Adapting a fluorescence microscope to perform surface enhanced Raman spectroscopy

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The methodology to modify a simple fluorescence microscope to perform sophisticated Surface Enhanced Raman Scattering (SERS) experiments, retaining the primary function of the microscope is discussed. Recent advancement in the use of SERS for the study of biologically important molecules for structural information, sensors and diagnostic applications, makes this an important development. Our method not only reduces the cost of acquiring a Raman spectrometer, but also facilitates an ability to perform experiments without major modifications to the sample preparation and mounting techniques that exist in the laboratory.

Ever since the Raman effect was discovered, it has played a significant role in the field of vibrational spectroscopy. Although Raman scattering acts as an effective tool to probe molecular structure, it has some disadvantages like fluorescence overlap, small cross-sections and low sensitivity. Of late, due to the emergence of nanotechnology and improvement in sensitivity of optical instruments, Surface Enhanced Raman Scattering (SERS) has played an important role in overcoming the above-mentioned disadvantages. In this phenomenon, the Raman mode intensity of a molecule is enhanced by several orders of magnitude ($10^5$ to $10^9$) upon adsorption of the molecule to noble metal surfaces, which exhibit atomic scale roughness. Ever since its discovery, SERS has been utilized as an effective analytical tool to study molecules of biological interest and detection tool to probe different aspects of biology. Most importantly, it has been used to study protein-drug interaction and DNA complexes with a fair amount of success. Recently, we have shown that SERS can also be used to unveil structural information of proteins, such as p300, which are not accessible to crystallographic techniques. It has also been shown that SERS is equipped with a high degree of sensitivity to detect single molecules within a confined volume.

One of the most important aspects of the SERS study is the experimental set-up used to probe and detect the analyte under supervision. Although commercial Raman microscopes have played a key role in the success of SERS, most of them are expensive, sophisticated and lack flexibility. There have been a few successful attempts to build Raman microscopes previously, but most of them require specialized knowledge for construction. Therefore, it is important to design an

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**Fabrication of SERS microscope**

For the present work, we have selected a Nikon Eclipse 50i (Nikon, Japan) microscope with an epifluorescent attachment as the main part of the collection optics for the Raman spectrometer. It should be noted that any other fluorescence microscope could be used for the same purpose. The epifluorescent attachment, which is used for fluorescent imaging, contains the bayonet mount for placing the white light source. The laser of desired wavelength is launched through this mount. An aluminum disc with a 1 mm hole in the centre, sitting snugly on the epifluorescent lamp attachment, could help align the laser beam along the optical axis. The adjustable field diaphragm present in the microscope, which restricts white light illumination on the area of the specimen being viewed, was used as the second aperture to assist the alignment of the laser beam along the optical axis. The diaphragm can be used to focus the laser beam onto the sample under observation. The fluorescence microscopes are provided with dichroic mirror-cube holders. For fluorescence measurements a dichroic mirror is used, which is selected based on the excitation and emission band of the chromophore. In the case of Raman spectroscopy, this would effectively block a large region of the Raman spectrum ($\approx 200$ cm$^{-1}$) close to the Rayleigh scattering. There is an added disadvantage of using the dichroic mirror as it could also cutoff the high-frequency Raman spectra. In order to overcome this, we have replaced...
the dichroic mirrors of the fluorescence microscope with a special mirror designed by us, and fabricated by Axcon Technologies, Singapore. The mirror has an Ag coating of 2 mm diameter at the centre of a 25.2 x 35.6 (±0.2) mm fused silica substrate of 1.1 mm thickness. The mirror had a reflection band between 400 and 900 nm, with reflectivity greater than 95%. With this modification, we were able to record the low-frequency Raman spectrum up to ~50 cm⁻¹, as shown in the case of silicon in Figure 1. The diameter of the laser beam was ~1 mm, which was completely reflected by the mirror of 2 mm diameter.

The microscope was equipped with an additional camera port, which is used for imaging the field-of-view. We have mounted a digital camera on this port. A 200 μm multimode single core optical fibre with a bandpass of 400–1000 nm was used to collect the scattered light. The length of the optical fibre could be anywhere between 1.5 and 5 m. In order to optimize the collection of the scattered light into the fibre, a microscope objective was used at the camera port of the trinocular of the microscope. Details of the construction are given below (Figure 2).

Figure 2 shows the schematic of the micro-Raman system. The Raman excitation light (532 nm) provided by a solid-state frequency-doubled Nd:YAG laser (Model G-SLM-015, Suwtech Inc., China), which traversed a bandpass filter (LL01-532-12.5, Semrock, UK), was used as the excitation source. The 632.8 nm laser source from a He-Ne laser (Model No. 30994, Newport, USA) with appropriate bandpass filter (LL01-633-12.5, Semrock) was also used in some of the experiments. In order to reflect the laser beam by 45° onto the sample, a special mirror was used (as discussed earlier). The scattered light passes through an edge filter (LP03-532RS-25, Semrock) placed at the output camera port of the trinocular. The scattered light was focused onto the optical fibre using an objective lens with a numerical aperture (NA) of 0.4–0.5, as shown in the Figure 2. The other end of the optical fibre was f-number matched at the factory to a 0.55 m spectrograph (Jobin-Yvon 550 Triax, Instruments SA, Inc., NJ, USA) attached with a liquid nitrogen-cooled CCD detector. The f-number matching could also be achieved using a couple of achromatic doublet lens with appropriate focal lengths and the clear apertures.

