國立交通大學
National Chiao Tung University

出國報告（出國類別：■ A類、考察訪問
□ B類、出國短期研究
□ C類國際會議）

題目：至東京大學、筑波大學考察，並商討計畫事宜

服務機關：應化系
姓名職稱：重藤真介 助理教授
前往國家：日本東京　東京大學　筑波大學
出國期間：100/06/09~06/13
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備註：出國報告書審核程序如下
一、初閱：各學院教師A、B、C類及其他行政單位A類由單位主管，研究生由指導教授；中心計畫及學群A、B、C類由各中心計畫主持人。
二、複閱：經費所屬之一級單位；中心計畫及學群A、B、C類由頂尖計畫執行長。

一、摘要（200-300 字）

In this trip, I visited the Hamaguchi group at the University of Tokyo and the Nomura group at the University of Tsukuba. The primary objective of the visit to the University of Tokyo was to discuss with Professor Hiro-o Hamaguchi our revised paper submitted to Chemical Communications and possible ways to squeeze out our students' travel support for the International Summer School, which will be held in Japan next month. In addition, I attended a regular group meeting of the Hamaguchi group. During my visit to the University of Tsukuba, I met with our collaborator, Professor Nobuhiko Nomura. We discussed new collaborative projects on Raman imaging studies of nitrate bacteria and *Lactobacillus* as well as some preliminary results of our previous project, that is, vibrational spectroscopy of bacteria-derived alginates.

於此次訪問中，我拜訪東京大學的濱口宏夫教授實驗團隊，及筑波大學的野村教授團隊。訪問東京大學的主要目的在於和濱口教授討論發至 Chemical Communicaitons 的論文的修正版，同時也討論關於 7 月於日本舉辦的 International Summer School 旅費一事，此次會議我們亦會派學生出席。除此之外，我也參加濱口教授實驗室的正規 meeting。在參訪筑波大學中，我和計畫的參與執行者野村暢彥教授會面。我們討論新的合作計畫：拉曼成像術在亞硝酸細菌和乳酸菌研究，及先前研究的初步成果，即振動光譜中細菌衍生之藻酸鹽。

三、本文

（一）目的

The purpose of the visit to the University of Tokyo is to discuss with Professor Hiro-o Hamaguchi our paper in revision for publication in Chemical Communications and the International Summer School held in Izu, Japan, during July 15–17. The visit to the University of Tsukuba is meant to have a discussion with Professor Nobuhiko Nomura about our ongoing collaborative projects and possible future plans.

1、出訪東大目的：（1）討論預計發表在 Chemical Communications 的論文更新版；（2）7/15~7/17 在日本伊豆舉辦的 International Summer School。

2、出訪筑波大學目的：和野村暢彥教授討論進行中的合作計畫，及可能的發展方向。

（二）過程

The schedule of my visit is summarized as follows:

6/9	14:30	Arrival at Tokyo (Narita)
6/10		**Stay at the University of Tsukuba**
	10:30–15:00	Discussion with Prof. Nomura and his postdoctoral researcher and student
6/13		**Stay at the University of Tokyo**
	10:00–12:00	Attend group meeting
	12:00–13:00	Luncheon meeting
	13:00–14:00	Discussion with Prof. Hamaguchi
	18:10	Leave for Taiwan

June 10. I met with Prof. Nomura of the University of Tsukuba. He is one of our collaborators in Japan, who is working on quorum sensing (cell-cell communication) in bacterial biofilms. We initiated our collaboration in 2008, and since then, we have been seeking for a new platform of biofilm studies at the molecular level, in which Raman microspectroscopy and imaging are combined with microbiological approaches. The outcome of the collaborative project on Raman imaging of *Escherichia coli* biofilms has been accepted for publication in Journal of Raman Spectroscopy (see the attachment). I gave a follow-up report on this project and ongoing Raman studies of *Rhodococcus sp.* SD-74 biofilms. Then we discussed future projects including Raman imaging of

nitrate bacteria and of *Lactobacillus*. Given that the oxidation of ammonia to nitric acid (HNO_3) through nitrous acid (HNO_2) by ammonia oxidizing bacteria and nitrate bacteria is a crucial step in wastewater treatment, it will be environmentally very important to chemically visualize the localization of nitric acid and nitrous acid. These bacteria samples will be sent to us soon.

We continued our discussion during lunch with Dr. Masanori Toyofuku, a postdoctoral research in the Nomura group, and Ms. Kaori Ono, an undergraduate student of Prof. Nomura. I explained the experimental details of Raman microspectroscopy to Ms. Ono; she is working on the effects of amino acids on the formation of *E. coli* biofilms found in our Raman imaging study.

