Characterization and Manufacturing QC

The NIIMBL Vaccines roadmap[10] noted that vaccine product release time is on the scale of weeks to months, rather than the one to two days that might be possible with the right analytical tools. Vaccines have historically been relatively simple formulations, with one or multiple vaccine component (live-attenuated vaccines, subunit, polysaccharide, conjugate, or toxoid) with a diluent, and perhaps an adjuvant (a component that enhances the immune response). With the events of the last 18 months, vaccine technology has made huge advances in the fight against the Coronavirus pandemic, and mRNA vaccines, and the use of viral vectors has proliferated. The ViewSizer has been used as an analytical tool in the analysis of adjuvants.[11] However, the focus of this section will be on describing an approach for QC for vaccine manufacture that might meet the NIIMBL goal of one to two days for product QC.

One of the struggles for standard vibrational spectroscopic approaches is that vaccines tend to be formulated at very low concentrations. Raman spectroscopy without resonant enhancement struggles to characterize protein samples under ~1 mg/ml. Although Surface Enhanced Raman (SERS) can be used to enhance signals, it is challenging to fully characterize the impact that SERS substrates might have on the vaccine components. A-TEEM has been shown to have very low limits of detection, recently calculated to be ~0.15 µg/mL for a well-characterized vaccine formulation.[12] For these same low concentration vaccine samples, A-TEEM has shown the ability to readily detect post-translational modifications, as well as differentiate between samples with simple amino acid substitutions, even those of non-fluorescent amino acids. In addition to the low limits of detection, A-TEEM has also been shown to characterize complex mixtures, differentiating and quantifying components against strong backgrounds, most notably in wine.[13]

To demonstrate the suitability of A-TEEM to the analysis of vaccines, we tested four over-the-counter multicomponent canine vaccine products. We used the SOLO-JEC brand multi-component canine vaccines (Table 2) from Boehringer Ingelheim VetMedica that were available at a local retail store (Tractor Supply Store). We tested SOLO-JEC 5 ("protection against five common canine infectious diseases"); SOLO-JEC 6 ("delivers the same protection as SOLO-JEC 5 plus additional protection against coronavirus); SOLO-JEC 9 ("delivers additional protection against four types of leptovirus that are known to infect dogs"; and SOLO-JEC 10 ("delivers the same protection as SOLO-JEC 9 plus additional protection against coronavirus")..

The vaccines were reconstituted according to the package directions, diluted with distilled water in a ratio of 1:61 and added to a cuvette for measurement. To ensure repeatability, six individual measurements were collected for each vaccine, and this was done on two separate days. The calibration data set therefore consists of 48 measurements. To test reproducibility, a third unique set of validation samples were collected on a different instrument by a different operator, with 5 repeat measurements for each vaccine formulation, resulting in a validation set with 20

Table 2 OTC multicomponent vaccine products tested for this study

| | Basic Program Solo-Jec 5&6 | | Lepto Included Solo-Jec 9&10 | |
|---|---|---|---|---|
| Disease | Solo-Jec 5 | Solo-Jec 6 | Solo-Jec 9 | Solo-Jec 10 |
| Distemper | ● | ● | ● | ● |
| Hepatitis | | ● | ● | ● |
| Adenovirus 1 | ● | | | |
| Adenovirus 2 | ● | ● | ● | ● |
| Coronavirus | | ● | | ● |
| Parainfluenza | ● | ● | ● | ● |
| Parvovirus | ● | ● | ● | ● |
| Lepto (4 types) | | | ● | ● |
| Gentamicin | ● | ● | ● | ● |
| Amphotericin 8 | ● | | | |
| Thimerosal | | ● | ● | ● |
| Adjuvant | ● | ● | ● | ● |

Table 3 Samples used for Calibration and Validation

| Vaccine | Calibration set (n=48) | Validation set (n=20) |
|---|---|---|
| Spectra 5 | S5 | U5 |
| | S-5 | |
| Spectra 6 | S6 | U6 |
| | S-6 | |
| Spectra 9 | S9 | U9 |
| | S-9 | |
| Spectra 10 | S10 | U10 |
| | S-10 | |

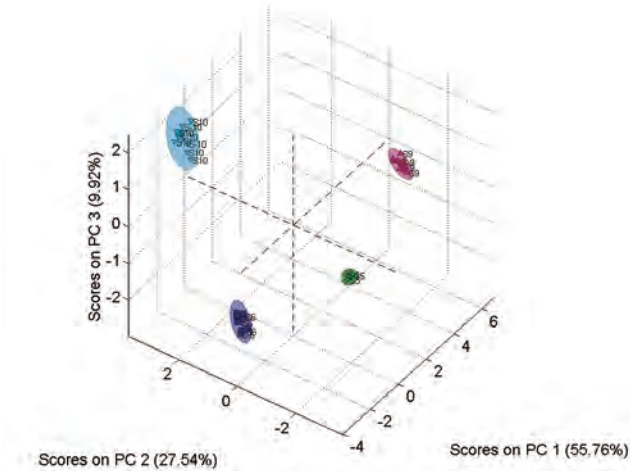



Figure 5 PCA scores scatter plot showing clear differentiation between 4 vaccine products, and excellent reproducibility for multiple measurements of a single vaccine type.

measurements, as shown in Table 3. A simple principal component analysis (PCA) was able to easily differentiate between the 4 vaccine products, with tight clustering of the calibration data.

In order to classify "unknowns" from the validation samples, a discriminant analysis model was created using Extreme Gradient Boost - Discriminant analysis. A-TEEM was able to identify and validate "unknown" samples with 100 percent certainty, as shown in Figure 6.

A-TEEM is the only spectroscopic approach that can perform this level of analysis on low concentration and complex vaccine formulations. Standard chromatographic QC approaches for vaccine batch analysis take days to complete. The results presented here demonstrate the potential for an on-line, rapid, sensitive and reproducible spectroscopic approach for vaccine QC, offer a potential solution to meet the goals for vaccine release that NIIMBL has set.

## Conclusion - The Future of Biopharma Characterization

HORIBA Instruments Inc. has a unique portfolio of analytical solutions that offer rapid, cost-efficient alternatives to chromatography, manual titer, qPCR, Analytical Ultra Centrifugation and other traditional analytical approaches. From A-TEEM to ViewSizer and with Raman as a complementary approach, these tools have the potential to

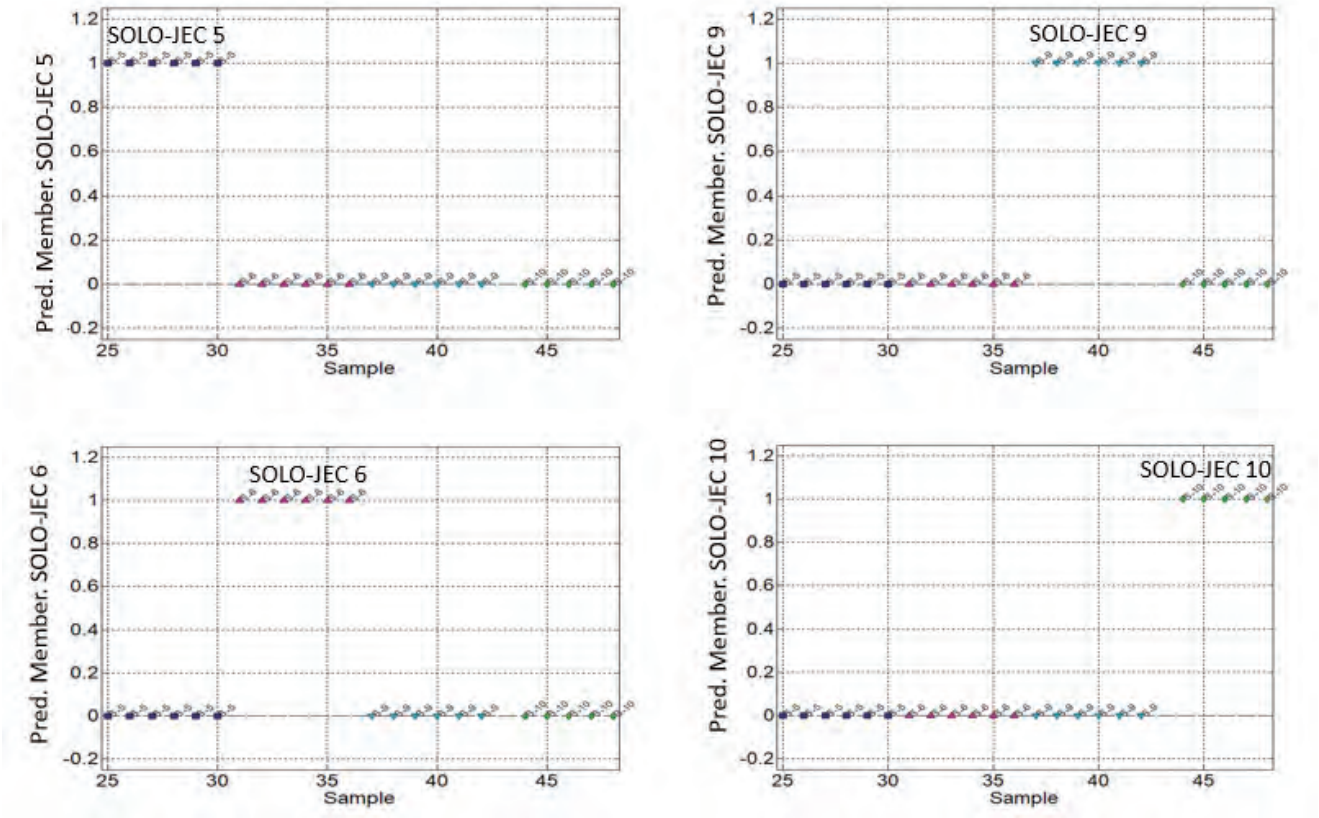



Figure 6 100% success in correctly identifying the vaccine product for unknown samples

shorten manufacturing times, and improve product safety with the ability to identify unknown components. The applications presented here are not meant to be an exhaustive compilation of the value that HORIBA tools provide to life science, but to highlight how spectroscopic and optical approaches offer a robust solution, as biopharmaceutical production methods scale to meet growing demand, and more information is needed more quickly. Starting with cell culture media, the fundamental raw material for all bioprocessing, both Raman and A-TEEM have proven utility in characterizing the quality of this material. Viral vectors are used to deliver novel vaccines and the components that make cell and gene therapies successful; ViewSizer and A-TEEM can be used to speed up analytical characterization steps that are needed in their manufacture. Finally, vaccines production has taken on new urgency, and A-TEEM has the potential to reduce QC time from weeks to hours. Other contributions in this version of Readout will explore other aspects and applications, including characterization of up-and-coming biotechnologies, like exosomes, and how HORIBA technologies are critical for the characterization of bioprobes.

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

## References


[1] NIIMBL. https://niimbl.force.com/s/about-niimbl.

[2] About Cell and Gene Therapy Catapult. https://ct.catapult.org.uk/about-us.

[3] Editors, B. P. I. (2021, June 18). Horiba Joins CGT Catapult Consortium to Accelerate Development of PAT. BioPharm International. https://www.biopharminternational.com/view/horiba-joins-cgt-catapult-consortium-to-accelerate-development-of-pat.

[4] Gilmore, A. M. & Tong, X. (2014, December 2). System and Method for Fluorescence and Absorbance Analysis, US Patent 8,901,153.

[5] Peter P. Mortensen; Rasmus, Bro. Real-time monitoring and chemical profiling of a cultivation process. Chemometrics and Intelligent Laboratory Systems, Volume 84, Issues 1-2, 1 December 2006, Pages 106-113.

[6] Adam M. Gilmore. How to Collect National Institute of Standards and Technology (NIST) Traceable Fluorescence Excitation and Emission Spectra. National Center for Biotechnology Information. March 27, 2014.

[7] Li et al., "Fluorescence Excitation-Emission Matrix (EEM) Spectroscopy for Rapid Identification and Quality Evaluation of Cell Culture Media Components," Applied Spectroscopy volume 65, Issue 11, 2011.

[8] Li et al., "Rapid Characterization and Quality Control of Complex Cell Culture Media Solutions Using Raman Spectroscopy and Chemometrics." Biotech & Bioeng. Volume 2, Issue 107, 2010.

[9] Alvin Togonon, Adam Gilmore, Ben Yang, Karoly Csatorday and Marinella Sandros. Monitoring Cell Culture Media Variability using A-TEEM™

[10] The National Institute for Innovation in Manufacturing Biopharmaceuticals Vaccines Roadmap

[11] P. Singh et al., "Particle size analyses of polydisperse liposome formulations with a novel multispectral advanced nanoparticle tracking technology", International Journal of Pharmaceutics, Vol. 566, 2019, pp 680-686.

[12] Unpublished results

[13] R. K. R. Ranaweera, "Authentication of the geographical origin of Australian Cabernet Sauvignon wines using spectrofluorometric and multi-element analyses with multivariate statistical modelling", Food Chemistry 335 (2021) 127592



Linda Kidder YARLOTT, Ph. D.

HORIBA New Jersey Optical Spectroscopy Center
HORIBA Instruments Incorporated



Adam M. GILMORE, Ph. D.

HORIBA New Jersey Optical Spectroscopy Center
HORIBA Instruments Incorporated



Sean TRAVERS, Ph. D.

Technical Sales Representative and Consultant for HORIBA Scientific
Anatom Technology

# Fluorescent Bioprobes for Life Science Applications

Florian FORMANEK

Bioprobes, and especially fluorescent bioprobes, have become essential tools for the detection and monitoring of important biological species and their related biological processes in live samples. Key applications of bioprobes fall into two main categories. The first one is bioimaging, from the cellular level, up to the organ and even small-animal level, *in-vitro* but also under *in-vivo* conditions. The second is biosensing, where probes are employed to detect various entities like ions, reactive oxygen species (ROS) or macromolecules, to help detect cancer, to observe drug internalization, and much more. This featured article will detail the typical characterization needs for the development and the use of the most common families of bioprobes: 1) determination of optical emission profiles; 2) measurement of size; 3) assessment of elemental composition or molecular signature. The article will showcase how HORIBA Scientific instruments are used by chemists all over the world to obtain rapid feedback on the best direction to follow to improve their synthesis processes.

key words
Fluorescence, Bioprobes, Sensing, Bioimaging

## Introduction

Bioprobes are fluorophores, fluorescent chemical compounds that re-emit light upon light excitation, intended for Life Sciences applications. Bioprobes can be called many things, tags, labels, sensors, reporters, or sensors, but they always refer to same thing. They are sometimes used alone, as tracers in fluids, as dyes for staining, as


Figure 1 General families of fluorescent bioprobes.

indicators (when their fluorescence is affected by environmental factors such as polarity, temperature, viscosity, or pH), or bound to macromolecules, serving as a marker for affinity or bioactive reagents.

Bioprobes are employed in a variety of domains from biomedical research to clinical applications and diagnostics. The first application is biosensing, which is the detection of biomolecules using an analytical device (or biosensor) combining a biological component with a detector. Small point-of-care devices used for diagnostics often rely on fluorescent bioprobes for their optical readout. This is also the case for devices intended for the monitoring of anthropogenic pollutants in various water matrices and aquatic environments.

Bioprobes are widely used to stain tissues, cells, or other biomaterials in a variety of analytical methods, including fluorescence imaging and spectroscopy. For instance, for *in-vivo* bioimaging in cancer research, to help with guided surgery in combination with other multimodalities. For that purpose, many groups are currently working on the development of near-infrared (NIR) emitting tags which are better adapted to deep-tissue imaging, taking advantage of reduced interference from photon absorption and scattering in this optical window.

High-content screening (HCS) systems used in the pharmaceutical domain for drug discovery also rely on fluorescent probes. The most common analysis workflow involves labeling proteins with fluorescent tags, while changes in cell phenotype are measured using automated image analysis in multiwell plates, in order to optimize synthesis processes or to confirm if a drug has the expected effect on its target.

Flow cytometry, which is used for biomarker detection, cell counting and sorting, or microorganism identification, is based on labels for measuring the physical properties of cells or particles. In most cases, fluorescent bioprobes with characteristic excitation and emissions peaks are designed to attach to a specific biological structure, for example a surface protein, to allow sorting cells in a fluidics setup, based on the fluorescence measured in different detection channels.

A new and rapidly growing field is the clinical application of bioprobes for photodynamic therapy (PDT), which shows great promises for cancer treatment by killing cell with the heat produced locally by the particles upon light absorption.

Here, research focuses on finding dyes with properties like good biocompatibility, specific targeting, or strong two-photon absorption.

Bioprobes can be grouped into 3 main categories, as illustrated in Figure 1. Organic dyes (e.g., fluorescein, rhodamine), biological fluorophores (e.g., green fluorescent protein, phycoerythrin, allophycocyanin) and fluorescent nanostructures or nanomaterials (e.g. quantum dots, AIEgens).

Organic dyes are small molecules mostly synthetized through chemistry, but which can also be found in nature, like in plants. Common tags employed for confocal fluorescence microscopy are found in this category.

Biological fluorophores on the other hand are large molecules, typically fluorescent proteins, the most famous of all being GFP (Green Fluorescent Protein). DNA, which falls into this category does not have intrinsic fluorescence but can be used as biotags once coupled to a fluorophore.

Finally, we have nanomaterials that are engineered with specific chemical functions or tailored structures to generate unique light emission. This class of material encompasses a wide range of objects, like metallic nanoclusters, core-shell fluorophores, carbon dots, supramolecule assemblies and many more.

## Characterization needs

### Simultaneous Absorbance and Fluorescence

When studying novel bioprobes with unknown characteristics

or with emission properties impacted by solvent effects, having a complete fluorescence profile of a given material over a large range of wavelengths is key to avoid reperforming additional measurements at a later stage. An Excitation Emission Matrix (EEM), which is basically a three-dimensional scan that results in a contour plot of excitation wavelength vs. emission wavelength vs. fluorescence intensity, can provide such information (Figure 2).

Of importance as well when characterizing concentrated solutions (typically above 0.1 to 0.2 absorbance units), which is often the case when synthetizing bioprobes, is to account for the Inner Filter Effect (IFE). The IFE is a well-known phenomenon that distorts the measured fluorescence spectrum of a molecule due to absorption within the optical path length of the liquid sample, traditionally measured in a cuvette. IFE can significantly affect fluorescence emission spectral profiles, distorting their general shape, shifting the spectral position of the peak maximums, and decreasing the emission intensities, preventing from performing true quantitative analysis.

IFE can be corrected using absorbance data, and the HORIBA Duetta two-in-one spectrometer is unique in that it combines absorbance and fluorescence measurements on the same platform, allowing real-time correction for the IFE, on the same sample at the same time, as illustrated in Figure 2. It also takes advantage of an extended wavelength detection up to 1100 nm to cover the useful NIR biological transparency window.[1]

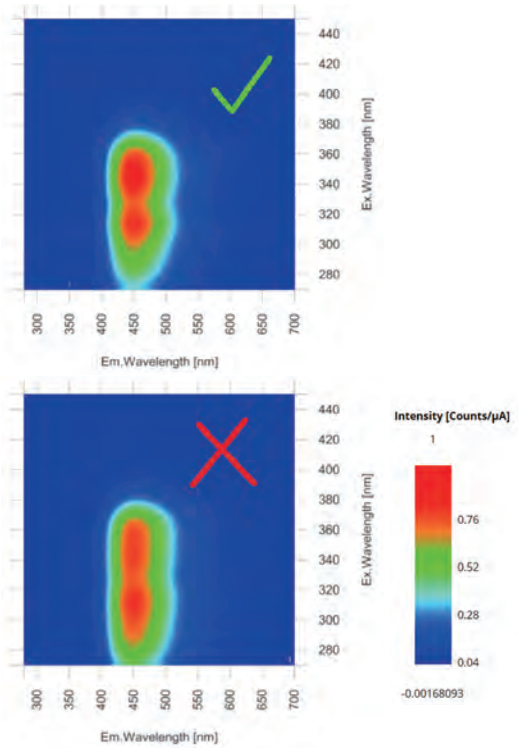



Figure 2 Fluorescence EEM profiles obtained with a HORIBA Duetta spectrometer, with (top) and without (bottom) inner filter effect correction.

## Quantum Yield

The fluorescence quantum yield (QY) is defined as the ratio of photons emitted to photons absorbed by a fluorophore, basically an indicator of its brightness. This parameter is crucial for fast bioimaging consideration, as samples are often light sensitive or present rapid time kinetics.

