Development of Total Internal Reflection Raman Imaging for Non-intrusive Quantitative Visualization of Near-wall Concentration

Reiko Kuriyama¹,*, Tetsuro Tateishi¹, Yohei Sato¹

¹: Dept. of System Design Engineering, Faculty of Science and Technology, Keio University, Japan
* correspondent author: kuriyama@tfe.sd.keio.ac.jp

Abstract The present study proposes a total internal reflection (TIR) Raman imaging technique for non-intrusive and quantitative visualization of near-wall concentration distributions in micro- and nanofluidic devices. The measurement principle relies on the chemical specificity of spontaneous Raman scattering and surface-selective excitation with an evanescent wave which decays exponentially from the surface. A two-prism-based illumination system was employed to generate the evanescent wave at a glass-solution interface. The Raman scattering arising from near-wall molecules was two-dimensionally captured by an EM-CCD camera through an optical bandpass filter. Since the intensity of the Raman signal is proportional to the number of molecules being illuminated, the Raman scattering images can be converted into concentration distributions via calibration data. In this study, mixture solutions composed of H₂O and D₂O were used as sample liquids focusing on their distinct Raman signals arising from O-H and O-D stretching vibrations. According to the TIR Raman spectra of these solutions, two bandpass filters were selected for Raman imaging in order to visualize the concentration of each species. Prior to the measurements, a calibration experiment was carried out with each filter under uniform concentration distribution. For compensating the inhomogeneity of the evanescent wave illumination, raw intensities were normalized by the reference intensity value at 50 mol% D₂O concentration. The calibration curve showed a linear relationship between the normalized intensity and the H₂O or D₂O concentration. Applying the calibration result, a near-wall concentration measurement was performed in the mixing flow field in a T-shaped microchannel. The lateral spatial resolution was 1.6 × 1.6 µm² and the total acquisition time was 30 seconds. The concentration distribution of each species was visualized in the evanescent wave spot positioned at the junction of the channel where pure H₂O and D₂O merged. These results demonstrate that TIR Raman imaging offers the potential to visualize near-wall molecular distributions quantitatively.

1. Introduction

Recent advances in microfluidic systems promise efficient approaches for miniaturizing and integrating various chemical processes involved in biological analysis and chemical engineering, which has led to the development of micro-total analysis systems or lab-on-a-chip devices (Arora et al. 2010). In such miniaturized systems, interfacial phenomena have a noticeable effect on the liquid’s flow structure and chemical properties due to the large surface-to-volume ratio. For example, an ionic layer called electric double layer (Probststein 1994), which is formed at liquid-solid interfaces, produces electrokinetic flow and affects the solution composition.

For further development of micro- and nanofluidic devices, many experimental efforts to probe these surface-related events have been reported to date. Total internal reflection fluorescence (TIRF) microscopy, which utilizes an evanescent wave to excite fluorophores only in the vicinity of the surface area, is often used as a well-established diagnostic tool (Axelrod 2001). Using TIRF microscopy, Kazoe and Sato (2003) developed a nanoscale laser induced fluorescence (nano-LIF) technique to measure the two-dimensional distribution of the zeta potential which is a dominant parameter of electroosmotic flow. The measurement principle takes advantage of the fact that fluorescent ions are distributed by electrostatic forces related with the wall zeta potential. By applying the nano-LIF technique, color mapping of the zeta potential distribution was successfully obtained at the channel wall with surface modification pattern (Kazoe et al. 2009). For near-wall flow velocity measurement, Zettner and Yoda (2003) first demonstrated nano-particle image velocimetry (nano-PIV), in which evanescent wave illumination was used for tracer-based velocimetry. Nano-PIV has been applied to various flow fields to study fluid mechanics in interfacial regions, including steady electroosmotic flows in a fused silica microchannel (Sadr et al. 2004) and transient electrokinetically driven flow with non-uniform wall zeta-potential (Kazoe et al. 2010). While these fluorescence-based methodologies offer sufficient sensitivity and high spatiotemporal resolution for the investigation of various
near-wall phenomena, they have an inherent disadvantage of using extrinsic fluorescent labels which can potentially affect the measurement results.

