研 究 報 告 書

「In-situ laser photo-fabrication of metallic nanoprobes inside living cells for spectroscopic analysis of biofunctions」

研究タイプ：通常型
研究期間： 平成 21 年 10 月～平成 25 年 3 月
研 究 者： Nicholas Isaac Smith

1． 研究のねらい

This project aim is to develop new nanotechnology and apply it to analytic bioimaging. Nanoparticles have been of great interest to scientists due to their unique abilities to act as nanoprobes inside biological cells. A local antenna effect allows nanoparticles to report the local chemical environment inside a cell, unattainable by other means. For example, with the single molecular detection sensitivity that comes with such local enhancement, it is in principle possible to discover exactly which molecules are involved in complex cellular signaling pathways. Single molecule detection has already been established for some biological molecules using these methods. However, until now there has been no way to put a particle at a location of interest in the cell. Cellular uptake and final destination of particles in the cell could not be controlled. This project overcame these limitations by using light-based direct fabrication of nanoparticles at any location of interest inside the cell. Using photoreduction of gold ion solution, gold nanoparticles and nanostructures could be formed. Nanoparticles had previously been observed to form in cells over long periods of time. This project used light to drive the reduction reaction and create targeted particles. Using different light sources, including linear and nonlinear photon processes, the project aimed to establish the best procedure to photofabricate intracellular structures. The project further aimed to establish such fabricated gold structures as probes for surface-enhanced Raman scattering (SERS) spectroscopy, and to develop methods for detecting weak signals from SERS-active regions from amongst the thousands of spectra that are recorded in a 3-dimensional Raman hyperstack of data.

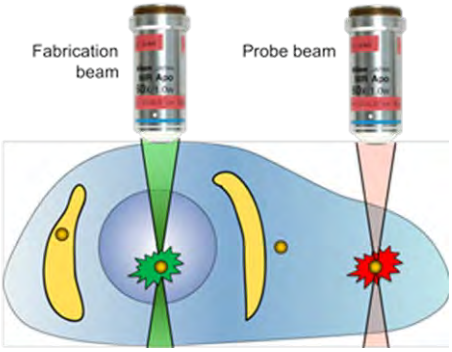

Figure 1: Fabrication beam creates nanoparticles inside the cell whereby cellular molecular information can then be read by a separate probe beam.

2． 研究成果

（1）概要

The project outcomes can be separated into three areas. The first is the findings on fabrication and the properties of the particles, the second is additional outcomes related to the use of nanoparticles in cells or the detection of Raman signals, and the third area is not yet

open and includes patents and publications currently under submission. At the beginning of the project fabricated structures were quite large and not likely to be useful as probes inside cells. The subsequent solution to this problem using 532 nm laser light produced nanoparticles of between 2 and 20 nm in size, which are smaller than usually used in cell nanoparticle applications but large enough to satisfy the requirements of the project (submitted for publication). A wide range of chemical and optical parameters were varied and the optimum procedure for fabricating particles was determined. A problem was to observe whether the particles would generate significant heat under the influence of the incident laser. To measure this, nanoparticles were irradiated by 532 nm laser and the thermal-induced shift in the scattering spectra of particle coatings were measured (Opt Express 2010). Other important findings were made on immunological effects of particles on cells (Particle 2013). During the measurement of surface-enhanced Raman signals it was determined that even with targeted laser fabrication, it is not trivial to find locations with enhanced Raman signals. To this end, a number of algorithms were developed to search for and identify SERS spectra from within an imaging zone (J. Biophotonics 2012). This proved to be a necessary development for the progress of the overall project and significantly improved the data generated by laser fabricated probes.

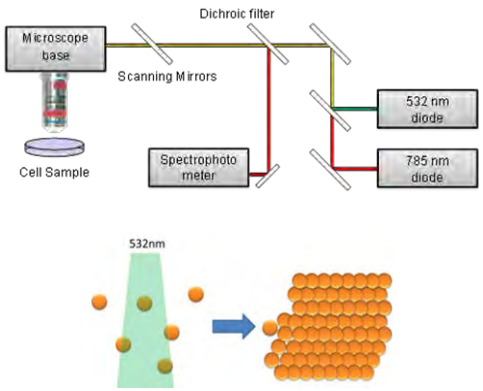

Figure 2: Optical setup for fabrication and schematic of reduction and aggregation of gold into nanoparticles.