6/10　我和筑波大學的野村暢彥教授見面，野村教授是我們實驗團隊在日本的計畫和作者，他的研究領域是在細胞生物膜的「聚量感應」(細胞-細胞間訊息傳遞)。我們實驗室於 2008 年開啓了彼此的合作，自此之後，我們開始尋找在分子標準上從事細胞研究的平台，在此平台上，結合拉曼顯微鏡和成像系統與微生物方法。此項使用拉曼顯微術於大腸桿菌的合作成果已被「拉曼光譜術期刊」接受。我同時也將此計畫的後續發展成果及目前正在進行的拉曼光譜在 *Rhodococcus sp.* SD-74 菌株的研究。之後，我們討論未來計畫的主題，包括亞硝酸細菌和乳酸菌的拉曼成像。藉著氨氧化菌和亞硝酸細菌，將氧化的氨水通過硝酸（HNO_2）加至亞硝酸(HNO_3)，這是廢水分解處理上的關鍵步驟，同時這在環保上也是很重要的，因爲能看出其硝酸和亞硝酸之侷限性。同時，細胞樣本也將寄至我們團隊。

午餐時間同時也進行討論，我們和 Dr. Masanori Toyofuku，野村教授實驗室的博後研究員、Ms. Kaori Ono，野村教授的大學部學生，我向她解釋拉曼成像系統的細節，她同時使用我們實驗室在拉曼成像研究中發現的成型 *E. coli* 生物薄膜的氨基酸進行研究。

June 13. I paid a routine visit to the Hamaguchi laboratory at the University of Tokyo. In the morning, I attended the weekly group meeting. I happened to meet Mr. Minami, an alumnus of the Hamaguchi group. He is currently working for a company developing embedded Linux systems and was invited to talk about the software that he developed for multivariate analysis of a large number of Raman spectra. Following Mr. Minami's presentation, Mr. Kotatsu Bito, a Ph.D. student of the Hamaguchi group, gave a progress report. He showed preliminary results of simultaneous measurements of both coherent Stokes and anti-Stokes Raman scattering (CSRS and CARS, respectively) spectra with the help of supercontinuum light. Finally, Mr. Masanari Okuno gave a practice talk for his poster presentation in the 15th International Conference on Time-Resolved Vibrational Spectroscopy. He presented a time-resolved CARS imaging study of the dissolution process of mammalian cells. I was much

impressed by the speed of CARS imaging.

After the morning meeting, I had lunch with Prof. Hamaguchi, Mr. Minami, and Mr. Chikao Onogi. We discussed in more detail the application of the multivariate analysis software developed by Mr. Minami to our Raman data. I was excited by the power of that analytical software and plan to test it as soon as possible.

Furthermore, I had a one-to-one discussion with Prof. Hamaguchi about our revised manuscript submitted to *Chem. Commun.* and about the financial support for my students' travel to attend the International Summer School.

6/13

當天爲定期至濱口宏夫教授實驗室訪問的行程。早上我參加定期的週間討論。在討論會上，我巧遇 Mr.Minami，他是之前由濱口教授實驗室畢業的校友，目前在就職的公司發展嵌入式 Linux 系統。濱口教授實驗室邀請他演講其所開發的軟體，此軟體可分析大量的拉曼光譜數據。在 Mr.Minami 之後，則由濱口教授實驗室博士班學生 Mr. Kotatsu Bito 發表目前的進度報告。他發表初步研究在於使用超連續光譜以同步監測同調史托克拉曼散射和同調反史托克拉曼散射的成果。最後，Mr. Masanari Okuno 練習預計在第十五屆時析振動光譜會議發表的海報講演。他發表使用同調史托克拉曼散射光譜研究哺乳類細胞的分散過程，我對於其速度留下深刻印象。

在討論之後，我與 Prof. Hamaguchi, Mr. Minami, and Mr. Chikao Onogi 用餐，我們討論更多的細節在於申請這項可以統計分析的軟體，同時也希望能早點測試這套分析軟體。

甚者，我也和濱口教授討論修正已投至 *Chem. Commun.* 的文章，並且討論學生參加 summer school 的經費支援。

（三）心得及建議

All the discussions that I had during this visit are of great help for ongoing research in my laboratory; in particular, the fact that we will be able to use multivariate analysis software for Raman data is very good news to us. The discussion with Prof. Nomura brought me an idea to establish a research center (or association) in Taiwan for spectroscopic investigations of biofilms oriented to environmental sciences. As always, I highly appreciate kind support from the ATU plan.