Different approaches based on Raman spectroscopy can be a powerful tool for surface-selective and chemically-specific measurement. Surface-enhanced Raman spectroscopy (SERS) is a widely-used technique, taking advantage of the local enhancement of the electromagnetic field at a nano-structured metal surface due to excitation of surface-plasmon resonances (Kudelski 2009). Since SERS produces highly increased Raman signal from molecules in contact with or in the proximity of the surface, it has been applied to various fields of studies such as catalysis (Kim et al. 2010), biophysical chemistry (Hu et al. 2007) and molecule detection in microfluidic sensors (Piorek et al. 2007). However the enhancement effect can be achieved only with appropriately designed SERS-active metal surface; it makes the technique less versatile or impractical in some applications.

Another methodology, total internal reflection (TIR) Raman spectroscopy, realizes non-intrusive measurement without the need for any external markers or resonators by utilizing spontaneous Raman scattering excited by an evanescent wave (Woods and Bain 2012). TIR Raman spectroscopy has long been overshadowed by SERS since it was first demonstrated by Ikeshoji et al. in 1973, but modern instrumentation makes it a practical alternative in applications where SERS is undesirable (Woods and Bain, 2012). Nickolov et al. (1993) investigated the water structure adjacent to a hydrophobic surface by measuring TIR Raman spectra of OH stretching vibrational band of water. Tyrode et al. (2008) evaluated the adsorbed amount of surfactants on a hydrophilic silica surface from the integrated intensities of TIR Raman scattering. Although these studies have proved the effectiveness of TIR Raman spectroscopy, they have been limited to single-point detection through a spectrometer.

The objective of the present study is to develop a non-intrusive and planar visualization technique of near-wall molecular distributions. Total internal reflection Raman imaging is proposed by combined use of spontaneous Raman imaging (Takahashi et al. 2012, Rinke et al. 2012) and evanescent wave illumination generated at solid-liquid interface. Using this technique, Raman scattering from molecules in the vicinity of a microchannel wall was two-dimensionally detected by a camera and converted into a concentration distribution. H$_2$O and D$_2$O mixtures were utilized as sample liquids and a near-wall concentration map of each species was visualized in the mixing flow in a T-shaped microchannel.

2. Measurement Principle

2.1 Evanescent Wave Illumination

When light passes from one medium (with higher refractive index, $n_1$) to another (with lower refractive index, $n_2$), some is reflected back into the first medium and the other is transmitted into the second medium. If the incident angle, $\theta_i$, is smaller than the critical angle $\theta_c$, most of the light propagates into the lower index medium at a refractive angle $\theta$, which is given by Snell’s law. For supercritical condition ($\theta_i > \theta_c$), all of the light is reflected, i.e., it undergoes total internal reflection. Even in this case, however, the electric field penetrates through the interface and propagates parallel to the surface (in Figure 1). This field is termed evanescent wave and its intensity, $I_{eva}$, decays exponentially with the perpendicular distance $z$ from the interface, as expressed by

$$I_{eva}(z) = I_{eva}(0) \exp \left( -\frac{z}{z_p} \right),$$

where

$$z_p = \frac{\lambda}{4\pi n_1 \sin^2 \theta_i - n_2^2}. \tag{2}$$

The characteristic length $z_p$ is called the penetration depth and the parameter $\lambda$ is the wavelength of the incident light in vacuum. Since $z_p$ is generally in the order of $\lambda$ or smaller except for supercritical $\theta_i \rightarrow \theta_c$. 

---

- 2 -
(where $z_p \to \infty$), the evanescent wave can selectively excite the molecules in the vicinity of the interface without exciting the region farther from the interface (Axelrod 2001).

![Evanescent wave and penetration depth](image)

**Fig. 1** (a) Generation of evanescent wave and (b) its intensity distribution

### 2.2 Spontaneous Raman Scattering

When monochromatic light is incident on a sample, it is partially transmitted, absorbed and scattered. Most part of the scattered light has the same frequency as the incident light ($\nu_0$) due to the elastic process called Rayleigh scattering. A small fraction of the scattered light (about $10^{-6}$ of the incident beam) occurs however, as a result of inelastic collisions of photons and molecules, and thus is observed as a frequency-shifted emission ($\nu_1 = \nu_0 \pm \nu_i$). This inelastic scattering event is known as Raman scattering. Raman shift ($\nu_i$) is equal to the vibrational or rotational frequency of a molecule and its intensity has dependence with the number of molecules being excited. Therefore chemically-specific quantitative analysis can be realized by measuring Raman scattering intensity at the characteristic Raman shift.