(2)詳細

研究テーマA「In-situ fabrication of gold nanoparticles and nanostructures inside biological cells」

Particles were fabricated in cultured cells using the 532 nm beam of a diode laser, as shown in Figure 2, following incubation in gold ion solution. The beam was directed to the microscope by scanning mirrors, collinear with the 785 nm probe beam so that either beam could be directed to the sample by computer controlled mirrors. The focusing target in the cells is chosen by a separate set of scanning mirrors. Gold ions in the solution are reduced and aggregated by the 532 beam. Previously, multi-photon fabrication of silver nanoparticles (outside cells) had been shown, where the multiphoton effect serves to localize the reduction to

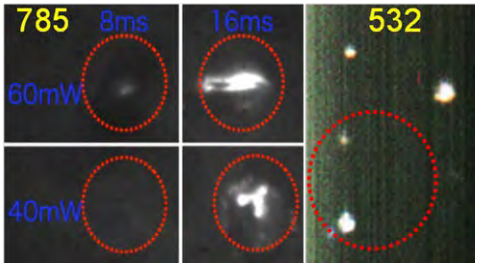

Figure 3: Luminescence measured from regions irradiated by 785 nm pulsed laser and 532 nm. The red circle in each case shows a 10 μ m region for size comparisons. Results show strong nonlinearity with 785 pulsed irradiation.

the area around the focal spot. Therefore, initially, a separate 785 nm fs pulsed laser (not shown in Figure 2) was trialed before moving to 532 nm setup..

Proof of particle fabrication was performed using a number of different methods. The presence of particles was confirmed with electron microscopy imaging, with chemical characterization of gold properties performed by X-ray spectroscopic analysis. Figure 3 shows luminescence images from fabricated regions. The 785 nm pulsed irradiation regions were found to result in strongly nonlinear formation, with a doubling in exposure time causing a large difference in the amount of fabrication occurring. Since this is difficult to control, the laser was changed to a 532 nm beam. Figure 3 also shows fabricated regions using the 532 nm beam. Direct comparison of the two regimes (532 vs 785) is not possible, but the 532 nm fabrication uses tightly focused Gaussian beams of several milliwatts of power, with the laser focus inside the cell. In contrast to the 785 pulsed regime, the 532 nm single-photon process was found to produce repeatable generation of particles at the laser focus. The selection of fabrication photon absorption process should lead to an interesting number of possibilities in fabrication whereby a mixture of the single and multiphoton absorption processes may control the photo-induced growth to the experimentalist requirements.

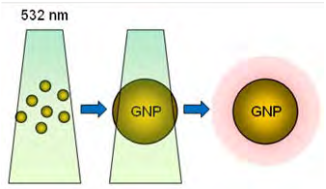

Fig 4: Upon aggregation of gold nanoclusters which lead to growth of the nanoparticle, the 532 nm beam can cause heating effects.

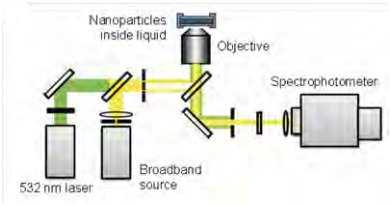

Fig 5: Schematic for measuring heating effects of laser irradiation on nanoparticles in a liquid.

The presence of gold ion solution was determined to halt some cell metabolism and therefore preclude long-term viability, with the results that experiments should be carried out rapidly after fabrication. For long-term viability of samples, additional steps are required.

Most research done using nanoparticles and lasers has not treated the question of the direct laser effect on the nanoparticle, including heating effects. Though difficult to measure, a critical question is whether the 532 nm beam can heat the newly formed particles. To solve this, nanoparticles were coated by an organic polymer with thermally sensitive refractive index and immersed in water to simulate the conditions of nanoparticles inside a cell. The scattering spectrum from a broadband light source was then observed to be shifted (Optics Express, 2011). This was the first direct measurement of such type of laser-nanoparticle effect, and the results showed that the laser heating effect is limited to around one degree with the type of 532 nm irradiation used here. Such low thermal effect indicates laser fabrication of particles occurs by photochemical means.