A reliable method for recording the QY is a comparative method which involves the use of well characterized reference standards with known properties.

A second approach compatible with liquid samples is based on lifetimes and different concentrations of a

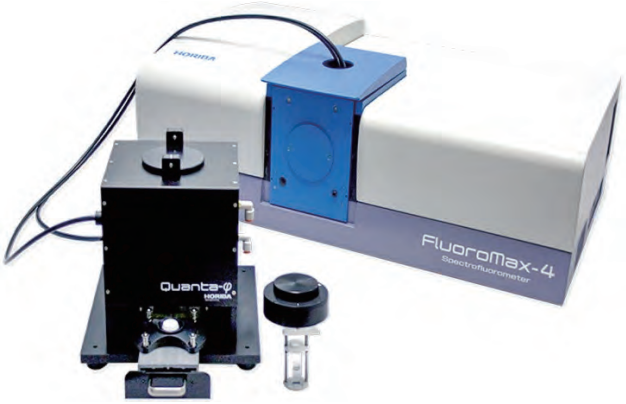



Figure 3 Quanta-φ integrating sphere coupled to a FluoroMax-4 spectrometer by HORIBA for quantum field determination.

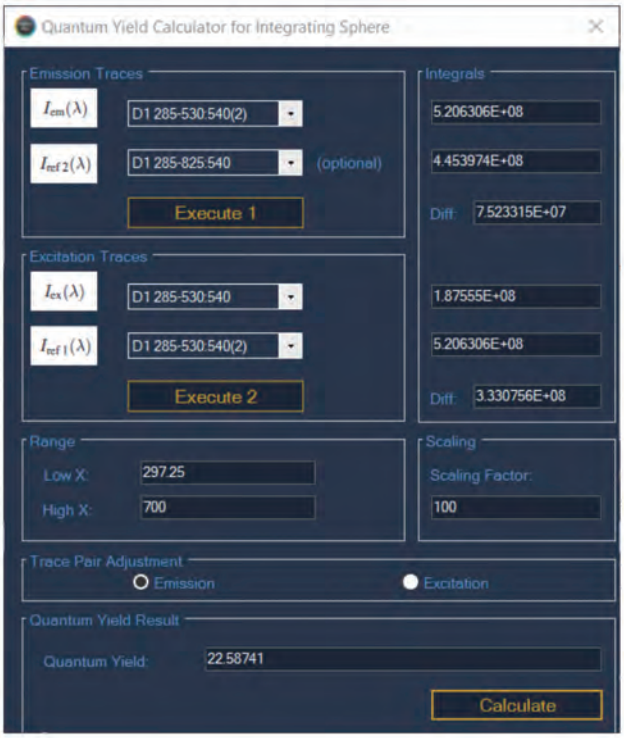



Figure 4 Interface window of the FelixFL software on the HORIBA Fluorolog-QM spectrometer with integrated quantum yield calculator.

quencher to calculate the QY of a molecule in accordance with the Stern-Volmer equation. HORIBA customers from the Swiss Federal Laboratories for Materials Science and Technology (EMPA) recently reported on this method for their work on polyimide-based photoemitters for oxygen biosensing.[2]

However, the most accepted method to determine the absolute QY is to employ an integrating sphere (Figure 3), a sphere coated with an all-reflective surface to capture all the light going in and out.

A recent example of the use of the HORIBA Quanta-φ integrating sphere by scientists from the University of Chile explains the synthesis of an anthracene derivative dye with solvatochromic properties and highlights the importance of designing bright bioprobes for microscopy experiments on living cells.[3] Accurate quantum yield determination can be made easier using a dedicated application embedded in most HORIBA software, as seen in Figure 4.

## Lifetime

The fluorescence lifetime is a photophysical property of bioprobes, providing complementary information to the simple steady-state monitoring of fluorescence intensity. The technique has gained popularity thanks to its high sensitivity to microenvironmental parameters and changes in molecular conformation such as temperature, viscosity, pH, and ion concentration. At the same time, lifetime measurements are largely independent of fluorophore concentration and do not require calibration steps like intensity-based experiments.

Förster Resonance Energy Transfer (FRET) sensors, which are widely used to monitor protein-protein interactions to elucidate cellular signaling, disease progression and drug efficacy, also benefit from a lifetime monitoring, especially for *in-vivo* applications. Figure 5 illustrates the lifetime decay for a solution of Qtracker 655 quantum dots, collected on a HORIBA DeltaPro spectrometer equipped with a photomultiplier and a DeltaDiode485L pulsed laser source.

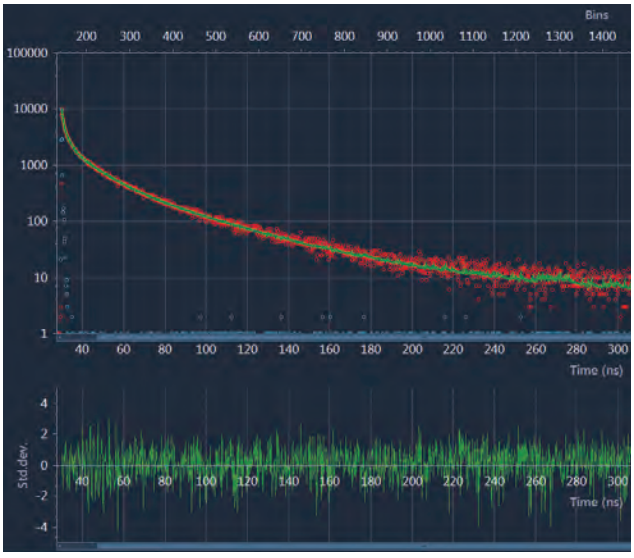



Figure 5 Lifetime decay (red) and fit (green) to a 5-exponential decay equation, expressed in photon counts. The lower graph shows the standard deviation error residuals from the fit to the fitted data.

## Polarization

Fluorescence anisotropy or fluorescence polarization is a measurement of the changing orientation of a molecule in space, with respect to the time between the absorption and emission events. Anisotropy is an excellent tool for understanding changes in macromolecule shape, as well as molecular binding.

Figure 6. exemplifies the confirmation of effective binding between an antibody and the bronchodilator drug Theophylline labeled with a fluorescein dye and mixed with an antiserum in pH 7.2 buffer, then incubated for 3 minutes. The free and the complexed drug were distinguished through polarization measurements.

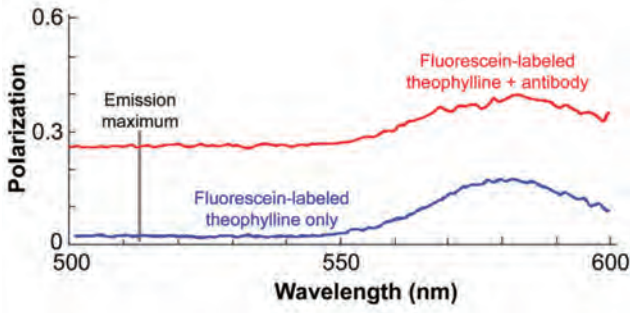



Figure 6 Polarization emission scan for labeled theophylline with (red) and without (blue) antibody (λexc = 485 nm). The emission maximum for fluorescein-labeled theophylline is marked by a gray line.

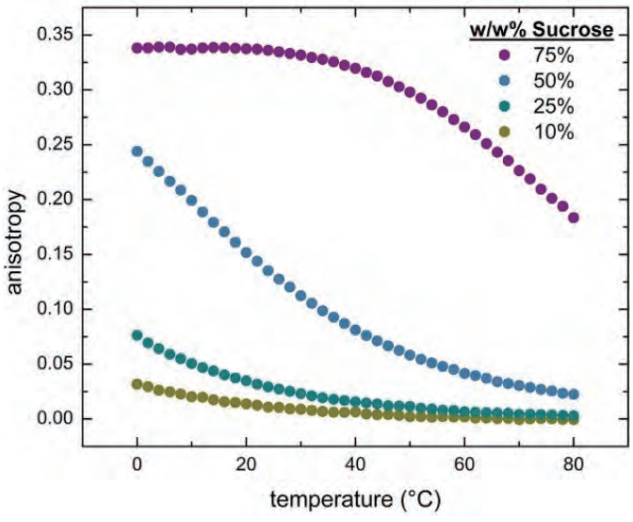



Figure 7 Fluorescein dissolved in sucrose solutions. With increased temperature, viscosity decreases, yielding faster rotation times and lower anisotropies.

Figure 7 shows additional polarization measurement of fluorescein dye dissolved in four aqueous solutions, performed with a HORIBA Fluorolog-QM spectrometer.

This indirect measure of the local viscosity gives information on sample aggregation, structural changes, molecular binding, and other mechanisms.

### Bioimaging

Fluorescent bioprobes, especially small organic molecules, have become indispensable tools in modern biology. For research on living cells, these probes need to fulfill specific requirements like high permeability and good intracellular solubility, good affinity and specificity for the target, photostability, as well as low toxicity.

Microscopes with epifluorescence illumination are routinely used to observe the penetration and distribution of labelled targets within cell organelles to provide information on their structure and functions. Figure 8 illustrates cell staining with multiple probes, Texas Red as a marker of proteins, DAPI as an indicator of membrane viability, and BODIPY to reveal neutral lipids.

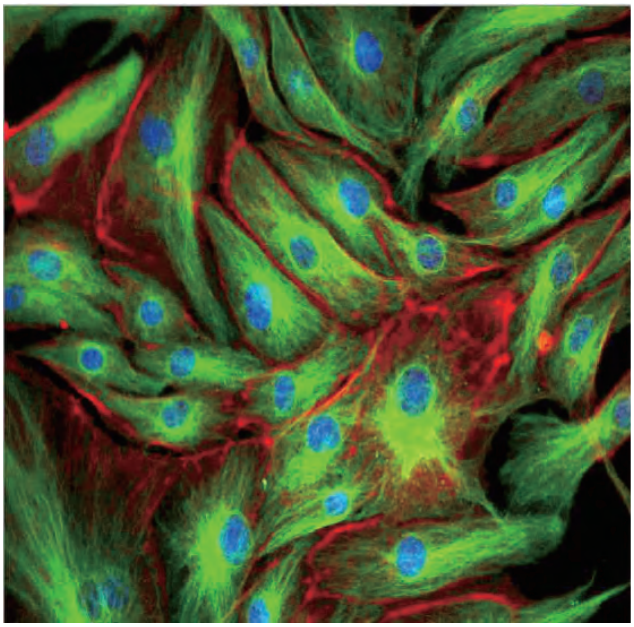


Figure 8 Epifluorescence imaging of Bovine Pulmonary Artery Endothelial cells (BPAE Line) with Anti-α-Tubulin antibody, stained with BODIPY, Texas Red and DAPI fluorophores, using a HORIBA XploRA microscope.

The influence of environmental parameters on intra- and intermolecular interactions at the cellular level can also be obtained with microscopy instruments by monitoring the fluorescence lifetime of fluorophores instead of their intensity.

HORIBA now offers a new concept of wide field imaging, called FLIMera, capable of video-rate lifetime determination, based on time-correlated single photon counting technique (TCSPC) realized independently in each pixel.[4]

Figure 9 highlights research done with the FLIMera camera by HORIBA application teams in collaboration with Glasgow Caledonian University (UK). Saccharomyces cerevisiae, a single-celled fungus microorganism, was stained with a viscosity sensitive mitochondrial fluorescent probe called DASPMI to assess its viability. The cells displaying a longer average lifetime for DASPMI are associated with cells having a higher microviscosity, which is indicative of cell death (Figure 9 Right).

In addition, the decay associated spectra were determined for yeast cells suspended in growth medium, to investigate the photophysics of the fluorescence emission (Figure 9 Left). The use of the two-color FUN-1 stain, which transports into cell vacuoles, revealed the condition of plasma membranes through the formation of cylindrical intravacuolar structures.[5]

## Research based on HORIBA instruments

HORIBA Scientific is proud to count world-leading research teams among its customers. This following section will highlight a selection, far from exhaustive, of their recent publications in the field of bioprobes.

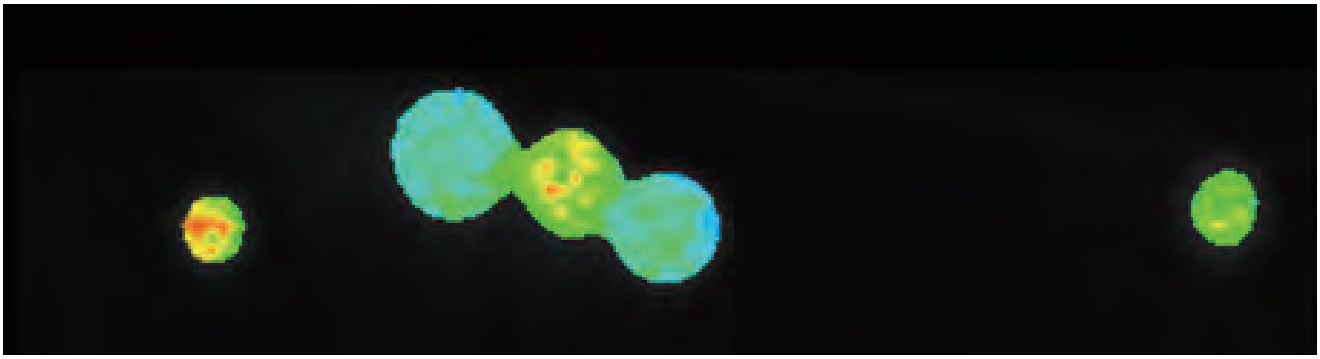


Figure 9 Left: Decay associated spectra for the FUN-1 dye in yeast cells grown in different growth media, also showing the sum spectrum that relates to the steady state emission. Right: FLIM measurement of DASPMI stained Saccharomyces cerevisiae in a polysaccharide polymer grown.

### Photodynamic therapy

Photodynamic therapy refers to treatments which involve light-sensitive therapeutics which can be switched "on" with a light source to destroy abnormal cells and treat various types of diseases.

The development of multifunctional bioprobes for simultaneous specific near-infrared (NIR) imaging and phototherapy of tumors being of great significance. For this purpose, our Fluoromax-4 spectrometer users at the City University of Hong Kong (China) reported on the integration of protoporphyrin IX (PpIX) into nanoscale metal-organic frameworks (NMOFs).[6]

Another example can be found in a recent publication in the journal Nanomedicine: Nanotechnology, Biology and Medicine, where a fluorophore called indocyanine green was co-encapsulated with a CT (Computed Tomography) contrast agent within nanoliposomes for imaging guided treatment. For this work, a Fluorolog-3 instrument from HORIBA recorded the fluorescence spectra in the NIR range and helped with the formulation of the particles.[7]

### Bioluminescence

Recently, our European laboratory, based South of Paris (France) on the Paris-Saclay campus, was closely involved in research with the famous Institut Pasteur.[8] Time-dependent chemiluminescence properties of core-modified coelenterazine luciferin analogues were recorded in the range of 250 to 800 nm using a HORIBA Aqualog spectrometer. These small molecules generated in bioluminescent organisms show great promise for bioimaging and therapeutics, by eliminating the need for external optical illumination and avoiding autofluorescence background in existing fluorescence techniques.

A recent publication highlights the application of such probes for the detection and imaging of peroxynitrite, a reactive nitrogen species (RNS), in live cells and mice *in-vivo*.[9] A HORIBA Fluoromax-4 spectrofluorometer was used to measure the bioluminescence emission of the synthetized compounds.

### Ion sensing

As detailed in the introduction, a major application for bioprobes is biosensing, especially to monitor inorganic ions which are essential for cellular activity.

Researchers from the College of Pharmacy at Seoul National University (Korea) have developed novel fluorescence cellular iron ions sensors to better understand cell death processes.[10] Their work required advanced characterization tools. A HORIBA FluoroMax-4 spectrometer was used to study the fluorescence response of their chemosensors in the presence of various metal ions. The brightness of the probes was assessed by measuring the absolute quantum yield with an integrating sphere assembly. Finally, kinetics information was obtained through fluorescence decay curves and analyzed using the DAS6 decay analysis software.[11]

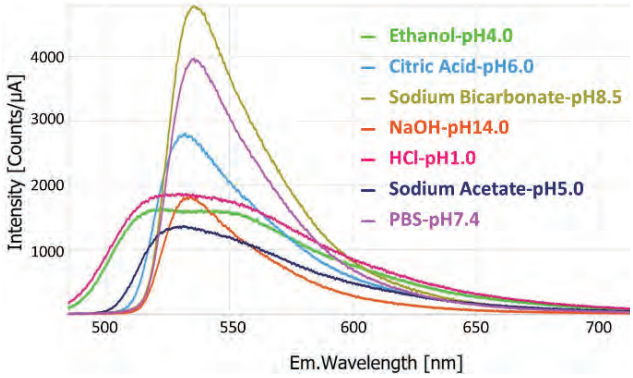



Figure 10 Fluorescence spectra of the widely used FITC dye (Fluorescein isothiocyanate) in different solvents and under varying pH conditions.

Another group from the College of Chemistry and Materials Science from Nanning (China) proposed an efficient fluorescent sensor to detect Ag+ but also L-cysteine in complex biological fluids and living cells using nitrogen, boron, sulfur co-doped carbon dots. The fluorescence emission profile, quantum yield, as well as lifetime of those nanostructures were determined using a HORIBA QM-8075 QuantaMaster instrument.[12]

### Microenvironment

Bioprobe fluorescence spectra are very sensitive to local environment, including solvent polarity, pH and temperature. This sensitivity is often leveraged in cell physiology research as many processes (metabolism, cell proliferation, membrane potential, etc.) are affected by changes in these parameters. Figure 10 shows the evolution of FITC emission properties under different pH conditions.

Besides pH, other local environmental factors can be assessed with specific tailored probes. Scientists from the National Academy of Sciences of Belarus have for instance designed carboxyfluorescein bifluorophores as viscosity sensors in model lipid bilayers or internal membranes of live cells.[13] Indeed, viscosity is one of the key parameters that controls the diffusion rate of molecular species and affects the reaction speeds of diffusion-controlled processes at the microscopic scale.

For their research, they have used a HORIBA Fluorlog-3 spectrometer to measure the fluorescence spectra and degree of polarization of the molecules.

## A focus on quantum dots

An important class of fluorescent nanomaterials is nanoparticles of semiconductors called quantum dots or QDs. QDs are artificially designed nanostructures which display unique emission properties depending on their composition and shape. They offer several advantages over organic fluorophores, among them brighter emission, photostability, broader excitation spectra combined with sharper emission peaks to avoid signal overlap for multi-color labelling.

As quantum objects, QDs emission wavelengths directly relates to their dimension (Figure 11. a), therefore controlling their size at different steps of their production is key. Using a HORIBA SZ-100 analyzer relying on dynamic light scattering (DLS), CdTe QDs with a passivation coating layer were measured in aqueous solution in a microcuvette and revealed a monodisperse distribution centered around 9.9 nm with a standard deviation of 3.4 nm (Figure 11. d)

Because of their brightness, QDs emission profiles can readily be measured with any spectrofluorometer. However, we have seen before that due to inner filter effects (IFE), the fluorescence of bioprobes may not coincide with the true fluorescence emission, even for low concentrations.[14] This is visible in Figure 11.b where InP/ZnS QDs were measured with the HORIBA Duetta instrument capable of simultaneous IFE correction. Besides the obvious difference in fluorescence intensity, we see that the position of the peak maximum is blue shifted by 15 nm.

Apart from controlling the expected optical emission properties, verifying the elemental composition of synthetized or purchased QDs is important to validate chemical synthesis routes, but also for safety and ecological consideration. This information can be easily obtained by using X-Ray Fluorescence (XRF) spectroscopy. Figure 11. c. shows a XRF spectrum of commercially available CdTe QDs functionalized with an organosulfur compound, characterized under solid form with a HORIBA XGT-9000 analyzer.