Figure 2 shows the typical Raman spectra from bulk H$_2$O/D$_2$O mixtures. These mixtures present distinct spectral shapes depending on their composition. The two broad Raman bands with peak intensities at 2500 cm$^{-1}$ and 3420 cm$^{-1}$ can be observed, which originate from O-D and O-H stretching vibrations, respectively. In the present study, these H$_2$O/D$_2$O mixtures were used as sample liquids to demonstrate the ability of TIR Raman imaging to probe the species concentrations in solutions containing multiple components.

![Raman spectra of bulk H$_2$O/D$_2$O mixtures](image)

**Fig. 2** Spontaneous Raman spectra of bulk H$_2$O/D$_2$O mixtures

### 2.3 Total Internal Reflection Raman Imaging

For non-intrusive visualization of near-wall concentration, TIR Raman imaging technique was proposed for capturing Raman images from liquid molecules in contact with a solid surface. Figure 3 illustrates the concept of the technique. The use of an evanescent wave enables the surface-selective excitation of spontaneous Raman process at the solid-liquid interface. The Raman signal arising from near-wall liquid
molecules is spectrally filtered by an optical filter and two-dimensionally captured by a camera. Since this technique employs direct imaging of Raman scattering in the measurement area, there is no need for traversing the light source or the sample. The resultant image is a distribution of the Raman scattering intensity integrated over the transmittance wavelength of the filter, as shown in Figure 3. In order to ensure the detection of only the desired Raman signal, the optical filter should be selected to transmit the wavelength range that corresponds to the characteristic Raman signal of intended species. The expression for the measured Raman intensity for the i-species, \( I_i \) [photons s\(^{-1}\) m\(^{-2}\)], is

\[ I_i = k \times I_0 \times \Omega \times \left( \frac{d\sigma}{d\Omega} \right)_i \times N_i, \tag{3} \]

where \( I_0 \) [photons s\(^{-1}\) m\(^{-2}\)] is the excitation laser intensity, \( \Omega \) [sr] is the solid angle of signal detection, \( d\sigma/d\Omega \) [cm\(^2\) molecule\(^{-1}\) sr\(^{-1}\)] is the absolute differential Raman cross-section and \( N_i \) is the number of i-species molecules being illuminated. The constant \( k \) is introduced to express influencing parameters such as transmission of the optical components and collection efficiency of the detector. The concentration of i-species (\( C_i \)) can be obtained by exploiting the linear dependence of the Raman intensity (\( I_i \)) on the number of molecules (\( N_i \)). Since the Raman intensity also depends on the excitation intensity (Eq. (3)), the measured Raman intensity is normalized by that at the reference concentration (\( I_{i\text{, ref}} \)) as follows:

\[ I_{\text{ratio}} = \frac{I_i}{I_{i\text{, ref}}} = \frac{k \times I_0 \times \Omega \times \left( \frac{d\sigma}{d\Omega} \right)_i \times N_i}{k \times I_0 \times \Omega \times \left( \frac{d\sigma}{d\Omega} \right)_{i\text{, ref}} \times N_{i\text{, ref}}} = f \left( C_i \right). \tag{4} \]

By determining this Raman intensity ratio, the influence of the inhomogeneity of the evanescent wave intensity can be cancelled out.

![Schematic concept of the near-wall concentration measurement by TIR Raman imaging](image)

**Fig. 3** Schematic concept of the near-wall concentration measurement by TIR Raman imaging

### 3. Measurement System for TIR Raman Imaging

#### 3.1 Experimental Apparatus

The experimental setup is illustrated in Figure 4. A two-prism-based illumination system (Kazoe and Sato 2007) was employed to generate an evanescent wave at the glass-solution interface in a microchannel. This configuration has two advantages compared to the popular objective-based system (e.g., Axelrod 2001); the first is a larger field of view and the second is wide selectivity of the channel substrate.

