Graduate School of Advanced Science and Engineering
Waseda University

博　士　論　文　概　要
Doctoral Dissertation Synopsis

論　文　題　目
Dissertation Title

Detection and localization analysis of secondary metabolites in microorganisms using Raman microspectroscopy

顕微ラマン分光法を用いた微生物内における二次代謝産物の検出と局在解析

申　請　者
(Applicant Name)
Shumpei HORII
堀井　俊平

Department of Advanced Science and Engineering
Research on Life Science and Medical Bioscience

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Biomolecular imaging is an important tool for elucidating biological phenomena. In the field of life science, basic research on biomolecules, such as genes and proteins, has been actively conducted at the molecular level through genome and gene expression analyses. However, analysis of only individual molecular functions is not sufficient to elucidate complex life phenomena. One of the most important processes is the visualization of the spatiotemporal intracellular dynamics of biomolecules during various biological phenomena, such as cell growth and differentiation. Particularly in microbial research, there is an increasing demand for methods to detect and visualize biomolecules to commercialize enzymes and antibiotics. For the practical application of microbial products, understanding the metabolic mechanisms of microorganisms with respect to their growth environment will contribute to improving the production efficiency of these microbial products. In addition, microorganisms effectively maintain their biological activities by transmitting proteins and signals to other cells or symbiotic hosts through cell-to-cell contact and the release of extracellular vesicles (EVs). Therefore, biomolecular imaging is an effective method for understanding the communication between microorganisms and their external environment.

Raman spectroscopy is a vibrational spectroscopy technique with molecular sensitivity that can be applied to the in vivo molecular analysis of biological samples. It provides characteristic information on the molecular structure of biomolecules and does not require sample pre-treatment. Furthermore, it enables rapid and minimally invasive observations and can be used to detect proteins and lipids that constitute living cells. Moreover, Raman microspectroscopy, specifically the combination of Raman spectroscopy and optical microscopy, allows for the construction of molecular distribution images with a high spatial resolution (~300 nm) at the single-cell level. On the other hand, Raman spectra from biological samples are typically composite spectra of multiple biomolecules as well as fluorescence from biological samples or compounds. Furthermore, it is difficult to detect biomolecules, such as secondary metabolites, whose biodistribution is unknown and in trace amounts. Appropriate analytical methods for detecting biomolecules in biological samples have not yet been established. Thus, in this thesis, I established a Raman spectroscopic analysis platform for the detection and visualization of various biomolecules in biological samples and applied it to visualize the intracellular spatiotemporal distribution to discuss the mechanism of microbial secondary metabolite formation.

In Chapter 1, Raman microspectroscopy is compared with other biomolecular imaging techniques, and its status and associated challenges with respect to biological sample measurements are outlined. The purpose and significance of Raman microspectroscopy is described within the context of biological samples.

In Chapter 2, I established a Raman spectroscopic analysis platform using fungi as a model system for biological samples. A procedure for the measurement of biological samples to detect single biomolecules is provided. Furthermore, a multivariate curve resolution-alternating least squares (MCR-ALS) method was applied to the Raman spectra of fungi to detect secondary metabolites. In the MCR-ALS method, spectral decomposition is thoroughly applied without prior knowledge through the optimization of calculations under physiochemically reasonable constraints. The spectra of individual biomolecules that can be molecularly identified are obtained. The Raman spectrum of penicillin G overlaps with that of proteins, which makes its detection inside microorganisms difficult. MCR-ALS analysis of the bacterial spectrum revealed successful detection of penicillin G. Maps of the molecular distribution within the cells were reconstructed from an intensity profile matrix. Localized “particle-like” features were apparent in the distribution map of penicillin G. It has been suggested that penicillin G is biosynthesized within peroxisomes. There are no reports of the direct visualization of penicillin G in mycelial cells.

In Chapter 3, the spatiotemporal subcellular localization of secondary metabolites during morphological differentiation, which is a characteristic of actinomycetes, is visualized. The filamentous bacterial genus *Streptomyces*, which produces approximately 70–80% of clinically useful antibiotics, has been reported to undergo complex morphological differentiation in its life cycle, which is closely related to the production of secondary metabolites. Generally, during the life cycle of *Streptomyces* under solid culture conditions, seeded spores germinate through the growth of compartmentalized mycelia (MI) into the agar medium. After this initial growth period, multinucleated mycelia (early MII) and aerial mycelia (late MII) were formed. Similarly, all stages of differentiation and growth occur under liquid culture conditions, except in the late MII stage. In most *Streptomyces* species, aerial mycelium formation and sporulation do not occur under liquid culture conditions and growing mycelia form pellets/clumps. However, the induction of morphological differentiation to activate antibiotic production is largely unexplored because the temporal production and localization of secondary metabolites during morphological differentiation are not clear. Therefore, an evaluation of the spatiotemporal distribution of secondary metabolites during morphological differentiation of actinomycetes would improve antibiotic production. In this chapter, *Streptomyces avermitilis*, which produces the secondary metabolite avermectin, was evaluated over time in liquid (24 and 168 h) and solid (48–120 h) cultures. In liquid culture, avermectin was detected only in one of the samples collected after 168 h of incubation and was highly concentrated in certain locations at the center of the mycelial pellet. This indicates that MI mycelia

differentiated into early MII mycelia, resulting in the production of avermectin. It is also important to note that avermectin was not uniformly distributed in individual mycelia but was concentrated at certain mycelial locations. In the solid-state culture, samples were collected at different time intervals (24–120 h), progressively comprising different stages of differentiation. Inspection of the Raman images revealed that the chemical compositions of the substrate mycelia (SM) and spiral spore chains (SSCs) were characteristically different; specifically, carotenoids were more abundant in SSCs than in SM, and avermectin was present only in SSCs and not in SM. Mycelia that differentiated in the late MII stage had SM, spore-bearing mycelia (SBM), and SSCs. For detailed chemical analysis, SM, SBM, and SSCs were isolated on day 5 of solid culture, and each domain was analyzed via Raman imaging. Raman imaging showed that avermectin was localized to the SBM and SSCs, but not to the SM. The production of antibiotics during sporulation has been reported in several *Streptomyces* species, and antibiotics can protect spores from environmental factors prior to reactivation from dormancy. Spherical structures with a chemical composition different from that of spores were observed on the agar medium during sporulation, and Raman imaging revealed proteins and lipids as biological components with avermectin distribution in some EVs. The possibility that these EVs were extruded from the MII mycelia of *S. avermitilis* cannot be ruled out.