研究テーマ B「Detection of Surface-enhanced Raman from Fabricated Areas」

Since metallic nanoparticles are much smaller than the diffraction limit of normal microscopy, they are in effect a point-light source. Surface-enhanced Raman scattering was generated when the incident laser field is locally amplified, and molecular vibrations interact with the amplified field, producing frequency-shifted emission. In this way, the local enhancement from

the particles allows the determination of the amount and or type of molecule in proximity to the nanoparticle at single molecule sensitivity. This means that it is very important to be able to control the nanoparticles in the cell. The use of laser irradiation produced particles at chosen locations and surface-enhanced Raman was observed. Figure 6 shows such signals from irradiated regions inside a cell. These signals could be produced in the case of 532 nm but not in the case of 785 pulsed multiphoton fabrication due to the large size of structures. However, even using laser fabrication, the presence of nanoparticles is subject to statistics, and the resulting Raman hyperstack contains a very large number (typically more than 10,000 spectra), and the signals of interest are very difficult to find. The 3d hyperstack of spectra contains some molecular signatures but most data is enhanced background fluorescence or noise. A semi-automated method of detecting, processing, and characterizing the spectra was therefore needed. (Figure 7). 15 different algorithms were trained on test data and then applied to real cell spectra to determine the optimum process (details in J. Biophotonics, 2012) The results shown in Figure 8 allowed greatly improved surface-enhanced Raman data, with the relevant spectra extracted, and classified in spatial position (color overlays in Fig. 8), and grouped by relevance and self-similarity with other spectra (identified clusters in Fig. 8). Together, this was a significant step forward in the use of surface-enhanced Raman measurement, both for this project and also for other related works.

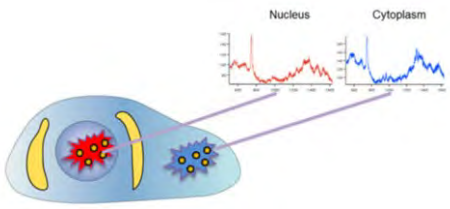

Fig 6: Surface enhanced Raman scattering from particles in the cell.

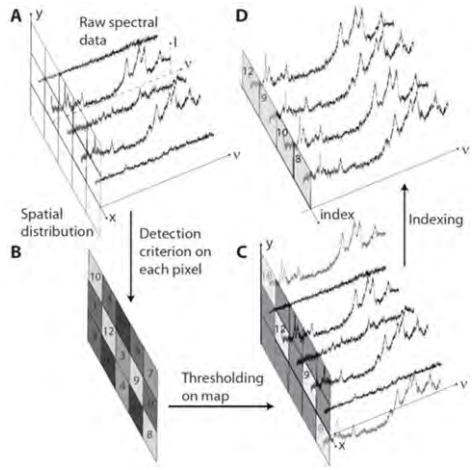

Fig 7: Schematic of approach to automated detection of SERS from fabricated regions in measured data.

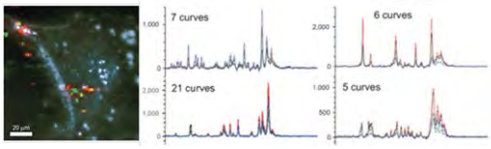

Fig 8: Automated Detection and clustering reports the spectra present in the data and identifies clusters of similar molecules.

3. 今後の展開

Fabrication of nanoparticles in cells has been shown and has been controlled by light. In the short duration of this project, only a few different applications were explored, and the applications in surface-enhanced Raman were deemed to be so important that they were main focus in applications. However, there are a large number of applications of nanoparticles, and these can be developed into separate projects. For example, even though the heating calculations

performed indicate that the thermal effects can be kept within one degree or a few degrees, by alternatively increasing the laser power we should be able to generate additional heat in the target cell. And the heat generation is localized to the area around the nanoparticle since its absorption is far higher than the rest of the cell. This should lead to interesting photodynamic therapy options.