Typical QDs have the disadvantage of showing potential cytotoxicity for biomedical applications, as their core-

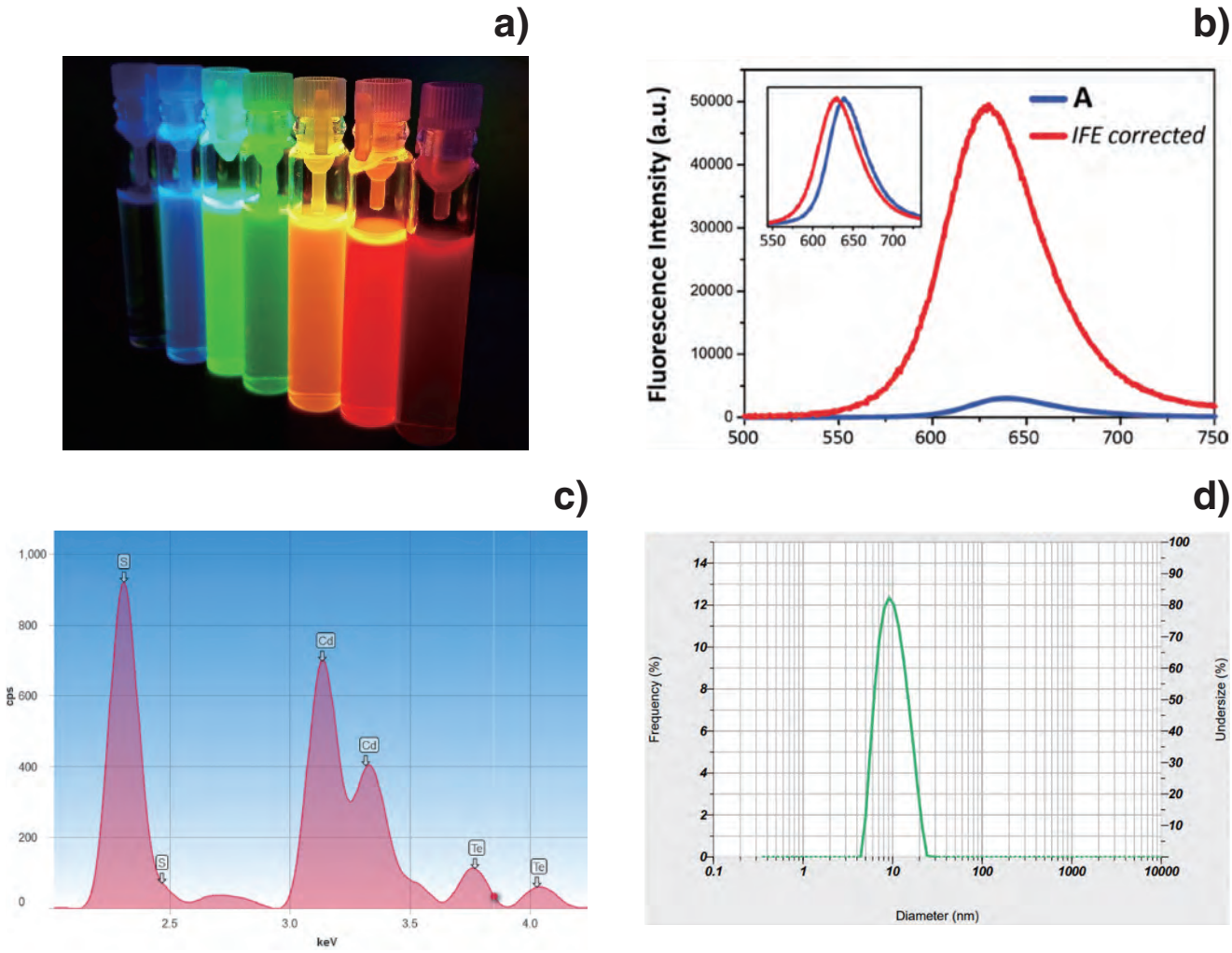



Figure 11 a) QDs of different sizes showing different emission colors. b) QDs fluorescence emission profile extracted from an Excitation Emission Matrix. c) QDs elemental composition determined by X-Ray Fluorescence spectroscopy. d) QDs size distribution obtained by dynamic light scattering.

shell structures often contain heavy metals in the form of binary chemical compounds such as lead sulfide (PbS), cadmium selenide (CdSe), gallium arsenide (GaAs), or zinc telluride (ZnTe), which can generate reactive oxygen species, which in turn damage cellular proteins, lipids, and DNA. Therefore, significant efforts have recently been made to find alternative biocompatible materials.

Such a structure, with low toxicity and chemical inertness can be found in the form of carbon dots. Researchers from the University of Madras (India) recently reported on carbon dot nanocomposites conjugated with chitosan, acting both as a nano-drug carrier (for dopamine encapsulation in this case) and as a delivery tracer through bioimaging.[15]

Besides photoluminescence characterization of the probes with a HORIBA Fluorolog-3 spectrometer, the authors also employed Raman spectroscopy with a HORIBA LabRAM HR microscope to confirm the intensity ratio of the D band (1378 cm-1) to the G band (1580 cm-1) to control the presence of defects or amorphous carbon.

Another approach to avoid QDs toxicity is to encapsulate them in order to prevent their degradation when internalized, and to control their release at the right target. Our FluoroMax user at the University of Leeds (UK) proposed "sweet" QDs capped with mannose, (a sugar monomer important in human metabolism), to probe multivalent interactions of HIV/Ebola receptors using a sensitive, ratiometric FRET assay. The detection scheme is based on the fluorescence intensity ratio measured at wavelengths of 626 nm and 554 nm.[16]

## Conclusion

We have seen that fluorescence bioprobes have become tools of choice for various applications in bioimaging and biosensing.

HORIBA Scientific offers a comprehensive suite of analytical tools capable of covering the variety of characterization needs required for the design and the optimization of chemical synthesis routes of such novel probes.

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

## References


[1] https://www.horiba.com/en_en/products/detail/action/show/Product/duetta-1621/

[2] *Reversible and Broad-Range Oxygen Sensing Based on Purely Organic Long-Lived Photoemitters*. Applied Polymer Materials, 3 (2020). https://pubs.acs.org/doi/10.1021/acsapm.1c00064

[3] *CAPRYDAA, an anthracene dye analog to LAURDAN: a comparative study using cuvette and microscopy*. Journal of Materials Chemistry B, 8 (2020). https://doi.org/10.1039/C9TB01738K

[4] https://www.horiba.com/en_en/products/detail/action/show/Product/flimera-1989/

[5] *Viability of Saccharomyces cerevisiae incorporated within silica and polysaccharide hosts monitored via time-resolved fluorescence*. Photochemical & Photobiological Sciences, 12 (2013). https://doi.org/10.1039/C3PP50202C

[6] *Harnessing combinational phototherapy via post-synthetic PpIX conjugation on nanoscale metal-organic frameworks*. Journal of Materials Chemistry B, 31 (2019). https://doi.org/10.1039/C9TB01154D

[7] *Co-encapsulating indocyanine green and CT contrast agent within nanoliposomes for trimodal imaging and near infrared phototherapy of cancer*. Nanomedicine, 29 (2020). https://doi.org/10.1016/j.nano.2020.102269

[8] https://research.pasteur.fr/fr/project/design-and-optimization-of-new-bioluminescent-systems-better-suited-to-in-cellulo-and-in-vivo-conditions/

[9] A Bioluminescent Probe for Imaging Endogenous Peroxynitrite in Living Cells and Mice. Analytical Chemistry, 90 (2018). https://doi.org/10.1021/acs.analchem.8b00198

[10] *Novel turn-on fluorescent biosensors for selective detection of cellular Fe3+ in lysosomes: Thiophene as a selectivity-tuning handle for Fe3+ sensors*. Dyes and Pigments, 169 (2019). https://doi.org/10.1016/j.dyepig.2019.05.008

[11] https://www.horiba.com/en_en/products/detail/action/show/Product/das6-1377/

[12] *Red emission nitrogen, boron, sulfur co-doped carbon dots for "on-off-on" fluorescent mode detection of Ag+ ions and l-cysteine in complex biological fluids and living cells*. Analytica Chimica Acta, 1035 (2018). https://doi.org/10.1016/j.aca.2018.06.051

[13] *Fluorescent Properties of Carboxyfluorescein Bifluorophores*. Journal of Fluorescence, 30 (2020). https://doi.org/10.1007/s10895-020-02535-w

[14] https://static.horiba.com/fileadmin/Horiba/Application/Materials/Material_Research/Quantum_Dots/How_IFE_Can_Affect_Fluorescence_Measurements.pdf

[15] *Luminescent chitosan/carbon dots as an effective nano-drug carrier for neurodegenerative diseases*. RCS Advances, 10 (2020). http://xlink.rsc.org/?DOI=d0ra04599c

[16] https://www.leeds.ac.uk/news/article/3841/quantum_dots_light_the_way_for_new_hiv_and_ebola_treatment



Florian FORMANEK

HORIBA Scientific, France
Global Life Sciences Market Manager
Ph. D.

# Providing Solutions for the Life Science Field Using Spectroscopic Analyzers

UCHIGASHIMA Mikiko

We provide solutions using surface plasmon resonance imaging device, particle tracking analysis device, Raman spectroscopic device, etc. for research on drug discovery and formulation of antibody drugs, quality control, and research on medical treatment and drug discovery using exosomes, which have been attracting attention in recent years. I will introduce the outline of these products and measurement examples, I will also introduce leading research and development in the life science field, which we are working on in collaboration with external organizations.

## Introduction

The HORIBA Group's scientific analysis device applications are employed in extensive areas of research and manufacturing. Aiming to expand the use of our applications and augment our provision of solutions in bioscience and life science in particular, HORIBA launched our commitment to these fields in 2014. Backed especially by the spectroscopic-technology-based products among all of our scientific instruments, we target the pharmaceutical, food, and cosmetic markets, with a special emphasis on pharmaceutical products. We seek to establish ourselves as a provider of measurement instruments used in all drug-related settings, from drug discovery and formulation studies through manufacturing. By "measurement," HORIBA contributes to not only conventional small-molecule drugs but also diversifying modalities, such as antibody drugs, nucleic acid drugs, cell therapy drugs, and gene therapy drugs. Here I share with you some examples of our business deployment with our spectroscopic technologies in the pharmaceutical market and also our new engagement in the life science field based on collaboration with external organizations.

## Technologies and products

HORIBA's measuring technologies are employed in various settings in the life science market as well. Recently drawing much interest in particular is the use of surface plasmon resonance (SPR) imaging, particle tracking analysis (PTA), and Raman spectroscopy for medical research and development and drug quality control purposes.

SPR allows monitoring of label-free molecular interactions. Parameters such as the association constant, dissociation constant, and affinity/binding activity can be assessed based on refractive index changes caused by the interaction between molecules immobilized on the metal film sensor surface and analyte molecules that are injected over the surface. Interactions between not only the same types of biomolecules (e.g., protein-protein, DNA-DNA) but also different types (e.g., DNA-protein) are analyzable, and so are interactions on the cell surface, such as antibody-microorganism interactions. HORIBA's SPR imaging (SPRi) system tracks binding of molecules immobilized in an array format onto the sensor chip; the imaging capability makes simultaneous monitoring of multiple interactions possible.

PTA is a methodology for tracking the scattered light from each laser-illuminated particle. Since the rate of the Brownian motion of particles differs depending on their sizes, the diffusion rate of each particle can be used to determine each particle size and particle count in the sample. PTA thus enables both size measurement and counting of nanoparticles.

Ramon spectroscopy is a spectroscopic technique used to acquire information on the molecular structure and composition of the analyte of interest through detection of Raman scattering from the illuminated analyte. Being a highly molecular specific, non-destructive, and non-contact analysis with no special pretreatment of samples required, this technique is expected to be extensively applied in the bioscience and life science fields.

Here are some examples of antibody drug and exosome analyses to which applications of these technologies were employed.


Figure 1 OpenPlex

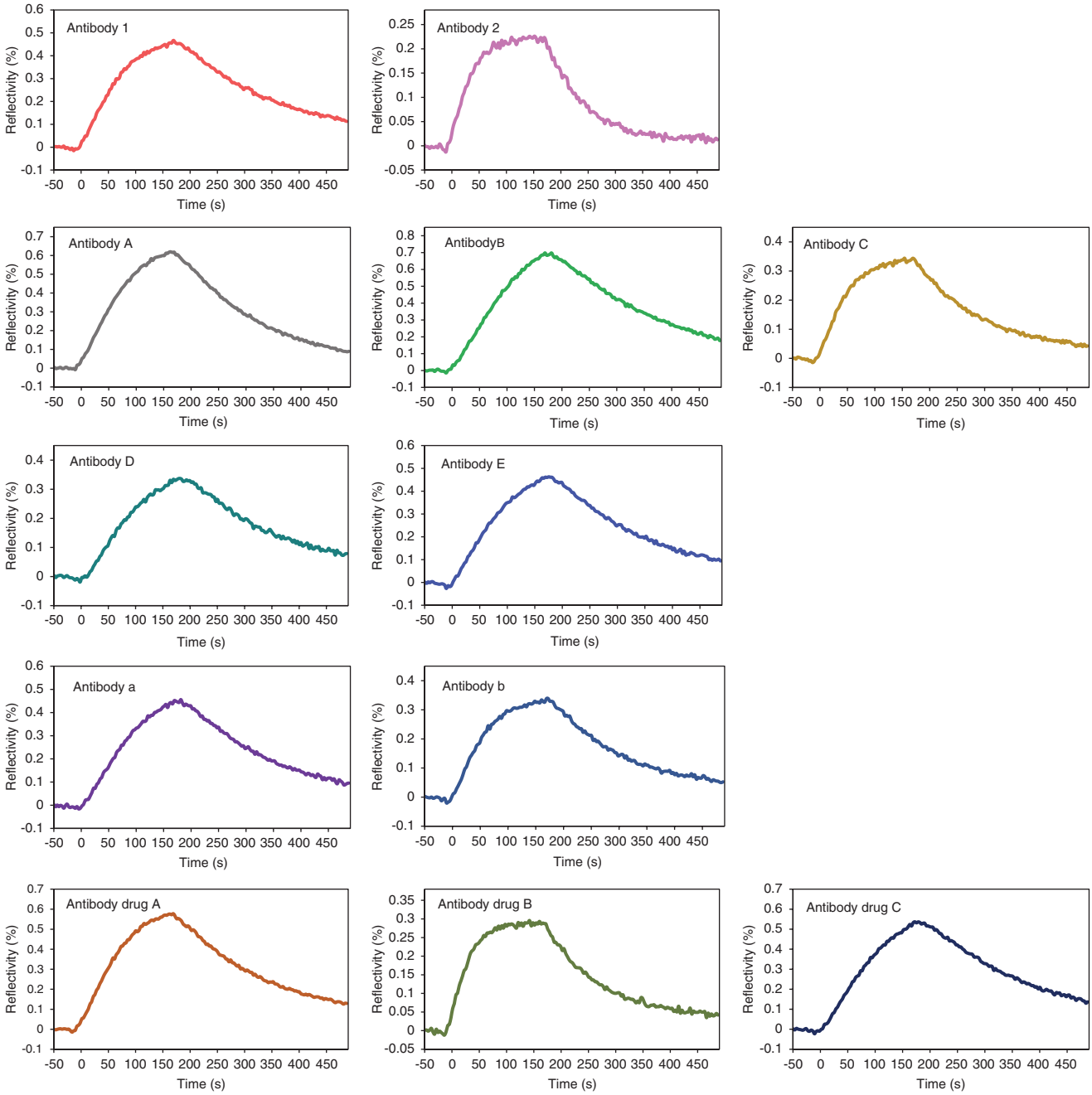



Figure 2 Measurement results with OpenPlex

## Applications

### Antibody drug analysis

Recent years have witnessed diversification of pharmaceutical forms with the emergence of biopharmaceuticals, taking over conventional small-molecule drugs. Possessing more complicated molecular structures, biopharmaceuticals require different methods for physicochemical property assessment from those for conventional drugs. For antibody drugs, in particular, which have been actively developed, it is essential to analyze and discuss their functional activity, structural stability, and colloidal stability. I would like to present our solutions to such demand using some measurement examples.

### (1)SPRi measurement example

The binding potency of antibodies can be analyzed using HORIBA's SPRi system (Figure 1). It enables kinetic analysis for interpretation of interactions between antibodies and target analytes, and its multichannel allows simultaneous measurement of multiple interactions. In a case where interaction analysis was performed with 12 types of antibodies immobilized on the sensor surface over which Fc receptors were injected (Figure 2), slight differences in kinetic parameter values due to antibody glycan structural differences were detected. This result suggests that it may be possible to perform assessment in a way suitable for each different mechanism of antibody-antigen interaction (Figure 2).

### (2)PTA measurement example

Antibody aggregation can not only reduce the drug effect but also trigger a side effect(s). Aggregates ranging in size from 100 nm to 10 μm in particular, called sub-visible particles (SVPs), are increasingly recognized as of importance, as reflected in the issuance of SVP guidelines by the U.S. Food and Drug Administration (FDA). PTA is a particle size analysis method with a competitive edge in this regard. Unlike conventional scattering methods, PTA is capable of quantitative colloidal stability analysis based on particle count. With its integrated temperature regulation system, ViewSizer 3000 (Figure 3), our PTA-based instrument, allowed monitoring of increases in particle count over the course of antibody solution heating at 50˚C for a certain time period (Figure 4). This demonstrates that PTA is useful in discussing the colloidal stability of antibody drugs.


Figure 3 ViewSizer 3000

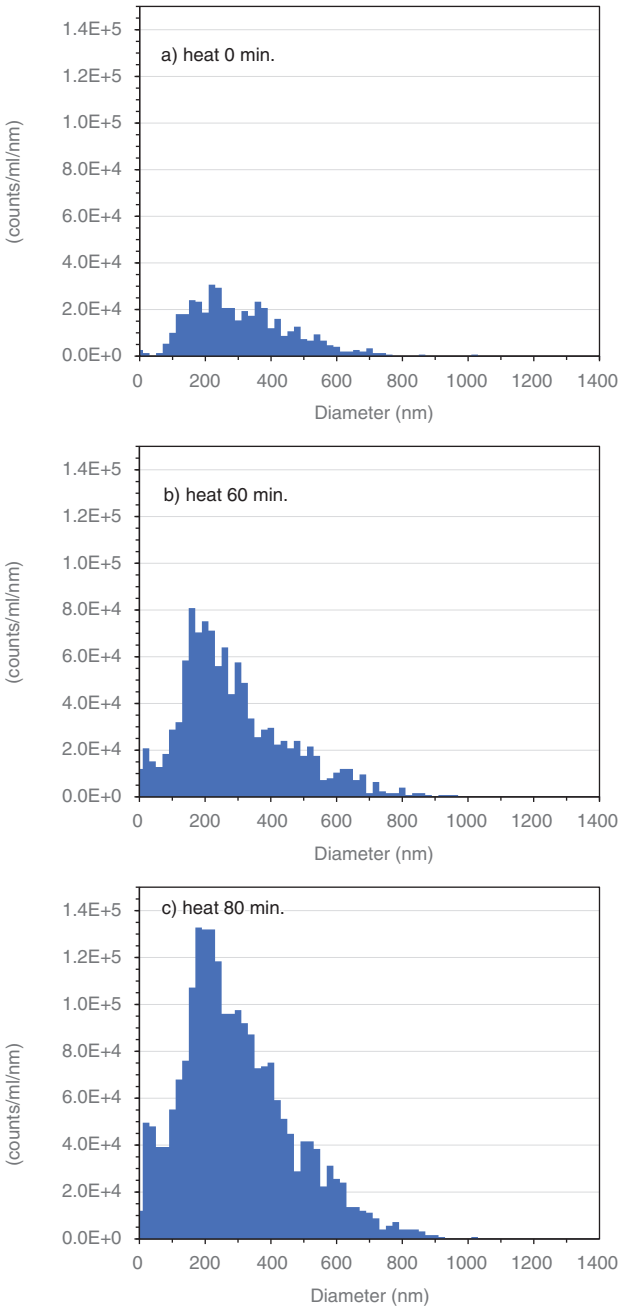



Figure 4 Measurement results with ViewSizer3000

### (3)Raman spectroscopy measurement example

In general, antibody drugs are formulated in high concentrations, ranging from several milligrams to several hundred milligrams per milliliter, and thus require search for formulation conditions that inhibit aggregate formation and selection of highly stable antibodies. Aggregates are evaluated using modalities such as liquid chromatography, small angle X-ray scattering (SAXS), static light scattering (SLS), or dynamic light scattering (DLS), all of which solely provide colloidal information. Moreover, it is challenging itself to measure protein solutions in high concentrations (antibody drugs). However, Raman spectroscopy allows direct measurement of high concentrated solutions, providing information that reflects the secondary and tertiary structures of antibodies. This spectroscopic method was expected to be applicable for structural stability assessment of antibodies under formulation conditions; thus, temperature dependence of Raman spectra was evaluated. The results indicate that antibodies' structural stability may possibly be analyzed based on Raman bands of proteins, aromatic amino acids or amides in particular. This may lead to useful findings in the formulation study of antibody drugs.[1]

The use of analysis systems like those describe above is expected to contribute to not only discovery and formulation studies but also quality control of antibody drugs.