在這段訪問期間所做的討論對我們實驗室正在進行的計畫有很大的助益。最特別的是，這套統計分析軟體對我們來說是項好消息。在和野村教授討論的過程中，他提供了一個想法在於未來也許能在台灣建立一處研究

中心是關於使用光譜研究生物薄膜導向至環境科學。此外，我們感謝頂尖大學計畫對此項訪問的贊助。

四、附錄

- Outcome of the collaboration with Prof. Nomura published in Journal of Raman Spectroscopy (IF 3.147; 7/39 in Spectroscopy)

Rapid Communication

Journal of RAMAN SPECTROSCOPY

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Leucine pools in *Escherichia coli* biofilm discovered by Raman imaging

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In structured communities of bacteria known as biofilms, a variety of biomolecules have been shown to play a unique role as signals and/or regulators in biofilm formation. Here, we report that high levels of the amino acid leucine (leucine pool) were detected, for the first time, within microcolonies in a 30-h-old *Escherichia coli* biofilm by Raman imaging. Localization of leucine revealed by multifrequency Raman images indicates leucine accumulation during the early stage of the *E. coli* biofilm formation, which may have resulted from physiological environment-specific metabolic adaptation. We demonstrate that our label-free Raman imaging method provides a useful platform for directly identifying still unknown natural products produced in biofilms as well as for visualizing heterogeneous distributions of biofilm constituents *in situ*.

Supporting information may be found in the online version of this article.

Keywords: Raman microspectroscopy; label-free imaging; biofilms; leucine

Introduction

Biofilms are structured consortia of sessile bacterial cells adherent to a surface that are encased in an extracellular matrix comprising exopolysaccharides, proteins, and DNA. While traditional microbiology has focused on planktonic bacteria, it is bacterial cells in biofilms that play a central role in many microbe-associated processes such as bacterial infections,[1,2] wastewater treatment,[3] and bioremediation.[4] In all these processes, various biomolecules serve as chemical signals, regulators, and structural components.[5] For example, in many species including *Pseudomonas aeruginosa*[6,7] and *Staphylococcus aureus*,[8] extracellular DNA has been shown to be required for initial biofilm formation. Very recently,[9] it has been reported that some D-amino acids trigger biofilm disassembly in *Bacillus subtilis* and other bacteria. These studies have been done mostly with biochemical assays and/or fluorescence imaging. However, the former often lacks space-resolved information, and the latter has only limited access to the information on molecular structures and microenvironments in biofilms.

As a first step to fully understand how the biomolecules in biofilms, regardless of whether they are already identified or not, fulfill their advanced functions, we used a label-free Raman imaging method to study the constituents of an *Escherichia coli* biofilm *in situ* and to visualize their distributions in the biofilm. We found that high levels of the amino acid leucine were localized within microcolonies in the *E. coli* biofilm.

Experimental

Space-resolved Raman spectra were recorded on a laboratory-built confocal Raman microspectrometer with 632.8 nm excitation (see the Supporting Information for details). Spatial resolutions of 300 nm in the lateral direction and 2.4 μm in the axial direction were achieved. The laser power was 3 mW at the sample point throughout this work. For the space-resolved Raman measurements, the exposure time was 60 s for the *E. coli* biofilm, 100 s for a planktonic cell, and 30 s for amino acids. For the Raman imaging measurements, the sample was translated at 0.5 μm intervals both in *X* and *Y* directions and the Raman spectrum was recorded at each point with a 1-s exposure time. To improve image contrast, we employed a numerical post-treatment based on singular value decomposition[10,11] (Supporting Information).

E. coli XL1-Blue was routinely cultured in LB medium at 37 °C. Biofilms were grown on a glass-bottomed dish at 37 °C under static conditions. After 30 h, excess LB medium was gently pipetted out from the edges of the dish. The sample was then transferred directly to the microscope stage for Raman measurements without any further pretreatment. All the amino acids were purchased from Sigma-Aldrich and recrystallized once.

Results and Discussion

Everywhere in the 30-h-old *E. coli* biofilm, microcolonies were observed as shown in Fig. 1 (inset). Figure 1(a)–(c) compares the Raman spectra recorded inside and outside the microcolony at ~3 μm above the substrate with that of a planktonic *E. coli* cell measured independently using optical trapping. The extracolonial spectrum was almost identical to the planktonic Raman spectrum with few exceptions including the Raman band at 1408 cm^{-1}. This band is due probably to the COO^- symmetric stretch

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