The selection of the fabrication photon absorption process by choosing either 532 nm or 785 nm pulsed irradiation should lead to interesting possibilities. While, during this project, the large structures shown in Figure 3 were avoided so as to enhance the possibilities of detecting surface-enhanced Raman, they show it is possible to make much larger structures. The combination of the two laser beams should allow separation of seeding and growth processes with independent control over each.

To widen the applicability of this developed technique and also other Raman measurements in general, a spectral database is currently underway to help analyze the molecular contents measured in the cells, and in combination with the analytic tools developed during this project, should be a large step towards making meaningful spectral measurements in a number of different cells.

The fabrication process is shown to provide localized antennas for laser energy deposition so that a new type of photodynamic therapy may become possible. This too, should be investigated.

4. 自己評価

From the main aims of the project, most were successful. The fabrication of particles was proved, and was developed into a reliable protocol. The application of these fabricated probes to measure the intracellular environment was demonstrated. Measurement of Surface-enhanced Raman was an important secondary outcome, which was not obvious whether it would work, and was successfully completed. The cell health was found to be not robust against the technique, so that instead of live applications, the applications shift more towards analysis of cellular composition and point towards related techniques such as photodynamic therapy. At the close of the project the key findings show the direction towards future use of light-driven creation of nanotechnology for previously impossible measurements..

5. 研究総括の見解

Smith-kenkyusha presented various data showing that Raman signal could be obtained from molecules inside a cell by using nano gold particles. As a main goal of this PRESTO project, he tried to locate chemically gold nanoparticles and to collect spectroscopic information. He did it and demonstrated that surface-enhanced Raman spectra could be obtained with those particles, however, the living cell is always unstable and cannot survive under that condition, and eventually the goal is left as a future subject. During the project he has encountered various problems for example longer-term viability in biological cells. I believe he will overcome these problems and demonstrate the usefulness of his original idea to fabricate gold particles inside cells very soon.

6. 主な研究成果リスト

（1）論文（原著論文）発表

1. D. Pissuwan, Y. Kumagai and N. Smith, "Effect of surface-modified gold nanorods on inflammatory cytokine response in macrophage cells", Part. Part. Syst. Charact. (2013), in press.
2. N. Pavillon, K. Bando, K. Fujita and N. I. Smith, "Feature-based recognition of Surface-enhanced Raman spectra for biological targets", J. Biophotonics (2012), in press
3. A. Hobro, A. Konishi, C. Coban and N. I. Smith, "Raman spectroscopic analysis of malaria disease progression via blood and plasma samples", Analyst (2013), in press.
4. Y. Kumamoto, A. Taguchi, N.I. Smith, and S. Kawata, "Deep ultraviolet resonant Raman imaging of a cell," J. Biomed. Opt. 17(7), pp. 076001-1-076001-4 (2012)
5. M. Honda, Y. Saito, N. I. Smith, K. Fujita, and S. Kawata, "Nanoscale heating of laser irradiated single gold nanoparticles in liquid," Opt. Express, Vol. 19, Issue 13, pp. 12375-12383 (2011).

（2）その他の成果（主要な学会発表、受賞、著作物、プレスリリース等）

・国際会議招待講演（件）

1. N. Smith (invited) "Advances in optical microscopy: Nonlinearity and high resolution imaging", Senri Life Science Foundation (千里ライフサイエンス技術講習会), Osaka, Nov.9th, 2011.

2, N. Smith (invited) "Laser irradiation as a tool to highlight details in cell imaging" Senri Life Science Foundation (千里ライフサイエンス技術講習会), Osaka, Nov.9th, 2011.

3, N. Smith (invited), K. Fujita, S. Kawata, and Y. Kumagai "Optical control of cell functions: using laser light to remote control signalling, contraction and action potentials in living cells" Conference on Lasers and Electro-Optics (CLEO-PR), Sydney, Australia, Aug 28, 2011.

Invited Commentary

N.I. Smith "A light to move the heart" Nature Photonics, Vol 4, September 2010, 587-589

Press coverage:

Focus on Osaka, Supplement in Science, pp. 8-9 Science, 18 February 2011