The spectrograph itself had a computer-controlled adjustable slit and a monochromator which holds three gratings for a range of measurements. For the present Raman studies, a 600 grooves mm⁻¹ grating was used along with the 200 μm spectrograph entrance slit setting, providing ~5 cm⁻¹ resolution. A digital camera (Nikon Coolpix 5400, Nikon, Japan) atop the microscope allowed for registration of the focused laser spot and focusing the image of the laser spot onto the optical fibre (by back-illuminating the optical fibre). Typically, for Raman studies on liquid samples, a 60x infinity-corrected water-immersion objective (Nikon Fluor, NA 1.00, Nikon, Japan) was used. The laser power was ~8 mW at the sample.

For SERS measurements we have used citrate-reduced Ag nanoparticles prepared using the standard Lee and Meisel method. The analyte of interest was mixed with the Ag nanoparticles in the ratio of 5:95 by volume and deposited over a glass slide before bringing the water-immersion objective in contact with it for measurements. The final concentration of the analyte was 1 μM. The spectral accumulation time was typically 1–30 s for all measurements.

**Performance of the constructed microscope**

In order to demonstrate that the micro-Raman instrument constructed by the above method can serve to detect small traces of biologically important molecules, we have performed SERS on a variety of molecules like imidazole (a small organic molecule), rhodamine 6G (dye molecule), adenosine triphosphate and macro-molecules such as haemoglobin and myoglobin at micromolar concentrations. Figure 3 shows the SERS spectra of these molecules with a typical integration time of 1–30 s. All the spectra obtained were in good agreement with previous reported results. The typical volume of the nanoparticle–analyte system used for such detection was 30 μl. It was found that 7 μl was the minimum volume of the composite which produced detectable Raman spectra. It should be emphasized here that all these molecules were also detected at nano-Molar concentrations with this set-up using different sample preparation techniques and excitation sources. Also, one could increase the detection sensitivity by adding a small concentration of NaCl solution, which acts as an aggregating agent for the Ag nanoparticles.

In order to quantify the SERS enhancement, a neat solution of thiophenol was used for recording the Raman spectra, and a 1 μM solution was used for SERS measurements. Figure 4a and b shows

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**Figure 2.** Schematic of the micro-Raman system built with a simple viewing microscope with an epi-fluorescent attachment. ST, Stage; SA, Sample; O, Objective lens; L, Laser; BPF, Bandpass filter; NDF, Neutral density filter; SM, special mirror; B1, B2, Beam splitters; M, Mirror; BINO, Binocular; EF, Edge filter; FL, Focusing lens; F, Optical fibre; SPEC, Spectrometer; CCD, Charged coupled device; COM, Computer; CAM, camera.

**Figure 3.** SERS spectra of (a) rhodamine 6G (R6G), (b) imidazole (Imd), (c) adenosine triphosphate (ATP), (d) haemoglobin (Hb) and (e) myoglobin (Mb). The concentration of all the molecules used was 1 μM. Laser power was around 8 mW at the sample. Typical accumulation times were 1–30 s.
Figure 4. Raman (a) and SERS (b) spectra of thiophenol.

Figure 5. Schematic of the water-immersion objective lens focused at two different spots on the glass slide with the analyte-nanoparticle composite solution used for SERS measurements. Faint line on the figure to the left corresponds to the focus of objective lens at the liquid–glass interface. The corresponding SERS spectra of the R6G-nanoparticle composite are shown, indicated by arrows.

the Raman and SERS spectra of thiophenol (TP). The Raman enhancement factor (or gain), was calculated for the 1080 cm\(^{-1}\) band of TP using the standard method\(^{29}\), and was found to be of the order 10\(^6\). Other molecules, such as nucleic acid bases, different dye molecules, proteins and small molecules (spectra not shown here) were detected at low concentrations using this instrument.

Role and usage of water-immersion objective in SERS

One of the most important aspects of the Raman instrumentation for SERS studies on biomolecules is the ability to perform experiments in aqueous phase for which one uses a water-immersion objective lens. In order to obtain high spatial resolution, high throughput, and tight focusing of the incident beam in SERS studies, it is necessary to use a high NA and a high magnification objective lens, like the 1.2 NA and 60x magnification lens used in the present experiment. This has been a common approach for single-molecule SERS experiments as well as confocal Raman imaging\(^{15}\). Through a simple experiment, we demonstrate the ability to spatially resolve two regions inside the aqueous solution along the focusing direction, which are separated by only a few micrometres. This reveals two important aspects, namely, ability to probe a very small volume, and a limited confocal imaging ability.

Figure 5 shows the schematic of the objective lens focusing at two different heights inside the liquid, which formed a drop over the glass slide. The SERS spectra recorded on these two spots are also shown alongside. R6G with Ag nanoparticles acts as a good test sample since it gives a strong SERS signal. The two spots selected were separated by about a few micrometres; one of them was on the glass–liquid interface and the other was within the liquid just above it. It is interesting to note that the intensity of the strongest mode of R6G (1373 cm\(^{-1}\)) increases by a factor of 7.5 in the case of the glass–liquid interface compared to the one within the liquid. The R6G-nanoparticle composite in the liquid is under constant Brownian motion and therefore, the Raman signal collected for a stipulated time is a resultant of a time-averaged signal of the composite residing in the probed volume of the laser beam. At the glass–liquid interface, there would be an aggregation of the R6G-nanoparticle composite due to sedimentation with time. These stationary composite particles would hence provide a large Raman signal due to increase in the Raman scattering probability. This behaviour was common to all the molecules studied using SERS, and hence could be an important tip for SERS experiments.

In conclusion, we have shown that a simple viewing microscope used for fluorescence imaging can be modified to perform SERS experiments with the functionality of a commercial Raman set-up. One can detect a small trace of important biological molecules using such a set-up. Using this instrument, we have been able to detect several biologically relevant proteins, and have provided structural information during small molecule–protein interactions. Replacing the manually controlled microscope stage with a motorized one, it is possible to convert this set-up into a Raman imaging spectrometer. Work is under progress in this direction.

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