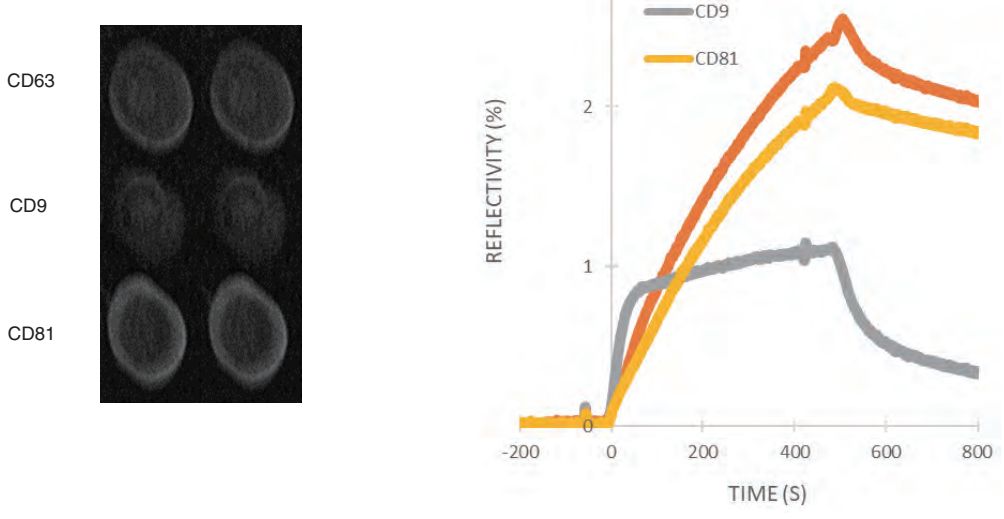
### Exosome analysis

Exosomes are extracellular vesicles (Ø50–150 nm) released from cells and are present in our body fluids like serum and urine. They bear surface lipids, proteins, and sugars and contain protein and nucleic acid inside; their constituents differ depending on the cells that release them. This means that exosomes released from disease-related cells exhibit molecular profiles specific to the disease, and such molecules in exosomes can be targeted in liquid biopsies. Exosomes are also studied for possible applications as a drug delivery system, attracting attention from both medical care (clinical testing and diagnosis) and drug discovery (therapeutic drug development) aspects. Here the applicability of SPRi as a method for exosome surface protein identification and that of PTA for exosome particle concentration measurement are discussed using studied examples.

### (1)SPRi measurement example

By immobilizing numerous ligands (192 at maximum) at specified spots on the biochip surface, OpenPlex enables simultaneous monitoring of many interactions, and its imaging function allows visualization of the interactions. Further, as another major advantage, analytes relatively in large sizes, such as cells, bacteria, and exosomes, are also assessable on OpenPlex.

Here is an example of SPRi measurement. Exosomes were purified from human serum by ultracentrifugation.




a) b)

Figure 5 Exosomes detection results with OpenPlex
a) Spot Imaging
b) Reflectance change

The biochip treated for low non-specific binding was used on which antibodies to exosome marker proteins (CD9, CD63, and CD81) were immobilized. An exosome solution 200 µL (≥1010 particles/mL) was injected over the biochip surface for analysis of interactions with the antibodies; interactions with all the antibodies were monitored by way of both imaging and percent changes in reflectivity (Figure 5). This result demonstrated the usefulness of SPRi as a means to identify exosome surface proteins.[2]

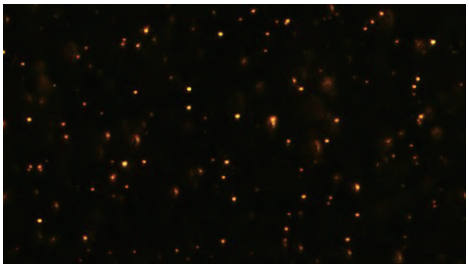
As stated above, OpenPlex is capable of identifying many different exosome surface proteins in a single run thanks to the use of a biochip with various ligands immobilized on its surface. This feature has gained OpenPlex a high reputation and expectations as a tool suitable for exploration and screening of novel markers and for quality control required in drug discovery and application.

### (2)PTA measurement example

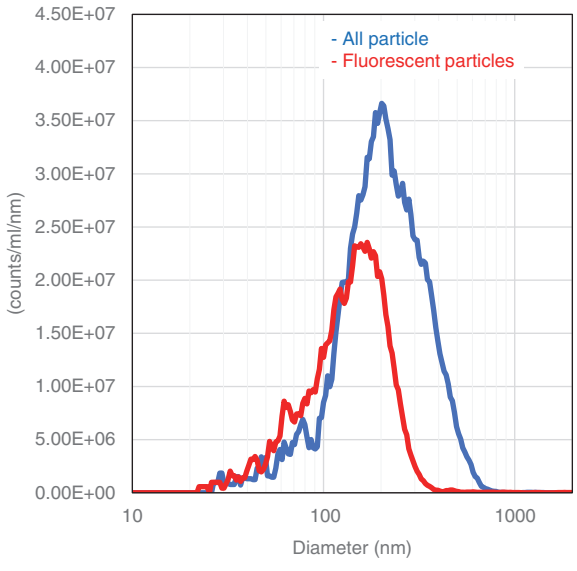
Equipped with three-wavelength laser light sources, ViewSizer 3000 uses for analysis a red laser for large particles and a blue laser for small ones and is thus capable of measurement of particles of a wide range of sizes. Fluorescently stained particles can also be measured by using fluorescent dyes of matching wavelengths.

Here is an example of exosome measurement by the ViewSizer 3000. Exosomes were purified from human serum using MagCaptureTM Exosome Isolation Kit PS (FUJIFILM Wako Pure Chemical Corporation, Osaka).[3] The resultant exosome solution was diluted 100-fold, and the target exosomes were stained with a fluorescent reagent. The size distribution of fluorescent particles was analyzed on ViewSizer 3000 in the fluorescence mode, and then all particles in the solution were counted. Approximately 40% of all particles were found to be stained ones (Figure 6). The above result indicates that ViewSizer 3000 is a useful tool for determining the proportion of exosomes among all particles. This instrument has thus won a reputation for being effective in detecting target exosomes and is expected to contribute to advancing drug discovery.

As described above, we are committed to disease-specific protein identification research, hoping to make contributions to basic medical science and drug discovery and development in which exosomes are involved. We also seek to contribute more to drug discovery and development for the aforementioned biopharmaceuticals and other various modalities by prompting further extensive use of our analyzers with advantageous features, such as OpenPlex's "multiplexed bioassays" and "analysis of interactions with cells and with bacteria" and ViewSizer 3000's fluorescence mode measurement employing three wavelengths.



a)




b)

Figure 6 Exosomes detection results with ViewSizer3000
a) Fluorescence imaging
b) Particle size distribution comparison

## Collaboration with external organizations

In addition to the above-stated provision of the spectroscopic-analysis-based solutions, we engage in collaborative work with external parties in the life science field.

### (1) LC-Raman: a high-performance liquid chromatograph-Raman spectrometer combined system

Shimadzu Corporation and HORIBA Ltd. initiated joint development of the LC-Raman system in 2020 and launched it in the market in June 2021. The high-performance liquid chromatograph separates a mixture sample into components, which are separately loaded into wells of the collection well plate; the Raman spectrum of each component is recorded using a Raman spectrometer. Since each component of a sample is separately analyzed by Raman spectrometry, it is possible to acquire molecular information on each component, which has been hard for mixture samples. This system is anticipated to contribute to the search for unknown natural functional components, biomarkers in biological samples, and more.

### (2) Moonshot Agriculture, Forestry and Fisheries Research and Development Project

"Building a platform for sustainable farming by environmental control based on the microbe atlas of the soil"

In line with Moonshot Goal #5 "Creation of the industry that enables sustainable global food supply by exploiting unused biological resources by 2050," established by the Cabinet Office of Japan, this project aims to define the requirements for a soil suitable for crop growing ("what is a healthy soil") and to create a sustainable food supply industry directed at agricultural activation and diet of the future, with eyes on "soybeans" as a future-oriented food. It is intended to establish a "platform for recycle-oriented cooperative agriculture" by applying technologies and software that enable such a form of agriculture on the basis of analysis and control of interactions between soil microorganisms, crops, and the environment. HORIBA Ltd. assumes the task of soil mineral ion measurement over the course of soybean growth, utilizing the electrochemical sensor technology that we have long nurtured. Linking soybeans' mineral ion values and molecular biological information may lead to the development of life-science-field solutions with the aid of not only spectroscopy but also electrochemical sensors as seeds for success.

### (3) Cross-ministerial Strategic Innovation Promotion Program (SIP)

"Technologies for smart bioindustry and agriculture"[4]

HORIBA takes part in the research for "technologies for smart bioindustry and agriculture" in SIP. The group for this research aims to establish a smart food chain that allows optimization of food distribution and processing based on data sharing, from production through distribution and consumption. HORIBA is tasked with assessment of residual chemicals in agricultural products. Control of residual agrochemicals is essential for food safety and security assurance. We are working on the development of an immunochromatographic kit suitable for simple on-the-spot measurement of residual chemicals in agricultural products, utilizing a monoclonal antibody for agrochemicals developed in-house.

### (4) Other

In addition to the above, we are pursuing various kinds of collaborative work related to pharmaceutical and cosmetic research and development with external partners, such as universities and research institutes. Here I have presented information primarily on biopharmaceuticals, but we are also active in the small molecule drug field, e.g., fluorescence spectroscopic evaluation of drug substances' crystalline nature[5] and Raman spectroscopy-based measurement of skin permeability of active ingredients, which is critical in dermal drug assessment.[6] Our expertise in measurement for the skin is garnering much attention from the cosmetic industry.

## Closing remarks

Besides the examples described herein, we are working on measurement instruments and applications suitable not only for drug discovery and development but also for production process and quality control, such as analysis of the active ingredient in a tablet using a transmission Raman spectrometer[7] and Raman spectroscopy- and fluorescence spectroscopy-based analysis of culture media in a mass cell culture bioreactor, which is required for the production of antibody drugs and gene therapy drugs.

We intend to accelerate our contribution to the life science field, including pharmaceuticals, foods, and cosmetics, by further continuing to provide various solutions.

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

## References


[1] "The Molecular Interaction of a Protein in Highly Concentrated Solution Investigated by Raman Spectroscopy" C. Ota, S. Noguchi, K. Tsumoto: Biopolymers, 103(4), 237–46 (2015)

[2] "Extracellular Vesicle (EV) Analysis by Surface Plasmon Imaging" [in Japanese] Y. Takada, T. Shibuta, D. Irikura, Y. Ono, Y. Hirooka, T. Umemura: The 66th Annual Meeting of the Japanese Society of Laboratory Medicine (Okayama), November 19–24 (2019)

[3] "Particle Tracking Analysis-based Comparison of Exosome Purification Methods" [in Japanese] K. Saihara, D. Irikura, Y. Yang, T. Kono, S. Komatani: The 7th Congress of the Japanese Society for Extracellular Vesicles (Tokyo), October 26–27 (2020)

[4] https://www8.cao.go.jp/cstp/gaiyo/sip/index.html

[5] "Assessing Amorphous State of Active Pharmaceutical I ngredient Using Fluorescent Fingerprint" [in Japanese] R. Haku, H. Arai, T. Takayama, T. Koide, T. Fukami. The 141st Annual Meeting of the Pharmaceutical Society of Japan (Hiroshima), March 26–29 (2021)

[6] "Advanced Formulation Design for Topical Creams Assisted with Vibrational Spectroscopic Imaging" Y. Ozawa, Y. Watanabe, D. Ando, T. Koide, T. Fukami: Chem. Pharm. Bull., 69, 271–277 (2021)

[7] "Raman Spectroscopic Approach in Production Process of Pharmaceutical Solid Formulation" [in Japanese] S. Kashiwagi, R. Ohashi: J. Jpn. Soc. Pharm. Mach. Eng., 27(2), 154–163 (2018)



UCHIGASHIMA Mikiko

Deputy Project Manager
Bio/Life Science Project, Sales Division
HORIBA, Ltd.

# History of HORIBA Medical Products to Contribute to the In Vitro Diagnostic Testing

NOMURA Naoyuki

Medical and health care is one of the greatest topic on development on society development and sustaining. Most of the medical and health care treatment is based on diagnosis based on the result from various testing, analysis and interviews. In vitro diagnostic medical devices are critical testing source to achieve necessary care to the patient. This article describes how HORIBA medical have been supporting the medical field by providing IVD medical device.

## Introduction

Globally, over several trillion dollars of annual spending is recorded for medical practices, with an increasing trend every year. Of the practices, while it is needless to say that diagnoses and treatments of diseases are essential parts, testing also plays a crucial role in supporting diagnoses, treatments and management of outcomes thereof.

In particular, data obtained on in vitro diagnostic (IVD) medical devices, which enable quantitative and qualitative reporting of intracorporeal signals that are invisible to our eyes, are considered highly important indicators in clinical practice.

Here, I describe IVD devices' roles and HORIBA Medical's engagement in hematology and clinical chemistry, basic but important fields in clinical testing, and also in the point of care testing (POCT) field, which places emphasis on speed and simple procedures in the recent ever-diversifying medical practices.

## Hematology field

### What is Hematology?

Blood circulating in the human body transports substances essential to maintain life and eliminates waste products. Depending on the physical state, substances found in blood for transportation or removal vary, making blood the most fundamental sample in understanding the body's condition.

Blood consists of a fluid component called plasma and blood cells, which travel through the plasma. Hematology in the context of clinical testing mainly refers to blood cell analysis, i.e., cell counting (white blood cells [WBCs], red blood cells [RBCs], and platelets) and RBC hemoglobin concentration, among others. Analysis of coagulation or hemostasis is related to hematology testing since these phenomena are activated by coagulation- or fibrinolysis-associated platelet reactions, but here hematology limited to blood cell counting is discussed.

### Hematology testing (significance)

Blood cell counting, i.e., complete blood cell count (CBC), refers primarily to RBC, WBC, and platelet counting.

Briefly, an increase or decrease in these blood cells reflects a certain state of the body: Anemia condition is reflected in the count of RBCs, transporters of oxygens throughout the body; the state of protection against foreign materials that have entered the body (i.e., immunity) in the count of WBCs, which have an immunological function; and the tendency to develop blood clots in the count of platelets, which can induce blood coagulation.

In combination with other test results, the disease state or condition is diagnosed. The CBC is the simplest test and is highly useful for screening that leads to a definitive diagnosis.

In principle, the most primitive method of blood cell counting is estimation by manual microscopic counting.

As a simple method using relatively readily available tools (a general microscope and a hemocytometer), microscopic counting is still employed nowadays in some situations; however, since around the 1950s, automated counting with higher efficiency has been increasingly in demand as the testing frequency has risen and the issue of measurement errors associated with laboratory staff skills has surfaced.

The advent of a blood cell counter based on the electrical resistance method, taking advantage of blood cells' dielectric property (also known as the Coulter method after the inventor of this device's basic principle), rapidly prompted further advances in cell counting instruments. The electrical-resistance-method-based technology allowed for analysis of cell sizes as well as cell numbers, hence mean RBC volume and hematocrit determination, making them reportable parameters. Concurrently, an improvement was added to the system to enable automated lysis and colorimetric analysis for quantification of hemoglobin in RBCs; further, by optimizing the lytic process, nucleus-size-based morphological WBC classification into lymphocytes, granulocytes, and others became possible (three-part WBC differential).

With various technological improvements incorporated into automated cell counter systems, it has become standard to analyze some morphological characteristics of cells and some clinical chemistry variables, beyond simple cell counting.

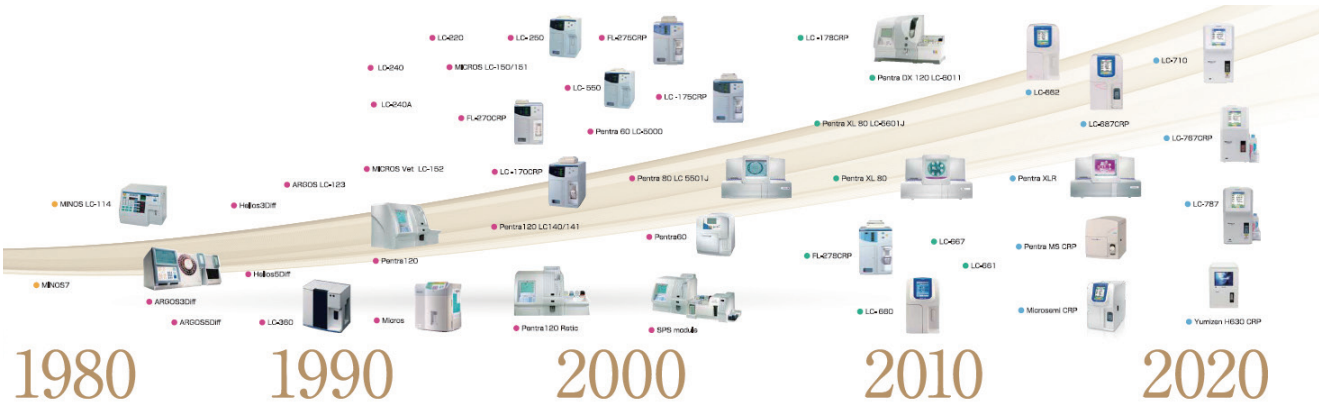
Along with the advancement in automated cell counters, their use further spread and their technological innovations progressed. With respect to WBC differentiation, which had remained a simplified classification, application of flow cytometry technology was promoted to enable a five-part differential as conducted by the manual microscopic approach. Manufactures attempted to produce hematology analyzers with distinctive features to achieve differentiation from competitors' devices, such as the addition of a screening flagging function for various hematological disorders.

Once the analysis technologies were established, the demand expanded, as a general trend, for improved operating efficiency based on fully automated hematology analysis and other laboratory workflow automation. The demand shifted from analysis-parameters- and test-performance-related aspects to solutions for higher operating efficiency, such as automated handling of large quantities of blood collection tubes (e.g., loading and unloading and post-analysis storage of analyzed samples) and within-hospital linkage of electronic patient records for smooth transition from testing to diagnosis and other in-hospital electronic information communication systems.

In parallel, smaller and simpler instruments are wanted so that hematology analysis that simply yields useful data for screening can be carried out with ease at primary care facilities, clinics and medical offices. This demand is described in the Point of Care section below.

## HORIBA's hematology business

The hematology analyzer business by HORIBA Ltd. dates back to 1996, when we acquired ABX S.A., a hematology analyzer manufacturer specialist of France. At that time, against the background of expanding demand for hematology analyzers, ABX was deploying




Note: The actual product launch year differs from the timing of regulatory certification. The time line is an idea based on Japan launch timing.

their business, focusing on compact analyzers for clinics, small hospitals, and small clinical laboratories to perform hematology analysis handily and readily. Figure 1 presents HORIBA Medical's hematology product portfolio in chronological order.

Of those, the three-part differential analyzers Micro Series and five-part differential analyzers Pentra Series have supported HORIBA Medical.

In earlier days, with the concept of micro-volume samples and small footprints, the Micro Series analyzers were deployed, mainly targeting countries having many clinics. In line with the market demand, HORIBA Medical launched an Open Tube model for flexible handling of sampling tubes and a Closed Tube model for analyzing samples in vacuum sampling tubes without the need for the operator to pierce the stoppers, which is expected to reduce the infection risk. HORIBA Medical also enhanced its computer software functions to support complicated day-to-day operation and data management at laboratories as the demand for such a tool grew greater.

For the past 30 years, the Micro Series has supported the brand image of HORIBA Medical's compact analyzers.