In Chapter 4, I summarize the study and describe future prospects. Biomolecular imaging using Raman microspectroscopy was used to visualize the localization of secondary metabolites in microorganisms with the subsequent discussion of their metabolic dynamics. A Raman spectroscopic analysis platform was established for the measurement of biological samples. In particular, the MCR-ALS method enables the detection and visualization of trace amounts of secondary metabolites when compared to biomolecules such as proteins and lipids. The antibiotics, penicillin G and avermectin, detected in this study were localized within mycelia, which is a new finding in terms of the production dynamics of secondary metabolites. The application of Raman imaging to the chemical profiling of microorganisms during mycelial differentiation was also demonstrated. The results revealed that the chemical composition of mycelia changed significantly during morphological differentiation. I believe that Raman imaging has the potential to help researchers investigate secondary metabolite production and regulation in microorganisms.

The Raman spectroscopic analysis platform established in this thesis can be applied to the measurement of various biological samples in biology, microbiology, and food science, thereby contributing to the advancement of biological research.

List of research achievements for application of Doctor of Engineering, Waseda University

Full Name : 堀井　俊平　　seal or signature

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Academic paper ○	Detection of penicillin G produced by *Penicillium chrysogenum* with Raman microspectroscopy and multivariate curve resolution-alternating least-squares methods, Journal of Natural Products, 83.11:3223-3229, 2020, Shumpei Horii, Masahiro Ando, Ashok Zachariah Samuel, Akira Take, Takuji Nakshima, Atsuko Matsumoto, Yoko Takahashi and Haruko Takeyama.
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Lecture	Shumpei Horii, Masahiro Ando, Takuji Nakashima, Ashok Zachariah Samuel, Yoko Takahashi and Haruko Takeyama. Subcellular localization of avermectin during morphological differentiation in *Streptomyces avermitilis* using Raman microspectroscopy, The 16th Symposium on Biorelevant Chemistry, 2022, Online, Poster, Poster Award.
Lecture	Shumpei Horii, Masahiro Ando, Takuji Nakashima, Ashok Zachariah Samuel, Atsuko Matsumoto, Yoko Takahashi and Haruko Takeyama, *In situ* detection of Penicillin and Avermectin in Microbes by Raman Microspectroscopy and Multivariate Analysis, Pacifichem 2021, 2021, Online, Poster.
Lecture	Shumpei Horii, Masahiro Ando, Ashok Zachariah Samuel, Takuji Nakashima, Naoko Shibata and Haruko Takeyama, *In situ* detection of secondary metabolites by Raman Microspectroscopy and Multivariate analysis, World Microbe Forum, 2021, Online, Oral.
Lecture	Shumpei Horii, Masahiro Ando, Takuji Nakashima, Ashok Zachariah Samuel, Atsuko Matsumoto, Yoko Takahashi and Haruko Takeyama, *In situ* detection of Penicillin and Avermectin in Microorganisms by Raman Microspectroscopy and Multivariate Analysis, The 101st CSJ Annual Meeting, Online, Oral.
Lecture	Shumpei Horii, Masahiro Ando, Takuji Nakashima, Ashok Zachariah Samuel, Atsuko Matsumoto, Yoko Takahashi and Haruko Takeyama, *In situ* detection of Penicillin and Avermectin in Microbes by Raman Spectroscopic Multivariate Analysis, 71st SBJ Annual Meeting, 2019, Okayama, Oral.
Lecture	Shumpei Horii, Takuji Nakashima, Ashok Zachariah Samuel, Akira Take, Masahiro Ando, Atsuko Matsumoto, Yoko Takahashi and Haruko Takeyama, *In Situ* Detection of Avermectin in *S. avermitilis* by Raman Microspectroscopy and Multivariate Analysis, The 2019 Annual Meeting of the Society for Actinomycetes Japan, 2019, Hokkaido, Oral and Poster, Poster Award.
Lecture	Shumpei Horii, Masahiro Ando, Akira Take, Ashok Zachariah Samuel, Takuji Nakashima, Atsuko Matsumoto, Yoko Takahashi and Haruko Takeyama, *In situ* Detection of Penicillin and Avermectin in Microbes by Raman Microspectroscopy and Multivariate Analysis, The 99th CSJ Annual Meeting, 2019, Okamoto, Oral.

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Others	
Academic paper	Raman Microspectroscopy Imaging Analysis of Extracellular Vesicles Biogenesis by Filamentous Fungus *Penicilium chrysogenum* , Advanced Biology, 2101322, 2022, Ashok Zachariah Samuel, Shumpei Horii, Takuji Nakashima, Naoko Shibata, Masahiro Ando and Haruko Takeyama.
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Publication	Yohei Nishikawa, Shumpei Horii and Haruko Takeyama, High-throughput platform for screening of novel bioactive compound producers, Precision Medicine, Vol.4, No.7, 2021.
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