The Pentra Series incorporates two novel technological features as additional appealing points: the multi distribution sampling system (MDSS), a dilution method to enable analysis of samples available in micro quantities, and the double hydrodynamic sequential system (DHSS), which enables simultaneous optical and electrical analysis of the inner cell structure and cell volume of WBCs as they pass through a specified path at a controlled rate. The following Pentra Series systems are available: Pentra 60, which performs 60 tests per hour; Pentra 80 with an autoloader, which automatically loads sample tubes placed in the rack into the analyzer and performs 80 tests per hour; and Pentra 120, whose throughput was increased to 120 tests per hour to meet the demand for higher processing speed.

For devices installed at laboratories handling massive quantities of samples, enhanced ranges of precision control and data assurance are demanded. With its products such as peri analytical Pentra ML Data Management software and the slide preparation system (SPS), which automatically prepares samples subjected to final manual review, HORIBA Medical has continued to offer solutions to satisfy the need for automated analysis systems and also support for better system operation with higher efficiency.

The technology platforms of the Micro Series and the Pentra Series have continued to be fostered after ABX joined the HORIBA Group as a hematology specialist. In parallel, driven by technological collaboration with and transfer from Japan-based HORIBA Ltd., HORIBA Medical has acquired the engineering technology and know-how of Japan, achieving a synergistic effect leading to stable analysis system operation, especially in the field of compact analyzers. Of special note, however, is the deployment of an innovative product that was jointly developed by HORIBA Ltd. and HORIBA Medical based on the concept of simultaneous analysis of C-reactive protein (CRP) with other tests. Availability of WBC count together with another inflammation marker, CRP data on a compact analyzer, is highly beneficial in clinical settings, and this concept has long supported the HORIBA Medical product brand. Being particularly common among private practitioners and clinics, this system was in operation at over 10,000 facilities as of 2020 in Japan. While basically an automated hematology analyzer, this


Figure 2 HELO Solution initiatives and Yumizen H1500/H2500 Automation

system is actually used more often for POCT in close proximity to patients. More details are provided below.

Aiming to expand its business into the large hospital and central laboratory environment based on a record of over a decade of Pentra Series operation in the market, HORIBA Medical released its new concept, HORIBA Evolutive Laboratory Organization (HELO) Solution (Figure 2), with the new brand Yumizen established to support the concept.

Product and service lineups for the HELO Solution are to be gradually enhanced, including Yumizen H1500 and H2500, the main analyzers; Yumizen T6000, an automated conveyor system that connects these analyzers; and a management system thereof, Yumizen P8000.

Capitalizing on its technological expertise and experience nurtured on compact hematology analysis systems, HORIBA Medical aims to achieve further growth by expanding its compact analyzer business into the POCT field and providing more extensively comprehensive solutions with its large-sized analysis systems in line with the HELO Solution concept.

## Clinical Chemistry Field

### What is clinical chemistry?

Clinical chemistry analysis deals with body fluid (e.g., blood, urine) components, for example, glucose, cholesterol, proteins, and enzymes. Such analysis enables estimation of the health state, presence of diseases and other abnormalities, and nutritious status. Like hematology, clinical chemistry analysis is deemed to be essential as routine IVD testing.

### Clinical chemistry testing

Clinical chemistry analysis employs colorimetry in principle; namely, a color reaction resulting from an enzymatic interaction between the enzyme used and the target analyte is analyzed spectroscopically. Since body fluid components are present in abundance, their analyzers had been in as much, if not greater, demand as hematology analyzers and were introduced into clinical settings earlier than were hematology analysis systems. The basic flow of clinical chemistry testing is as follows: a body fluid sample harvested in a predetermined amount is subjected to a reaction (or reactions, depending on the analyte) with the designated reagent and then spectroscopic analysis, followed by washing as a post-treatment. The differentiation points for this analysis system lie in the speed, stability, and spectroscopy unit performance sufficient for mass processing of samples. On the other hand, wavelength characteristics differ between different manufacturers' spectroscopy units, yielding different reaction characteristics with each reagent used; that is, performance can vary depending on analysis system and reagent combinations. Data assurance and performance validation are therefore required for individual combinations. This is a feature of clinical chemistry analyzers that is not applicable to hematology analyzers.

Figure 3 Pentra C400

It is of note that, once a system of an automated analyzer was established, the demand grew for simultaneous multi-parameter analysis for varying intended purposes, which led to a broader range of parameters reportable on clinical chemistry analyzers, including electrolytes $Na^+$, $K^+$, and $Cl^-$. These electrolyte parameters are measured using not the above-mentioned enzyme method but the ion selective electrode (ISE) method. Despite this difference in analysis principle, many clinical chemistry analyzers commonly have an electrolyte analysis unit as an integrated module since electrolyte analysis can be performed using the same sample as that for other tests, and electrolyte analysis is frequently conducted for clinical purposes.

Parameters tested on the clinical chemistry analyzer employing not the enzymatic but immunological-reaction-based colorimetric or turbidimetric method are on the increase. This immunological parameter analysis often requires a specific system because of the difference in the reagents used and reactions involved; this field is established as immunoassay. Demand for this assay, which directly identifies proteins and other substances involved in metabolic reactions, is rapidly growing as a useful tool for highly specific diagnoses.

### HORIBA's clinical chemistry business

The clinical chemistry business of HORIBA started with the launch of Pentra 400 (Figure 3) in 1999. It was

just after ABX joined the HORIBA Group, and with solely hematology analyzer systems in the product lineup, concern about weak sales strategies was raised, prompting us to challenge clinical chemistry, another highly demanding field in the IVD market.

What is notable is that it was possible for us to apply the grating technology of Jobin Yvon Inc.—which joined the HORIBA Group at around the time of our entry into the clinical chemistry analyzers market—and the electrode technology developed by HORIBA of Japan to the spectrophotometer, an important analysis unit as described above. In particular, Jobin Yvon's grating technology is of a world-class high standard and is adopted in world-leading clinical chemistry devices.

Our clinical chemistry business started using instruments developed and manufactured by HORIBA and reagents supplied by our original equipment manufacturer (OEM), which was an existing reagent manufacturer, and we currently engage in sales of our products mainly in the U.S. and Southeast Asia. As in our hematology business, we are continuously adding to reagent items and updating our software products in response to user demand.

Our business in the U.S. has been especially stably expanding since 2009, and in line with the HELO deployment, we needed to develop a product lineup to offer to customers handling massive quantities of samples. We chose OEM collaboration with JEOL Ltd., which was seeking a footstep into the U.S. market at that time, over taking the risk of developing analyzers for mass processing of samples in-house, and we decided to deploy our business with a model called Yumizen C1200 in the U.S. After clearing the hurdles for U.S. market entry and fully preparing for the approval for our reagent items, we initiated sales of this analyzer in the U.S. in February 2021.

Meanwhile, in January 2021, HORIBA acquired MedTest Holdings, Inc. of the U.S., a company focusing on the manufacturing and sales of reagents for clinical chemistry analyzers. We thus brought technology and accumulated data on reagents for clinical chemistry analysis into the group, as we had long considered. We aim to further expand our IVD business, capitalizing on three development bases with different fields of specialization located in Japan, France, and the U.S.

## POCT Field

### What is POCT?

POCT refers to testing on the spot, as the name suggests. In the IVD field, in many cases analysis samples are collected at a single site and processed in mass there for higher efficiency, whereas POCT is performed in close proximity to the patient for prompt provision of care needed.

### Significance of POCT

One beneficial example is blood glucose measurement. Strict blood glucose control is required for patients undergoing treatment for diagnosed diabetes. They usually need to measure their fasting, preprandial, and postprandial blood glucose levels not only for disease status management but also for judgment for the need of insulin administration. Especially for the latter reason, it is imperative for patients to keep themselves informed of their own glucose levels to prevent life-threatening hypoglycemia from occurring.

Demand for testing that suggests on-the-spot management suited for each instance has been on the rise annually to address concerns for pharmacotherapy's side effects and decision making for prompt care needed in the event of an acute disease.

As the utility of POCT has become increasingly recognized, its risk has also come to be widely argued. For example, blood glucose measurements are affected by the timing at which the glucose level is measured (e.g.,

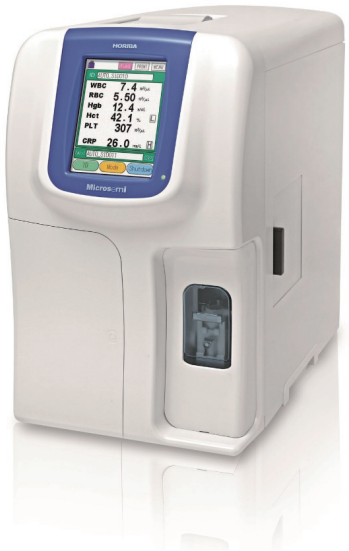



Figure 4 Microsemi CRP


Figure 5 HORIBA POCT Product: (Left) Yumizen M100 Banalyst, (Right) Antsense Duo

during fasting, before or after a meal) and by how it is measured (e.g., venous sampling, fingertip pricking, posture at the time of measurement). In the case of measurement on a clinical chemistry analyzer system at a laboratory, professional system management is required, including periodic maintenance for system behavioral assurance and routine precision control and calibration for assurance of obtained values. Namely, sample preparation and analyzer management should be undertaken by appropriately trained staff; however, such professionality may be compromised in POCT in exchange for quick test results, which then may lead to risk of medication misuse or misdiagnosis, as some argue.

To avoid such risks, many countries are preparing POCT practical guidelines and regulations on medical device handling. Because numerous diseases require prompt care, the demand for POCT will obviously grow further. However, POCT systems must be such that users are led to correct decisions; otherwise, confusion by misdiagnosis, or in the worst-case scenario, fatal consequences for patients could result. The growth of this field is deemed to depend on the promotion in tandem with regulations and standardization of POCT.

## HORIBA's POCT business

In the aforementioned hematology field, HORIBA Medical has proceeded with product deployment into POCT based on our compact analyzer expertise. Our core product in the POCT field is Micros CRP, an analyzer developed with an innovative concept of simultaneous CRP measurement.

WBC count and CRP levels are both known to fluctuate through the stages of the inflammatory response. WBCs promptly react to invading organisms at the injury site or via bacterial infection, while CRP is produced by hepatocytes in response to cytokines released in the immune reaction. WBCs and CRP thus differ in fluctuating timings and mechanisms, making these parameters useful in screening the inflammatory response at an early stage.

For instance, in the case of bacterial infection, WBCs increase rapidly in response to the invading bacteria, and then CRP levels begin to rise several hours later. In contrast, in the case of viral infection, it is not recognized as invasion by a foreign substance, resulting in negligeable changes in WBC and CRP levels. Antibiotic administration can lessen symptoms if they are caused by a bacterial invasion, but not those by a viral invasion. Yet, it is customary to give antibiotics in viral infection cases for the reason that a bacterial infection can occur or as a tentative measure. Recently, however, it is advised to avoid ungrounded antibiotic use because of the concern about the emergence of drug-resistant bacteria resulting from drug overuse. Simple screening has been shown to be useful in clinical settings.

Since WBC and CRP levels were separately measured on a hematology analyzer and a clinical chemistry analyzer, respectively, two types of samples (whole blood and plasma) were needed. Thus, analysis of this combination was cumbersome, although beneficial. Being a set of many tests run together as a panel, clinical chemistry used to be especially inconvenient for both reporting and utilization of test results for this combination. The novel technology enabling simultaneous WBC and CRP measurement in a single sample of whole blood got rid of the cumbersomeness and created an environment where simple analysis can be performed at appropriate timings. This simultaneous analysis system is considered to be an ideal tool for POCT operation.

The development of a product for simultaneous WBC and CRP assay accelerated our hematology analyzer development in Japan. The first renewed model of the Microsemi Series, customized in design and operability for the domestic general practitioners market, was launched in 2009 in Japan, and in 2013 an overseas model was launched abroad (Figure 4). The models for the domestic market were developed by incorporating the usability demanded by Japanese customers, as we learned from our past sales experience, in addition to performance and functional aspects. The improvements integrated into our analyzer systems, taking account of software and consumable exchange procedures, were also well accepted abroad, contributing greatly to our market expansion.

In Japan, since ABX joined the HORIBA Group, we have been exploring business deployment opportunities for products other than blood cell analyzers as well, setting our eyes on our POCT product portfolio augmentation. We inherited the business operations for the blood glucose meter model Antsense Series from Daikin Industries and Sankyo Co., Ltd. in 2000 and for the automated immunoassay analyzer Banalyst from Rohm Co., Ltd. in 2018 (Figure 5). There is not enough space for a detailed description of them herein, but they are products that will bring about the expansion of HORIBA Medical's product portfolio in the POCT field: Antsense Series as products that are expected to create synergy with the electrochemical and electrode technologies long possessed by HORIBA Ltd. as our core technologies, and Banalyst, a product which employs their original micro total analysis system (μTAS) technology, application of which is anticipated in more extensive areas.

## Closing remarks

HORIBA Medical shall continue to deploy products suitable for each field: hematology, on which we place emphasis, clinical chemistry, POCT, and although not described herein, coagulation. Furthermore, considering the shift in user needs from analysis itself to beyond analysis, we shall proceed with studies for solutions to offer our customers new added value, such as proposals for academic applications, data linkage via the Internet of Things, and services for supporting compliance with the relevant regulatory requirements.

Medical devices are strictly regulated by laws and regulations as important areas protecting people's health and lives. The manufacturers of such devices are responsible for applying a quality management system across the board, not limited to the validity and utility of the device performance. As a healthcare provider, we commit ourselves to user safety and security by abiding by the regulations while offering novel functions, good performance, and services. It is HORIBA Medical's mission to continuously contribute to medicine, healthcare, and patients' better quality of life through analysis operations.

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

**NOMURA Naoyuki**
General Manager
Medical Business Division
HORIBA, Ltd.

# Disruptive Technologies in Haematology for POCT Market

Damien ISEBE
Philippe PIEDCOQ
Benoit THOUY
Aurélien DAYNES
Michael BRUCKNER

A blood count is one of the most common blood tests that provides valuable physiological information about the patient's state. The tests are generally performed in specialized laboratories using "Gold standard" high-throughput diagnostic instruments. These automatic haematology analysers are bulky, need qualified operators for sample and data manipulation, and have more maintenance-related requirements. They also must respond to a growing demand for new complex parameters, complexities that are not required for urgent screening at the Point-of-Care. In this article, we present disruptive technologies for sample preparation and cell detection allowing the development of new generic platforms in haematology for a wide range of Point-of-Care applications.

key words
blood count, sample preparation, cell detection

## Introduction

Blood count is one of the first prescribed and most ordered tests by doctors as it generally constitutes the first step in the diagnosis and monitoring of many pathologies and treatments. It provides physicians with valuable information on the patient's physiological state, allowing them to make treatment decisions. Nowadays, most automated hematology laboratory analyzers are based on costly, bulky, reagent consuming and complex hardware systems for blood preparation and measurement. Moreover, highly qualified and specialized laboratory technicians are required to handle such instruments.

Today and tomorrow's world and society are shaping up new challenges for the healthcare systems such as the one generated by the Covid 19 pandemic. Therefore, for delocalized and personalized health care applications, there is an increasing need for automated, compact and transportable point-of-care blood cell counters, operable without requiring any training or maintenance. Such solutions would eliminate the need for patients to travel into lab testing facilities and shorten the time needed for doctors to make treatment decisions. Point-of-care devices can be suitably deployed at the bedside, private clinics, research laboratories, rural areas as well as in developing countries.

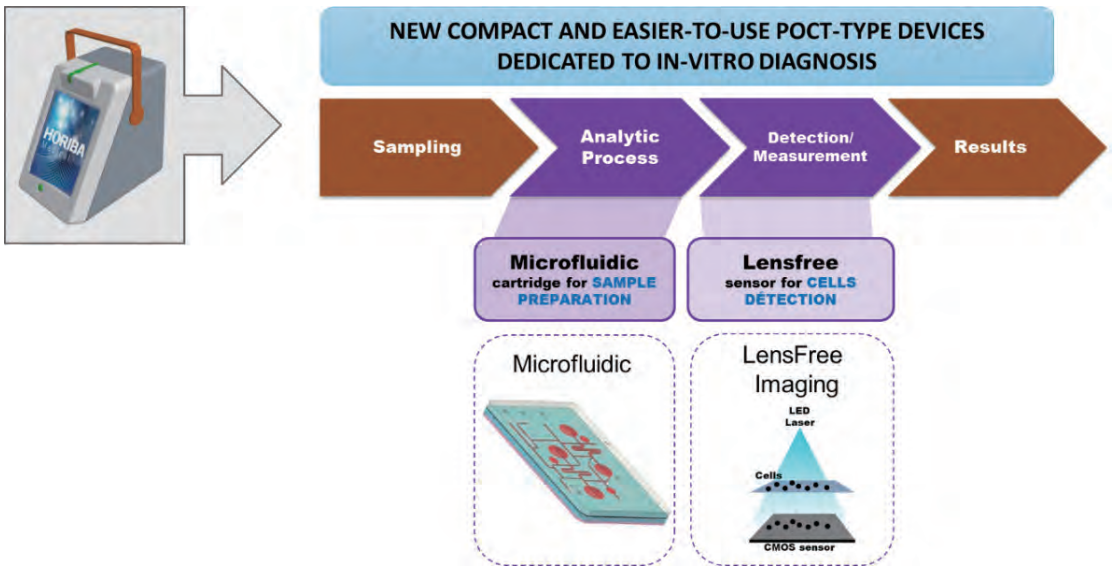



Figure 1 Scheme of the blood analyser principle based on innovative disruptive technologies

Using simple and smart technologies for blood preparation and cell measurement, low power and reagent consumption, these future low cost automated and miniaturized blood cell counters offer a genuine route towards a greater portability of hematology analyzers.

The work presented here is result of an "open innovation" collaborative program held since 2015 with a leading player in technologies development, the French Atomic Energy Commission (CEA), which is the biggest patent applicant in France. By combining their own expertise, both parties can accelerate the delivery of impactful technology solutions (Figure 1).

In this paper, we introduce first a new disruptive technology for cells detection, counting and differentiation, based on holographic lens-free imaging. Since all the optics and mechanical parts are removed, this low-cost technology, embedded in a device, should lead to ease-of-use with minimal operator training and remove the need for user maintenance. Secondly, we introduce an innovative microfluidic preparation cartridge for automated blood cell counters allowing low consumption and scaled-down integration. The whole blood preparation system embedded in these POCT devices answers different key challenges that need to be addressed regarding microfluidics technology: i) a large range of dilution, ii) dilution accuracy, iii) low cost per test and iv) maintenance free.

These two patented technologies[6, 7, 8] are well adapted to a POCT system, and, in the future, will enable us to propose a different approach to hematology, closer to the patient who needs it.

## Lens-Free Imaging

The Lens-Free Technology (LFT) is a simple imaging technique, developed in recent years and under continuing evolution and improvement. Based on in-line holography and numerical processing, LFT gives access to a reconstructed optical field revealing phase and absorption contrasts of the studied samples, which can be cells, particles, crystals, etc... It can generate highly contrasted images of biological objects on a large field-of-view with a micrometer-scale resolution using a very simplified optical setup without magnification. The components of a lens-free imaging system are uncomplicated, small and low-cost. In addition, the wide field of view, together with the high resolution of the sensor, leads to a count (> 10 000 cells) in a single-shot acquisition, consistent with the statistical performances required in haematology. The complexity of the system is transferred to digital processing requiring robust and powerful reconstruction algorithms. Hence, this technology allows the building of an automated, simple, cost-effective, robust, light weight and compact system meeting the requirements for screening point-of-care (POC) tests in human and veterinary markets (Figure 2).

In practice, the sample is simply positioned in a chamber between a light source and a high-resolution optical sensor, for example, a Complementary Metal Oxide Semiconductor (CMOS) (typically a resolution of around 10 MPix and 2 µm pixel size). This sample is illuminated from above by the point-like source of light and the sensor records interferences patterns (holograms) created by a combination of the light diffracted by the objects and the transmitted light. The raw data are then computerized with a numerical reconstruction algorithm, and dedicated

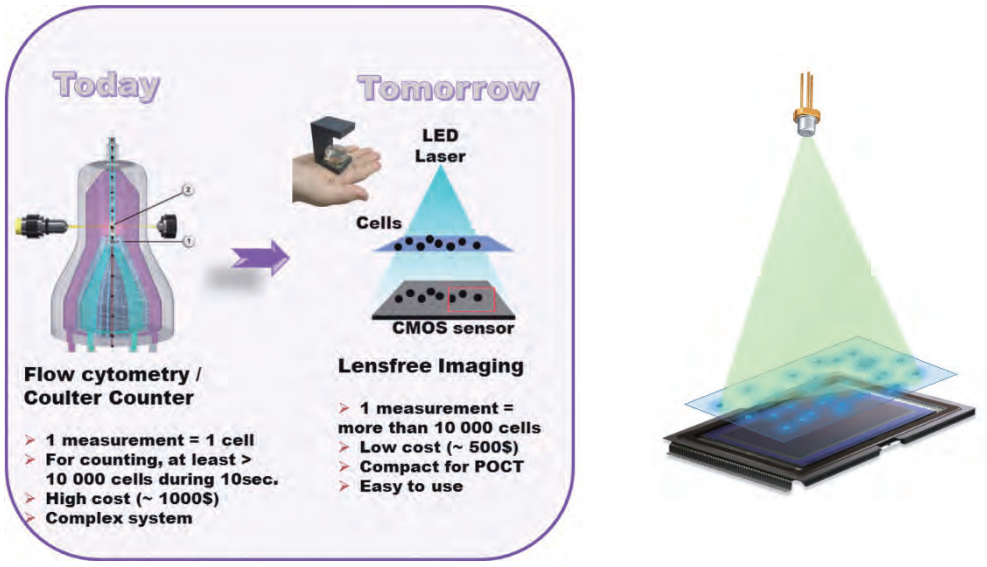



Figure 2 The advantages of the LensFree Imaging system, compared to classical Flow Cytometry setup

image processing workflows are specifically designed for each new application. Briefly, the reconstruction step allows, starting from the raw diffraction patterns acquisition, the reconstruction of an in-focus absorption map. From the acquired defocused hologram, classical gradient autofocus algorithms were applied to compute the position of the object plane (i.e. the focus plane). In this selected plane, an iterative reconstruction algorithm from a single image based on specific norm minimization is used. On this reconstructed image, grey-level thresholding is performed to detect the more contrasted objects. A binary mask is created: a 0 value is associated to the background, and a 1 value is assigned to the detected components. At this stage, there are between hundreds and thousands of components, depending on the sample. The detected components are sorted out based on morphometric characteristics or finer supplementary criteria using grey-level. This leads to the identification and characterization of most of the blood cells, depending on the considered application (Figure 3).

This new technique for measuring cells requires increased computation capacities. We implemented algorithms on an Nvidia Jetson Nano Graphical Processing Unit (GPU)

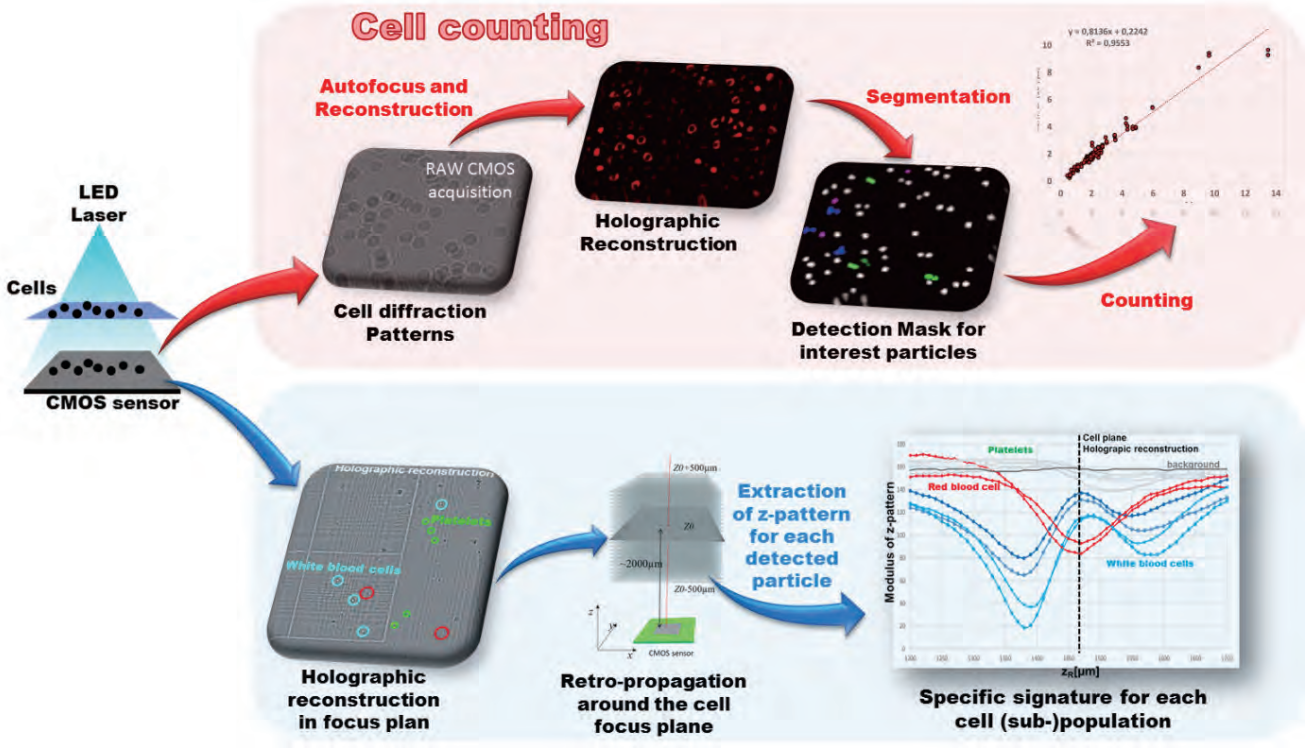



Figure 3 The steps of the algorithm for image reconstruction and cells detection and identification.

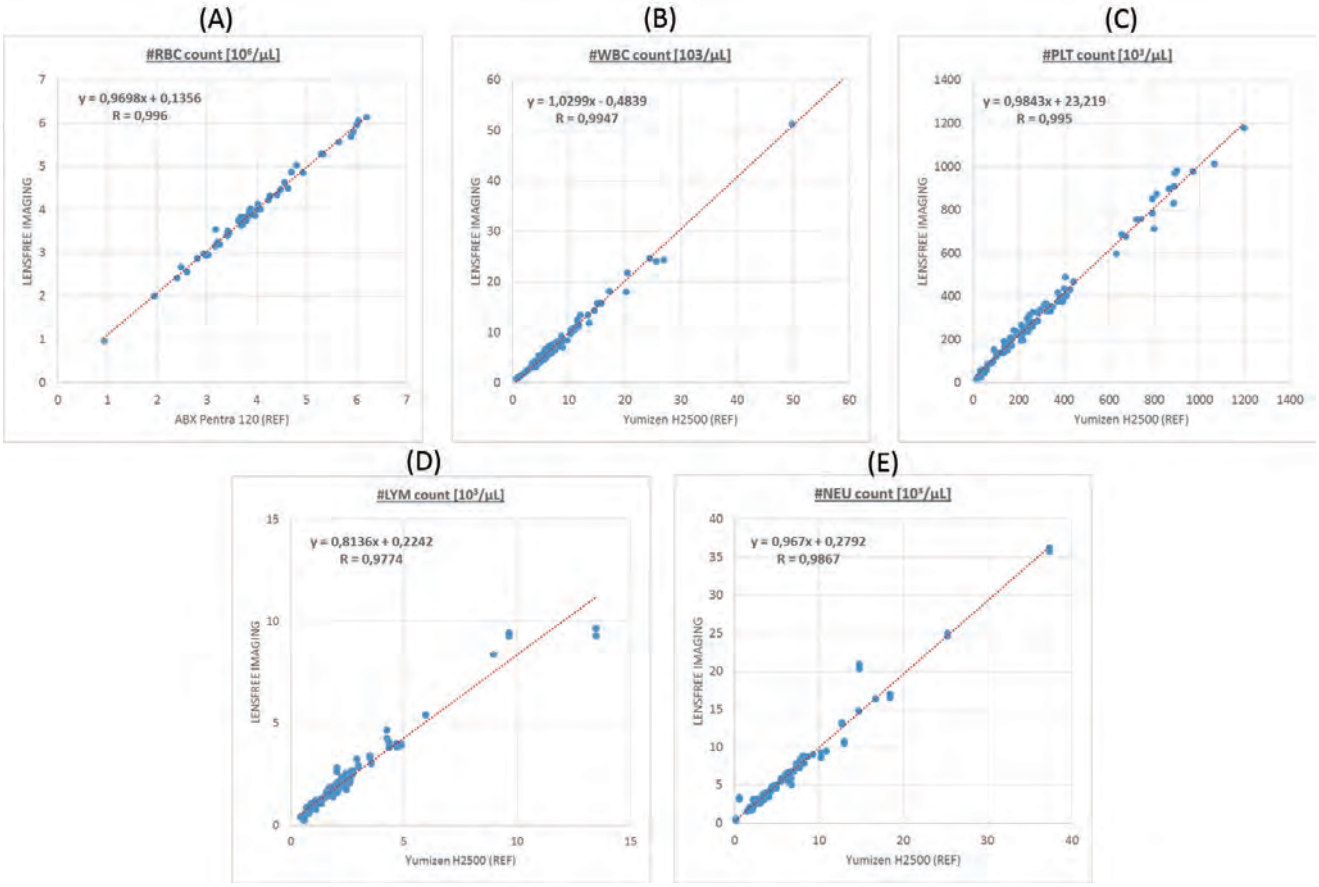



Figure 4 Summary of haematological analytical performances obtained with LFT in comparison with classical measurement systems embedded on current HORIBA Medical routine analysers

for massive parallelization. Increasing connectivity and the versatility of this sensor allow us to consider various and remote application fields.[2]

As a proof-of-concept, several applications have already been investigated with LFT, in the human and veterinary haematology field. We display, in Figure 4, performances obtained for red blood cells (RBC), white blood cells (WBC) and platelets (PLT) counting, as well as for lymphocyte (LYM) and neutrophil (NEU) identification, in comparison with results obtained with our reference HORIBA Medical analyzers (ABX Pentra 120 and Yumizen H2500).

Those preliminary results show good agreement between the LFT and conventional hematology analyzers on this set of parameters. No major discrepancies were observed among the different sample database evaluated (more than 100 normal and pathological samples). R correlation parameters are all located above 0.95.[1, 3, 4]

## Microfluidic Preparation

In this project, the main objective is to develop microfluidic cartridges allowing precise preparation of small samples with great accuracy and repeatability, and scaled-down integration for the associated analyzer. However, developing such a system with a small footprint comes with several challenges regarding microfluidics technology such as: sample introduction, reagent management, efficient fluid sample mixing, low cost-per-test and more importantly, large yet precise dilutions (Figure 6a).

To reach the desired high dilution ratios of the small blood samples and still achieve high precisions, we opted for a technology based on a hyper-elastic membrane to create collapsible chambers with large volumes and high aspect ratios (Figure 6b). Optimal configuration of these chambers and channels was reached through extensive Computational Fluid Dynamic (CFD) simulation studies using the YALES2BIO software (Figure 5). This technology offers a high-volume precision as the amount of used liquids is directly controlled by the chamber size. Futhermore, the microfluidic cartridge can be easily replaced by a “Plug & Play” system, leading to a maintenance free system.

For a first application case as a proof-of concept, the cartridge was designed to handle two precise dilutions simultaneously, one at 1/1000 ratio and another one at 1/10 ratio. The first dilution of the whole blood is performed for counting the RBCs and the second dilution for

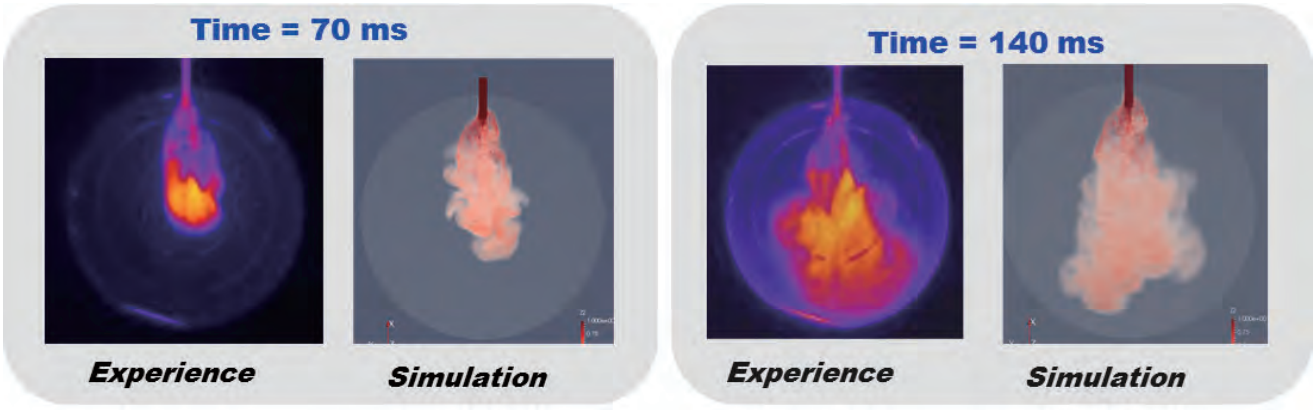



Figure 5 Comparison of sample injection on a dilution chamber. Experience confirms optimal results obtained by CFD computing.

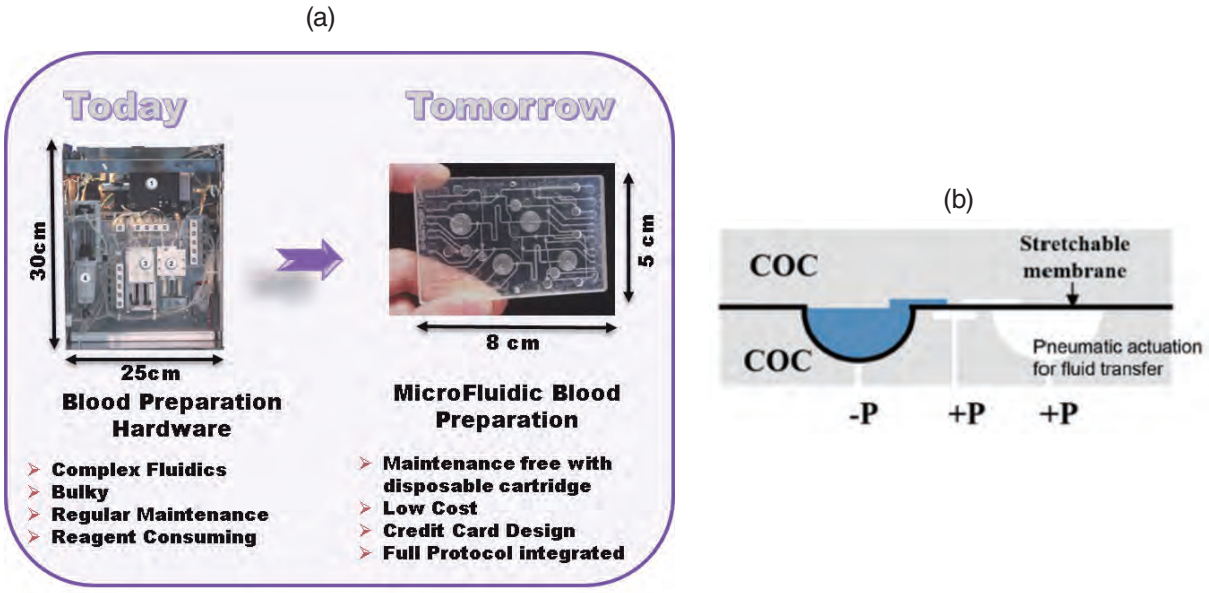



Figure 6 (a) The advantages of the Microfluidic preparation, compared to classical technology, (b) Principle of FlowStretch Technology. Chambers and valves are actuated using pneumatic pressure (COC : Cyclic Olefin Copolymer)

counting the WBC and the PLTs, and for the Hemoglobin measurement. During this second dilution, a lysis of RBCs is performed. This lysis step is necessary for two reasons: first, RBCs are numerous and can hinder the proper visualization of WBCs. Second, the lysis of RBCs allows the release of hemoglobin in the solution, and in this way the measurement of the hemoglobin concentration can be performed.[5]

To achieve the dilution protocol, a specific architecture and an associated protocol is designed. The several zones for different preparations are visible on Figure 7(a) one sample preparation for RBC counting (Zone I), and another one for WBC/PLT counting and Hemoglobin measurement (Zone II). The whole blood is first sampled in two successive cali-brated meandershaped channels (Figure 7(b)). Each meander channel is connected to two stretchable chambers that can be filled with reagents. By successive actions of the two opposed chambers, the calibrated blood sample is efficiently and quickly diluted and mixed with calibrated reagent (Figure 7(b)). The precise and repeatable dilution ratio is simply provided by the ratio volume of the meander channel and collapsible chamber. Thus, the resulting dilution ratio is generated solely by the geometry of the cartridge and does not depend on fluid properties. Repeatability performances obtained for blood parameters with microfluidic preparation are shown in Figure 8 (Left). In Figure 8 (Right), the correlation of Hemoglobin measurement obtained with mock-up coupling Microfluific sample preparation and LensFree Imaging sensor and the one obtained with classical routine analyser is depicted.

Figure 8 (Left) shows a good agreement between the expected statistical performances and the actual Coefficient of Variation (CV) measured during repeatability standard operating protocols. For each parameter, the actual value is slightly higher than the target. This is a normal effect of the variability introduced by the hardware such as the pneumatic driving of the actuated

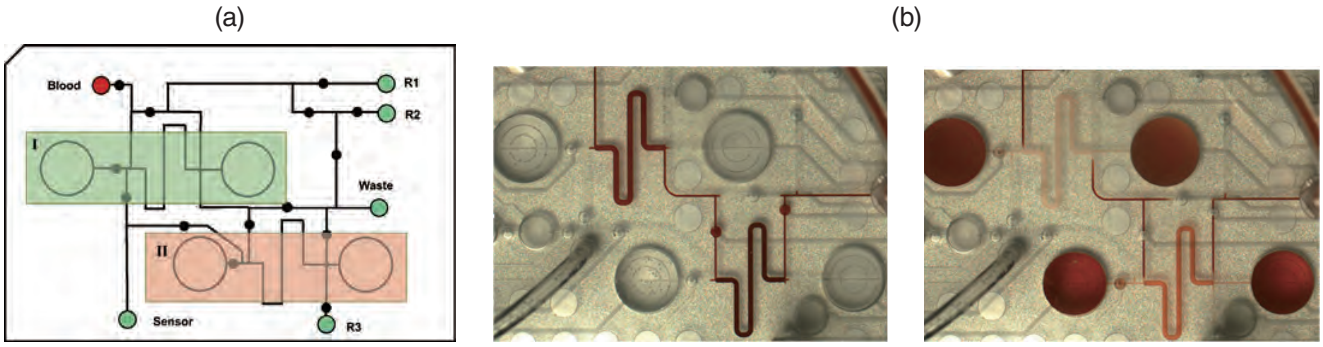



Figure 7 a) The architecture of the microfluidic cartridge with the two regions corresponding to the two performed dilutions. b) Representative images of the sampling and mixing steps in the meander shaped channel with the dilution chambers

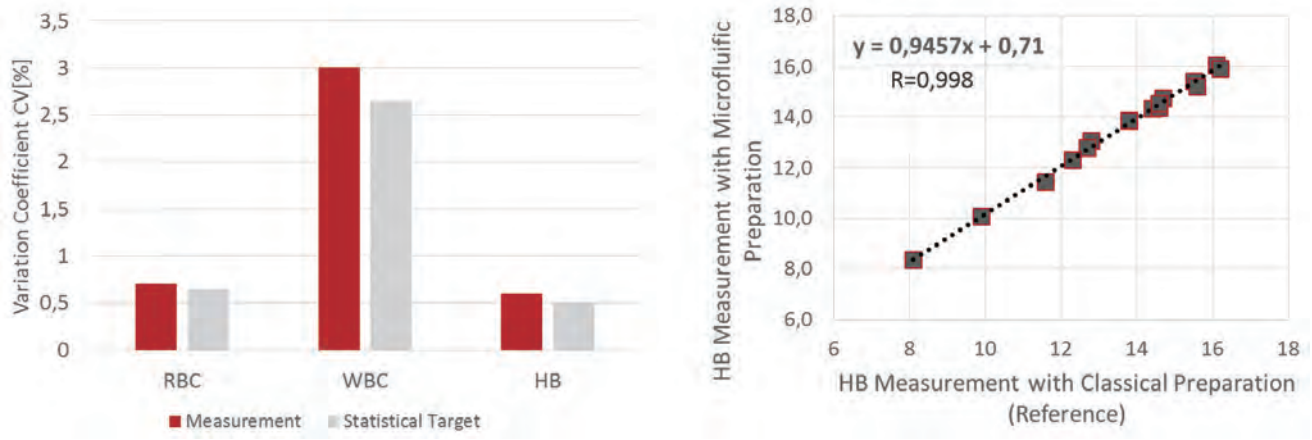



Figure 8 (Left) Repetability performances for blood parameters with microfluidic preparation - (Right) Performances on Hemoglobin Measurement with mock-up coupling Microfluific sample preparation and LensFree Imaging sensor

membrane. Likewise, the correlation on the hemoglobin parameter (Figure 8 Right) shows a good agreement with our qualified internal reference HORIBA Medical Yumizen H2500 It suggests an accurate preparation of the blood sample, from its sampling to the lysis, the dilution and the measurement of concentration.

## Conclusion

These innovative technologies pave the way to the development of new generic platforms for a wide range of POCT applications. The technology leads that we are following are opening a new way of performing diagnostics. By reducing the hardware complexity and the footprint of the instruments, we may now envision smaller, more reliable and more versatile devices. Whether it is at the doctor's office, closer the patient's bed at the hospital, or somewhere remote in the field, we propose two key assets for building more efficient ways of delivering the patient the care he/she needs. We have demonstrated a proof-of-concept feasibility on essential parameters. We now need to work on the enhancement of the algorithms involved in the computation of the LFT results. Indeed, since our primary goal is to deliver impactful insights to the physicians, we need to ensure now that we can address pathological conditions as well as healthy ones.

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

## References


[1] A. Ali-Cherif et al., International Conference on BioMedical Photonics | 16-17 March 2018, Towards a disruptive technology in Hematology: lens-free imaging for high precision red blood cell counting and sizing.

[2] A. Ali-Cherif, et al., MIFOBIO 2018 (GDR ImaBio) | 5-11 Octobre 2018 : Imagerie holographique grand champ pour l'hématologie.

[3] D. Isèbe, et al. "High accuracy platelet counting using LensFree imaging", Proc. SPIE 11075, Novel Biophotonics Techniques and Applications V, 110750O (22 July 2019);

[4] T. Lavabre and al., Detection of circulating microfilariae in canine EDTA blood using lens-free technology: preliminary results, Journal of Veterinary Diagnostic Investigation, 2021, vol. 33(3) 572- 576.

[5] D. Isebe, et al. Reusable microfluidic device for complete blood count applications, MicroTAS, October 2019

[6] Allier, C. Method for identifying blood particles using a photodetector US10379027 filed on March 23, 2016 and issued on August 13, 2019.

[7] Patent. Holographic method for characterizing a particle in a sample US10845286 filed on March 22, 2017 and issued on November 24, 2020

[8] Patent. Microfluidic sample preparation device offering high repeatability US17/291139 filed on November 07, 2019.



### Damien ISEBE, Ph.D.

Innovation Project Manager
Innovation Dept.
HORIBA ABX SAS

### Philippe PIEDCOQ, M.D.

Development Engineer
Innovation Dept.
HORIBA ABX SAS

### Benoit THOUY, M.D.

Algorithm Engineer
Innovation Dept.
HORIBA ABX SAS

### Aurélien DAYNES, Ph.D.

Scientific Expert
Innovation Dept.
HORIBA ABX SAS

### Michael BRUCKNER, Ph.D.

Innovation & Technology Manager
Innovation Dept.
HORIBA ABX SAS

# HORIBA World-Wide Network

## JAPAN

### HORIBA, Ltd.
2 Miyanohigashi-cho, Kisshoin, Minami-ku, Kyoto, 601-8510, Japan
Phone : (81)75-313-8121 Fax : (81)75-321-8312

**Biwako Factory**
1-15-1, Noka, Otsu, Shiga, 520-0102, Japan
Phone : (81)-77-548-6130 Fax : (81)-77-548-6193

**Tokyo Sales Office**
Kanda Awaji-cho Nichome Building, 2-6 Kanda Awaji-cho, Chiyoda-ku, Tokyo, 101-0063, Japan
Phone : 81-3-6206-4721 Fax : 81-3-6206-4730

**Hokkaido Sales Office**
6F Park East Sapporo, 1-3 Minami Ichijo Higashi, Chuo-ku, Sapporo, Hokkaido, 060-0051, Japan
Phone : 81-11-207-1800 Fax : 81-11-207-1802

**Tohoku Sales Office**
4-21-8 Izumichuo, Izumi-ku, Sendai, Miyagi, 981-3133, Japan
Phone : 81-22-776-8251 Fax : 81-22-772-6727

**Tochigi Sales Office**
1F Flora Building, 1-9-15 Higashishukugo, Utsunomiya, Tochigi, 321-0953, Japan
Phone : 81-28-634-7051 Fax : 81-28-634-6099

**Yokohama Sales Office**
1F ShinYokohama Mineta Building, 2-3-19 Shinyokohama, Kohoku-ku, Yokohama, 222-0033, Japan
Phone : 81-45-478-7017 Fax : 81-45-478-7029

**Nagoya Sales Office**
4F BIZrium Nagoya, 3-1-17 Noritake-Shinmachi, Nishi-ku, Nagoya, 451-0051, Japan
Phone*: 81-52-433-3450 Fax*: 81-52-433-3460

**Toyota Sales Office**
2-23 Tsukasa-cho, Toyota, Aichi, 471-0831, Japan
Phone : 81-565-37-8510 Fax : 81-565-37-8511

**Hamamatsu Sales Office**
221-1 Sanjino-cho, Minami-ku, Hamamatsu, Shizuoka, 430-0816, Japan
Phone : 81-53-468-7780 Fax : 81-53-468-7781

**Osaka Sales Office**
4F ShinOsaka UenoToyo Building, 7-4-17 Nishinakajima, Yodogawa-ku, Osaka, 532-0011, Japan
Phone : 81-6-6390-8011 Fax : 81-6-6390-8012

**Shikoku Sales Office**
9-9 Imazato-cho, Takamatsu, Kagawa, 760-0078, Japan
Phone : 81-87-867-4800 Fax : 81-87-867-4801

**Hiroshima Sales Office**
1F Furuta Building, 2-5-27 Miyanomachi, Fuchu-cho, Aki-gun, Hiroshima, 735-0005, Japan
Phone : 81-82-288-4433 Fax : 81-82-286-0761

**Kyusyu Sales Office**
1F Hakata Fukoku Seimei Building, 8-30 Tenyamachi, Hakata-ku, Fukuoka, 812-0025, Japan
Phone : 81-92-292-3593 Fax : 81-92-292-3594

**Tokyo Branch**
Kanda Awaji-cho Nichome Building, 2-6 Kanda Awaji-cho, Chiyoda-ku, Tokyo, 101-0063, Japan
Phone : 81-3-6206-4711 Fax : 81-3-6206-4720

**HORIBA Advanced Technology Center**
11-5 Hokotate-cho, Kamitoba, Minami-ku, Kyoto, 601-8116, Japan
Phone : 81-75-693-2300 Fax : 81-75-693-2350

**Kutsuki Training Center**
335-10 Tochu, Kutsuki, Takashima, Shiga, 520-1425, Japan
Phone : 81-740-38-3127 Fax : 81-740-38-3126

**Saiin Factory**
43-1 Nishimizosaki-cho, Saiin, Ukyo-ku, Kyoto, 615-0046, Japan
Phone : 81-75-323-7067 Fax : 81-75-323-7085

### HORIBA STEC, Co., Ltd.
11-5 Hokotate-cho, Kamitoba, Minami-ku, Kyoto, 601-8116, Japan
Phone : (81)-75-693-2300 Fax : (81)-75-693-2350

**Aso Factory**
Torikokogyodanchi, 358-11, Koumaibata, Toriko, Nishiharamura, Aso-gun, Kumamoto, 861-240, Japan
Phone : (81)-96-279-2921 Fax : (81)-96-279-3364

**Fukuchiyama Technology Center**
11-1 Miwa-cho Miwa, Fukuchiyama, Kyoto, 620-1445 Japan
Phone : (81)-773-59-2070 Fax : (81)-773-59-2074

**Tokyo Sales Office**
5F Kanda Awaji-cho Nichome Building, 2-6 Kanda Awaji-cho, Chiyoda-ku, Tokyo, 101-0063, Japan
Phone : 81-3-6206-4731 Fax : 81-3-6206-4740

**Tohoku Sales Office**
4-21-8 Izumichuo, Izumi-ku, Sendai, Miyagi, 981-3133, Japan
Phone : 81-22-772-6717 Fax : 81-22-772-6727

**Yamanashi Sales Office**
3F Daita Building, 2-14-13, Marunouchi, Kofu, Yamanashi, 400-0031, Japan
Phone : 81-55-231-1351 Fax : 81- 55-231-1352

**Nagoya Sales Office**
4F BIZrium Nagoya, 3-1-17 Noritake-Shinmachi, Nishi-ku, Nagoya, 451-0051, Japan
Phone*: 81-52-433-3451 Fax*: 81-52-433-3461

**Kyushu Chuo Sales Office**
Torikokogyodanchi, 358-11 Koumaibata, Toriko, Nishiharamura, Aso-gun, Kumamoto, 861-2401, Japan
Phone : 81-96-279-2922 Fax : 81-96-279-3364

### HORIBA Advanced Techno, Co., Ltd.
2 Miyanohigashi-cho, Kisshoin, Minami-ku, Kyoto, 601-8551, Japan
Phone : (81)-75-321-7184 Fax : (81)-75-321-7291

**Factory**
2 Miyanohigashi-cho, Kisshoin, Minami-ku, Kyoto, 601-8551, Japan
Phone : 81-75-321-1215 Fax : 81-75-321-1079

**Tokyo Sales Office**
Kanda Awaji-cho Nichome Building, 2-6 Kanda Awaji-cho, Chiyoda-ku, Tokyo, 101-0063, Japan
Phone : 81-3-6206-4751 Fax : 81-3-6206-4760

**Tohoku Sales Office**
4-21-8 Izumichuo, Izumi-ku, Sendai, Miyagi, 981-3133, Japan
Phone : 81-22-776-8253 Fax : 81-22-772-6727

**Nagoya Sales Office**
4F BIZrium Nagoya, 3-1-17 Noritake-Shinmachi, Nishi-ku, Nagoya, 451-0051, Japan
Phone*: 81-52-433-3452 Fax*: 81-52-433-3462

**Osaka Sales Office**
4F ShinOsaka UenoToyo Building, 7-4-17 Nishinakajima, Yodogawa-ku, Osaka, 532-0011, Japan
Phone : 81-6-6390-8211 Fax : 81-6-6390-8222

**Shikoku Satellite Office**
9-9 Imazato-cho, Takamatsu, Kagawa, 760-0078, Japan
Phone : 81-87-867-4841 Fax : 81-87-867-4842

**Kyusyu Sales Office**
1F Hakata Fukoku Seimei Building, 8-30 Tenyamachi, Hakata-ku, Fukuoka, 812-0025, Japan
Phone : 81-92-292-3595 Fax : 81-92-292-3596

**Kyushu Chuo Sales Office**
Torikokogyodanchi, 358-11, Koumaibata, Toriko, Nishiharamura, Aso-gun, Kumamoto, 861-240, Japan
Phone : 81-75-321-7184 Fax : 81-75-321-7291

### HORIBA TECHNO SERVICE Co., Ltd.
2 Miyanohigashi, Kisshoin, Minami-ku, Kyoto, 601-8305, Japan
Phone : (81)-75-313-8125 Fax : (81)-75-321-5647

**Tokyo Service Staition**
4F Kanda Awaji-cho Nichome Building, 2-6 Awaji-cho, Kanda, Chiyoda-ku, Tokyo, 101-0063, Japan
Phone : 81-3-6206-4750 Fax : 81-3-6206-4742

**Hokkaido Service Staition**
6F Park East Sapporo, 1-3 Minami Ichijo Higashi, Chuo-ku, Sapporo, Hokkaido, 060-0051, Japan
Phone : 81-11-207-1801 Fax : 81-11-207-1802

**Tohoku Service Staition**
4-21-8 Izumichuo, Izumi-ku, Sendai, Miyagi, 981-3133, Japan
Phone : 81-22-776-8252 Fax : 81-22-772-6727

**Fukushima Service Staition**
101 Office Tatsumi, 5-13-17 Saikon, Koriyama, Fukushima, 963-8862, Japan
Phone : 81-24-925-9311 Fax : 81-24-925-9312

**Tochigi Service Staition**
1F Flora Building, 1-9-15 Higashishukugo, Utsunomiya, Tochigi, 321-0953, Japan
Phone : 81-28-634-6098 Fax : 81-28-634-6099

**Chiba Service Staition**
1-8-12 Goichuo-higashi, Ichihara, Chiba, 290-0054, Japan
Phone : 81-436-24-3914 Fax : 81-436-24-0642

**Kashima Service Staition**
1-4-35 Kamisu, Kamisu, Ibaraki, 314-0143, Japan
Phone : 81-299-91-0808 Fax : 81-299-92-9561

**Tsukuba Service Staition**
1F Tsukuba Koken Building, 2-1-13 Umezono, Tsukuba, Ibaraki, 305-0045, Japan
Phone : 81-29-863-7311 Fax : 81-29-859-5221

**Saitama Service Staition**
1F Higashikawaguchi Garden Plaza, 1-6-1 Totsukahigashi, Kawaguchi, Saitama, 333-0802, Japan
Phone : 81-48-298-6871 Fax : 81-48-298-6880

**Nishitokyo Service Staition**
1F The-Macrocosm, 3-37-34 Izumi, Kokubunji, Tokyo, 185-0024, Japan
Phone : 81-42-322-3211 Fax : 81-42-322-3210

**Yokohama Service Staition**
1F ShinYokohama Mineta Building, 2-3-19 Shinyokohama, Kohoku-ku, Yokohama, Kanagawa, 222-0033, Japan
Phone : 81-45-478-7018 Fax : 81-45-478-7029

**Fuji Service Staition**
1F Suzuki Building, 4-7 Suzukawahigashi-cho, Fuji, Shizuoka, 417-0012, Japan
Phone : 81-545-33-3152 Fax : 81-545-33-3159

**Hamamatsu Service Staition**
221-1 Sanjino-cho, Minami-ku, Hamamatsu, Shizuoka, 430-0816, Japan
Phone : 81-53-464-1339 Fax : 81-53-464-6528

**Tokai Service Staition**
2-23 Tsukasa-cho, Toyota, Aichi, 471-0831, Japan
Phone : 81-565-37-3510 Fax : 81-565-37-3520

**Nagoya Service Staition**
1F Riverpage, 3-203 Kamiyashiro, Meito-ku, Nagoya, Aichi, 465-0025, Japan
Phone : 81-52-705-0711 Fax : 81-52-705-0710

**Hokuriku Service Staition**
10-14 Imaizumiseibu-cho, Toyama, 939-8281, Japan
Phone : 81-76-422-6112 Fax : 81-76-422-6446

**Mie Service Staition**
1F Taiyo-Seimei Yokkaichi Building, 1-1-18 Unomori, Yokkaichi, Mie, 510-0074, Japan
Phone : 81-59-340-6061 Fax : 81-59-340-6063

**Kyoto Service Staition**
2 Miyanohigashi, Kisshoin, Minami-ku, Kyoto, 601-8305, Japan
Phone : 81-75-325-5291 Fax : 81-75-321-5647

**Osaka Service Station**
4F ShinOsaka UenoToyo Building, 7-4-17 Nishinakajima, Yodogawa-ku, Osaka, 532-0011, Japan
Phone : 81-75-325-5291 Fax : 81-75-321-5647

**Hyogo Service Staition**
381 Nanjyo, Himeji, Hyogo, 670-0952, Japan
Phone : 81-79-284-8320 Fax : 81-79-284-8321

**Shikoku Service Staition**
9-9 Imazato-cho, Takamatsu, Kagawa, 760-0078, Japan
Phone : 81-87-867-4821 Fax : 81-87-867-4822

**Okayama Service Staition**
1-12-35 Tsurajima, Kurashiki, Okayama, 712-8012, Japan
Phone : 81-86-448-9760 Fax : 81-86-446-5637

**Hiroshima Service Staition**
1F Furuta Building, 2-5-27 Miyanomachi, Fuchucho, Aki-gun, Hiroshima, 735-0005, Japan
Phone : 81-82-283-3378 Fax : 81-82-286-0761

**Yamaguchi Service Staition**
1F 8th Matsumoto Building, 2-7-12 Miyanomae, Shunan, Yamaguchi, 746-0017, Japan
Phone : 81-834-61-1080 Fax : 81-834-62-7980

**Kyusyu Service Staition**
1F Hakata Fukoku Seimei Building, 8-30 Tenyamachi, Hakata-ku, Fukuoka, 812-0025, Japan
Phone : 81-92-292-3597 Fax : 81-92-292-3598

**Oita Service Staition**
1F Tsuruwa Building, 2-2-37 Hagiwara, Oita, 870-0921, Japan
Phone : 81-97-551-3982 Fax : 81-97-551-3889

**Kumamoto Service Staition**
Toriko-kogyodanchi, 358-11, Koumaibata, Toriko, Nishiharamura, Aso-gun, Kumamoto, 861-2401, Japan
Phone : 81-96-279-2985 Fax : 81-96-279-2986

* Phone and Fax numbers of Nagoya Sales Offices are valid from October 25, 2021.

# HORIBA World-Wide Network

## BRAZIL

### HORIBA Brasil Holding, Eireli
Rua Presbitero Plinio Alves de Souza, 645, parte A, Loteamento Multivias, Jardim Ermida II - Jundiai Sao Paulo - CEP 13.212-181 Brazil
Phone : (55)-11-2923-5400 Fax : (55)-11-2923-5490

### HORIBA Instruments Brasil, Ltda.
Rua Presbitero Plinio Alves de Souza, 645, Loteamento Multivias, Jardim Ermida II - Jundiai Sao Paulo - CEP 13.212-181 Brazil
Phone : (55)-11-2923-5400 Fax : (55)-11-2923-5490

### TCA/HORIBA Sistemas de Testes Automotivos Ltda.
Rua Goiás, 191 Vila Oriental, Diadema, São Paulo, Brazil CEP 09941-690
Phone : (55)-11-4224-0200 Fax : (55)-11-4227-3133

## CANADA

### HORIBA Canada, Inc.
Unit102, 5555 North Service Road Burlington, Ontario, Canada, L7L 5H7
Phone : (1)-905-335-0234 Fax : (1)-905-331-2362

**London Office**
347 Consortium Court, London, Ontario, Canada, N6E 2S8
Phone : (1)-519-668-6920 Fax : (1)-519-668-8437

### Clinitox Diagnostix, Inc.
5700 Timberlea Blvd Mississauga, Ontario Canada, L4W 5B9
Phone : (1)-905-629-8066

## U.S.A.

### HORIBA Americas Holding Incorporated
9755 Research Drive, Irvine, CA 92618, U.S.A.
Phone : (1)-949-250-4811

### HORIBA Instruments Incorporated
9755 Research Drive, Irvine, CA 92618, U.S.A.
Phone : (1)-949-250-4811 Fax : (1)-949-250-0924

**Ann Arbor Office**
5900 Hines Drive, Ann Arbor, MI 48108, U.S.A.
Phone : (1)-734-213-6555 Fax : (1)-734-213-6525

**Atlanta Field Office**
5871 Glenridge Drive, Suite 475, 30328 Atlanta, GA, U.S.A.

**Austin Office**
9701 Dessau Road, Suite 605, Austin, TX 78754, U.S.A.
Phone : (1)-512-836-9560 Fax : (1)-512-836-8054

**Chicago, IL Field Office**
554 Anderson Drive, Unit A Romeoville, IL 60446, U.S.A.
Phone : (1)-815-372-9076

**Houston Office**
5390 Bay Oaks Drive, Pasadena, TX 77505
Phone : 1-281-482-4334 Fax : 1-281-674-6058

**Novato Field Office**
359 Bel Marin Keys Blvd, #18, Novato, CA 94949

**HORIBA New Jersey Optical Spectroscopy Center**
20 Knightsbridge Rd, Piscataway, NJ 08854, U.S.A.
Phone : (1)-732-494-8660 Fax : (1)-732-549-5125

**Portland Office**
7007 S.W. Cardinal Lane, Suite 185, Portland, OR 97224, U.S.A.
Phone : (1)-503-624-9767 Fax : (1)-503-968-3236

**HORIBA Reno Technology Center**
3740 Barron way Reno, Nevada 89511, U.S.A.
Phone : (1)-775-358-2332 Fax : (1)-775-358-0434

**Sunnyvale Office**
430 Indio Way, Sunnyvale CA 94085, U.S.A.
Phone : (1)-408-730-4772 Fax : (1)-408-730-8975

**Troy Office**
2890 John R Road, Troy, MI 48083, U.S.A
Phone : (1)-248-689-9000 Fax : (1)-248-689-8578

### HORIBA UNITED STATES HOLDING, LLC
9755 Research Drive, Irvine, CA 92618, U.S.A.
Phone : (1)-949-250-4811 Fax : (1)-949-250-0924

### MedTest Holdings, Inc.
5449 Research Drive Canton, MI 48188, U.S.A.

### MedTest DX, Inc.
5449 Research Drive Canton, MI 48188, U.S.A.

### Pointe Scientific, Inc.
5449 Research Drive Canton, MI 48188, U.S.A.

### Medical Laboratory Solutions, Inc.
270 Rutledge Road, Unit D Fletcher, NC 28732, U.S.A.

## AUSTRIA

### HORIBA (Austria) GmbH
Kaplanstrasse 5, A-3430 Tulln, Austria
Phone : (43)-2272-65225 Fax : (43)-2272-65225-45

## BELGIUM

### HORIBA Europe GmbH, Antwerp Branch
Duwijckstraat 17, 2500 Lier, Belgium
Phone : (32)3 281 57 92 Fax : (32)3 230 06 95

## CZECH

### HORIBA Czech Olomouc Factory
Zeleznicni 512/7, 772 00 Olomouc, Czech Republic
Phone : (420) 588 118 365 'Phone : (420) 588 118 393

**HORIBA Czech Prague Office**
Prumyslova 1306/7, CZ-10200, Praha 10, Czech Republic
Phone : (420) 246 039 265

## FRANCE

### HORIBA Europe Holding SASU
14 Boulevard Thomas Gobert - CS 45002 - 91120 Palaiseau - France

### HORIBA Europe Research Center
14 Boulevard Thomas Gobert - Passage Jobin Yvon CS 45002 - 91120 Palaiseau - France
Phone : (33)-1-69-74-72-00 Fax : (33)-1-69-31-32-20

**HORIBA FRANCE SAS, Longjumeau Office**
16-18, rue du Canal, 91165 Longjumeau Cedex, France
Phone : (33)-1-69-74-72-00 Fax : (33)-1-69-09-07-21

**HORIBA FRANCE SAS, Villeneuve d'Ascq, Office**
231 rue de Lille, 59650 Villeneuve d'Ascq, France
Phone : (33)-3-20-59-18-00 Fax : (33)-3-20-59-18-08

**HORIBA FRANCE SAS, Lille Office**
455 avenue Eugène Avinée - 59120 LOOS - France
Phone : (33)-1-69-74-72-00 Fax : (33)-3-20-59-18-08

### HORIBA ABX SAS
Parc Euromédecine, rue du Caducée, BP7290, 34184 Montpellier Cedex 4, France
Phone : 33 (0)4 67 14 15 16

## GERMANY

### HORIBA Europe GmbH / Oberursel Office
Hans-Mess-Str.6, D-61440 Oberursel, Germany
Phone : (49)-6172-1396-0 Fax : (49)-6172-1373-85

**HORIBA Europe GmbH, Darmstadt Office**
Landwehrstrasse 55, D-64293, Darmstadt, Germany
Phone : (49)-6151-5000-0 Fax : (49)-6151-5000-3865

**HORIBA Europe GmbH, Dresden Office**
Hugo-Junckers-Ring 1, 01109 Dresden, Germany
Phone : (49)-351-8896807 Fax : (49)-351-8896808

**HORIBA Europe GmbH, Florsheim Office**
Mariechen-Graulich-Straße 10-12a, 65439 Flörsheim, Germany
Phone : 49 6145 37699-12

**HORIBA Europe GmbH, Hanover Office**
Frankenring 14, D-30855 Langenhagen, Germany

**HORIBA Europe GmbH, Korschenbroich Office**
Friedrich-Ebert-Str. 9-11, D-41352 Korschenbroich, Germany
Phone : (49)-2161-47537-0

**HORIBA Europe GmbH, Leichlingen Office**
Julius-Kronenberg-Str. 9, D-42799 Leichlingen, Germany
Phone : (49)-2175-8978-0 Fax : (49)-2175-897850

**HORIBA Europe GmbH, Munich Office**
Waldmeisterstr. 72-74/Robinienstr. 66, D-80935 Munich, Germany
Phone : (49)-89-2444779-0 Fax : (49)-89-2444779-10

**HORIBA Europe GmbH, Potsdam Office**
Dennis-Gabor-Str. 2, D-14469 Potsdam, Germany
Phone : (49)-3316-4900-70 Fax : (49)-3316-4900-74

**HORIBA Europe GmbH, Stuttgart Office (Boeblingen)**
Hanns-Klemm-Str. 56, D-71034 Boeblingen, Germany
Phone : (49)-7031-677-9440 Fax : (49)-7031-677-9450

**HORIBA Europe GmbH, Stuttgart Office (Neuhausen)**
Zabergaeustr. 3, D-73765 Neuhausen, Germany
Phone : (49)-7158-933-800 Fax : (49)-7158-933-899

**HORIBA Europe GmbH, Wolfsburg Office**
Klauskamp, Heinenkap II 38444 Wolfsburg , Germany
Phone : (49)-5361-38653-16 Fax : (49)-5361-38653-24

### HORIBA Jobin Yvon GmbH
Neuhofstrasse 9, D_64625, Bensheim, Germany
Phone : 49(0)62-51-84-750 Fax : 49(0)62-51-84-7520

### HORIBA FuelCon GmbH
Steinfeldstraße 1, 39179 Barleben, Germany
Phone : 49 39203 514 400 Fax : 49 39203 514 409

### HORIBA Tocadero GmbH
Johann-Hittorf-Str. 8 12489 Berlin Germany
Phone : 49 (0)30 6392 3150 Fax : 49 (0)30 6392 3151

### BeXema GmbH
Steinfeldstraße 2a, 39179 Barleben, Germany

## ITALIA

### HORIBA ITALIA Srl
Via Luca Gaurico 209 - 00143 ROMA
Phone : (39)-6-51-59-22-1 Fax : (39)-6-51-96-43-34

**HORIBA ITALIA SRL, Torino Office**
Via Feroggio, 30, 10151 Torino, Italy
Phone : (39)-1-19-04-06-01 Fax : (39)-1-19-00-04-48

**HORIBA ABX SAS, Italy Branch**
Viale Luca Gaurico 209/211, 00143 Roma, Italy
Phone : (39)-6-51-59-22-1 Fax : (39)-6-51-96-43-34

## MAURITIUS

### MIRA Engineering Service Mauritius Ltd.
9th Floor, Orange Tower, Cybercity, Ebene

## NETHERLANDS

### HORIBA Europe GmbH, Netherlands Branch
Science Park Eindhoven 5080 (Industrial park "Ekkersrijt") 5692 EA, Son, Netherlands
Phone : (31)-40-2900240 Fax : (31)-40-2900624

## POLAND

### HORIBA ABX Sp. z o.o.
Aleja Niepodleglosci 18
02-653 Warszawa (Warsaw), Poland.
Phone : (48)-22-673-2022 Fax : (48)-22-673-2026

## PORTUGAL

### HORIBA ABX SAS, Portugal Branch
Alfrapark - Estrada de Alfragide n° 67, Edificio F - Piso 0 Sul, 2610-008 Amadora, Portugal
Phone : (35)-12-14-72-17-70 Fax : (35)-12-14-72-17-89

## ROMANIA

### HORIBA (Austria) GmbH, Romania Branch
B-dul. Republicii, nr. 164, Etaj Parter, Birourile nr. 3 si 4, Pitesti, 110177, Judetul Arges, ROMANIA
Phone : (40)-348-807117 Fax : (40)-348-807118

## RUSSIA

### HORIBA OOO
Altufievskoe shosse, 13, building 5, 127106, Moscow, Russia
Phone : (7)-495-221-87-71 Fax : (7)-495-221-87-68

**HORIBA OOO, Zelenograd Office**
Office 106, 2nd West st., 1, build 1, 124460, Zelenograd city, Moscow, Russia
Phone : (7)-499-995-09-54

## SPAIN

### HORIBA ABX SAS, Spain Branch
Calle Apolonio Morales. Num. 6 (Bajos), 28036 Madrid, Spain
Phone : (34)-91-353-30-10 Fax : (34)-91-353-30-11

### HORIBA MIRA SPAIN, S.L.
Calle Oficios, nave 22, 04620 Vera (Almería)
Phone : (34)-950-39-11-53

## SWEDEN

### HORIBA Europe GmbH, Sweden Branch (Gothenburg)
Grimboasen 10 A, S-417 05 Gothenburg, Sweden
Phone : (46)-10-161 1500 Fax : (46)-10-161 1503

### HORIBA Europe GmbH, Sweden Branch (Sodertalje)
Sydhamnsvagen 55-57, SE- 15138, Sodertalje, Sweden
Phone : (46)-8-550-80701 Fax : (46)-8-550-80567

## TURKEY

### HORIBA Europe GmbH, Istanbul Office
Küçükbakkalköy Mah. Kayışdağı Cad., Flora Residence No:3/2504, 34750 Ataşehir/Istanbul Turkey
Phone : 90 216 572 1166 Fax : 90 216 572 1167

# HORIBA World-Wide Network

## UNITED KINGDOM

### HORIBA UK Limited
Kyoto Close Moulton Park Northampton NN3 6FL United Kingdom
Phone : 44 (0)1604 542500 Fax : 44 (0)1604 542699

### HORIBA Jobin Yvon IBH Ltd.
133 Finnieston St. Glasgow G3 8HB, United Kingdom
Phone : (44)-141-229-67-89 Fax : (44)-141-229-67-90

### HORIBA Test Automation Limited
Brook Court Whittington Hall Worcester WR5 2RX, United Kingdom
Phone : (44)-1905-359359 Fax : (44)-1905 359332

### Old HORIBA MIRA Certification Limited
Watling Street, Nuneaton, Warwickshire, CV10 0TU, United Kingdom
Phone : (44)-24-7635-5000

### HORIBA MIRA Limited
Watling Street, Nuneaton, Warwickshire, CV10 0TU, United Kingdom
Phone : (44)-24-7635-5000

**HORIBA MIRA Limited, Quatro Park**
Unit 1, Quatro Park, Paycocke Road, Basildon, Essex, SS14 3GH, United Kingdom
Phone : (44)-1268-290100

### HORIBA MIRA Certification Limited
Watling Street, Nuneaton, Warwickshire, CV10 0TU, United Kingdom
Phone : (44)-24-7635-5000

### MIRA Land Limited
Watling Street, Nuneaton, Warwickshire, CV10 0TU, United Kingdom
Phone : (44)-24-7635-5000

### MIRA Service Limited
Watling Street, Nuneaton, Warwickshire, CV10 0TU, United Kingdom
Phone : (44)-24-7635-5000

### MIRA Technology Park Limited
Watling Street, Nuneaton, Warwickshire, CV10 0TU, United Kingdom
Phone : (44)-24-7635-5000

## CHINA

### HORIBA INSTRUMENTS (SHANGHAI) CO., LTD
No.200, Taitao Rd, Anting Town, Jiading District, Shanghai, China 201814
Phone : (86)-21-6952-2835 Fax : (86)-21-6952-2823

### HORIBA Technology (Suzhou) Co., LTD.
No.1 building, Industry park, No.101 Chenmenjing Rd, Taicang, Jiangsu, China (215400)
Phone : (86)-0512-3306-6388

### HORIBA (China) Co., Ltd.
Room 1604, Building 1, No.185 Moyu Road, Anting Town, Jiading District, Shanghai, China, 201805
Phone : (86)-21-6289-6060 Fax : (86)-21-6289-5553

### HORIBA (China) Trading Co., Ltd.
Unit D, 1F, Building A, Synnex International Park, 1068 West Tianshan Road, 200335, Shanghai, China
Phone : (86)-21-6289-6060 Fax : (86)-21-6289-5553

**HORIBA (China) Trading Co., Ltd., Beijing Branch**
12F, Metropolis Tower, No.2, Haidian Dong 3 Street, Beijing, 100080, China
Phone : (86)-10-8567-9966 Fax : (86)-10-8567-9066

**HORIBA (China) Trading Co., Ltd., Guangzhou Branch**
Room 1611/1612, Goldlion Digital Network Center, 138 Tiyu Road East, Guangzhou 510620, China
Phone : (86)-20-3878-1883 Fax : (86)-20-3878-1810

**HORIBA (China) Trading Co., Ltd., Shanghai Technical Center**
No.200, Taitao Rd, Anting Town, Jiading District, Shanghai, China 201814
Phone : (86)-21-6289-6060 Fax : (86)-21-6289-5553

### HORIBA Precision Instruments (Beijing) Co., Ltd.
Chaoyang District, Bei Yuan Road 40, Beijing, 100012 China
Phone : 86-10 8492 9402 Fax : 86-10 8492 7216

### MIRA China Ltd.
Suite 501, Block B, Hongqiao Sunnyworld No. 1226 South Shenbin Road Shanghai, 201106, China
Phone : (86)-21-6220-6377 Fax : (86)-21-6220-6379

**MIRA China Ltd. Xiangyang Workshop**
A27-1, Jiahai industrial park, High-tech District, Xiangyang, Hubei, 441004, China
Phone : (86)-710-2578-268

## INDIA

### HORIBA India Private Limited
246, OKHLA INDUSTRIAL ESTATE, PHASE 3 NEW DELHI - 110020, India
Phone : 91-11-4646-5000 Fax : 91-11-4646-5020

**HORIBA India Private Limited, Bangalore Office**
No.55, 12th Main, Behind BDA Complex, 6th sector, HSR Layout, Bangalore South, Bangalore - 560102, INDIA
Phone : (91)-80-4127-3637

**HORIBA India Private Limited, Chennai Office**
No.9, 01 & 02 Floor, Ganapathy Colony, Thiru-Vi-Ka Industrial Estate, Guindy, Chennai , 600032 India
Phone : (91)-44-42077899

**HORIBA India Private Limited, Haridwar Factory**
Plot No. 26, Sector-7, IIE, SIDCUL, Haridwar, Uttarakhand - 249403, India
Phone : (91)-1334-239139

**HORIBA India Private Limited, Kolkata Office**
EK Tower/6th Floor/Office -4A, Action Area-II D, Newtown, Pin Code 700161, India
Phone : (91)-90073-63356

**HORIBA India Private Limited, Technical Center**
D-225, Chakan MIDC Phase-II, Bhamboli Village, Pune-410501, India
Phone : (91) 21-3567-6000

**HORIBA India Private Limited, Nagpur Factory**
Plot No B-3 & C-32 Butibori Phase 2, Industrial Area, Dist Nagpur, Nagpur, 441122, India

## INDONESIA

### PT HORIBA Indonesia
Jl. Jalur Sutera Blok 20A, No. 16-17, Kel. Kunciran, Kec. Pinang Tangerang - 15144, Indonesia
Phone : 62-21-3044 8525 Fax : 62-21-3044 8521

## KOREA

### HORIBA KOREA Ltd.
25, 94-Gil, Iljik-Ro, Manan-Gu, Anyang-Si, Gyeonggi-Do, 13901, Korea
Phone : (82)-31-296-7911 Fax : (82)-31-296-7913

**HORIBA KOREA Ltd., Ulsan Office**
613, Doosan We've the Zenith, 1877, Sinjeong-Dong, Nam-Gu, Ulsan-Si, 44679, Korea
Phone : (82)-52-275-0122 Fax : (82)-52-276-0136

### HORIBA MIRA Limited, Korea Branch Office
25, 94-Gil, Iljik-Ro, Manan-Gu, Anyang-Si, Gyeonggi-Do, 13901, Korea
Phone : (82)-70-4689-0680 Fax : (82)-31-296-7913

### HORIBA STEC KOREA, Ltd.
98, Digital valley-ro Suji-gu, Yongin-si Gyeonggi-do 16878 Korea
Phone : (82)-31-8025-6500 Fax : (82)-31-8025-6599

## PHILIPPINES

### HORIBA INSTRUMENTS (SINGAPORE) PTE LTD., MANILA OFFICE
27/F Tower 2 Enterprise Centre, 6766 Ayala Avenue cor Paseo de Roxas, Makati City, Philippines, 1226
Phone : 63 2 8885 8468

## SINGAPORE

### HORIBA Instruments (Singapore) Pte Ltd.
3 Changi Business Park Vista #01-01, Singapore 486051
Phone : (65)-6-745-8300 Fax : (65)-6-745-8155

**HORIBA Instruments (Singapore) Pte Ltd., West Office**
83 Science Park Drive, #02-02A, The Curie, Singapore 118258
Phone : (65)-6-908-9660

## TAIWAN

### HORIBA Taiwan, Inc.
8F.-8, No.38, Taiyuan St. Zhubei City, Hsinchu County 30265, Taiwan (R.O.C.)
Phone : (886)-3-5600606 Fax : (886)-3-5600550

**HORIBA Taiwan, Inc. Tainan Office**
1F., No.117, Chenggong Rd., Shanhua Township, Tainan County 741, Taiwan(R.O.C)
Phone : (886)6-583-4592 Fax : (886)6-583-2409

## THAILAND

### HORIBA Holding (Thailand) Limited
393, 395, 397, 399, 401, 403 Latya Road, Somdetchaopraya, Klongsan, Bangkok 10600, Thailand
Phone : (66)-2-861-59-95 Fax : (66)-2-861-52-00

### HORIBA (Thailand) Limited
393, 395, 397, 399, 401, 403 Latya Road, Somdetchaopraya, Klongsan, Bangkok 10600, Thailand
Phone : 66 (0) 2 861 5995 ext.123 Fax : 66 (0) 2 861 5200

**HORIBA (Thailand) Limited East Office**
850/7 Soi Lat Krabang 30/5, Lat Krabang Road, Lat Krabang, Bangkok 10520, Thailand
Phone : 66 (0) 2 734 4434 Fax : 66 (0) 2 734 4438

## VIETNAM

### HORIBA Vietnam Company Limited
Lot 3 and 4, 16 Floor, Detech Tower II, No. 107 Nguyen Phong Sac Street, Dich Vong Hau Ward, Cau Giay District, Hanoi, Vietnam
Phone : (84)-24-3795-8552 Fax : (84)-24-3795-8553

**HORIBA Vietnam Company Limited, Ho Chi Minh Branch Office**
Room 8.6- 8th Floor, Le Meridien Building, 3C Ton Duc Thang Street, Ben Nghe Ward, District 1, Ho Chi Minh City, Vietnam
Phone : 84 28 7305 4492 Fax : 84 28 6287 6269

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

Readout **HORIBA Technical Reports English Edition No.55**

Publication Date : October 19th, 2021
Publisher : HORIBA, Ltd.
Editor : NISHIKATA Kentaro
Associate Editor : NOMURA Satoshi
Publication Members : NAKATANI Shigeru, URAKAMI Chikako
MATSUDA Tetsuya, OHTAKE Yoshihiro, SAKAMOTO Junichi,SATAKE Hiromi
R&D Planning Center, Research & Development Division, HORIBA, Ltd.

Information
2, Miyanohigashi-cho, Kisshoin, Minami-ku, Kyoto 601-8510, Japan
Phone : (81)75-313-8121
E-mail : readout@horiba.co.jp
URL : https://www.horiba.com/us/en/publications/readout/

HORIBA

Explore the future