In the present study, a continuous-wave Nd:YVO\(_4\) laser (Coherent Inc., Verdi-V6, \( \lambda = 532 \) nm) was used for an excitation light source. A convex lens was inserted in the laser light path in order to focus the beam and to achieve the desired size of the evanescent wave spot. A microchannel fabricated from poly(dimethylsiloxane) (PDMS) and a silica glass slide was positioned on the prisms with index matching immersion oil. The focused laser beam was introduced into the silica glass through a prism and it traveled...
undergoing a certain number of total internal reflections with the incident angle of 75°. Afterwards the beam was thrown out from the prism in the opposite side. Evanescent wave spots were generated at the glass-solution interface and the molecules only in the vicinity of the interface were excited. The Raman scattering was collected through an objective lens (Nikon Corp., 10×, N.A. = 0.25), a dichroic mirror (> 555 nm) and an optical bandpass filter, and imaged by an EM-CCD camera (Hamamatsu Photonics K. K., C9100-13, 512 × 512 pixels, 16 bits). The selection of the bandpass filter will be described in detail below.

![Fig. 4 Schematic of the experimental setup utilizing two-prism-based system](image)

3.2 Microchannel

A major concern for detection of TIR Raman scattering is fluorescence and Raman signal arising from channel substrates where TIR of the laser occurs. Since these undesirable signals often dominate the measurement results, the substrate material should be carefully selected. This study focuses on the Raman scattering with relatively large Raman shift (between 2000–4000 cm⁻¹), thus silica glass which presents strong Raman peak mainly below 1300 cm⁻¹ (Tyrode et al. 2008) was used for the bottom wall of a microchannel. The channel structure was molded in a PDMS layer by soft lithography (Xia and Whitesides 1998) and enclosed by a 1 mm-thick and 50 mm-diameter silica glass slide by utilizing self-adherence of PDMS. A T-shaped microchannel with a height of 50 μm (Figure 5) was fabricated for a calibration experiment and concentration visualization.

![Fig. 5 Top and cross-sectional views of a T-shaped microchannel](image)

3.3 Solution
In the present experiments, five different mixture solutions composed of H$_2$O and D$_2$O were used as samples. The properties of these solutions are listed in Table 1. The penetration depth of the evanescent wave was calculated to be around 90 nm from Eq. (2), by substituting the laser wavelength ($\lambda$) of 532 nm, incident angle ($\theta_i$) of 75°, refractive index of silica glass ($n_s$) of 1.461 and that of the solution ($n_l$) measured by a refractometer (Atago Co., Ltd, PAL-RI).

| Table 1 Properties of H$_2$O/D$_2$O mixtures used for the calibration experiment |
|---------------------------|-----------|-----------|-----------|-----------|-----------|
| A  | B  | C  | D  | E  |
| H$_2$O concentration [mol%] | 100 | 75 | 50 | 25 | 0 |
| D$_2$O concentration [mol%] | 0 | 25 | 50 | 75 | 100 |
| Refractive index [-] | 1.332 | 1.331 | 1.330 | 1.329 | 1.328 |
| Penetration depth [nm] | 91.4 | 90.8 | 90.3 | 89.7 | 89.2 |

3.4 TIR Raman Spectroscopy for Filter Selection

Prior to the experiment of TIR Raman imaging, spectroscopic measurements were performed in order to choose the appropriate optical bandpass filters for concentration imaging, because it is important to separate the desired Raman signal from noise. Figure 6(a) shows the TIR Raman spectra from the H$_2$O/D$_2$O mixtures (in Table 1) and that from an empty channel (i.e., filled with ambient air). These were obtained by using the aforementioned illumination system, a grating spectrometer (Solar TII Ltd., 400 groove/mm) and the EM-CCD camera. The horizontal axis in the upper side is the wavelength of the scattered light excited at 532 nm. The total acquisition time per spectrum was 100 s and the spectral resolution was less than 0.57 nm (about 15 cm$^{-1}$). The nominal laser power was set to be 0.50 W. For noise reduction purposes, the measured spectra were smoothed with a 11-points, 3rd-order Savitzky-Golay filter (Savitzky and Golay 1964).

For all the spectra in Figure 6(a), broad Raman bands are observed at 1600 cm$^{-1}$ and 3700 cm$^{-1}$, which were not observed in the spectra from bulk mixtures (Figure 2). Since these bands are observed for the empty channel (shown as red line), they are attributed to silica glass being used as a channel substrate. In addition to these strong Raman bands, the relatively weak signals arising from O-H and O-D stretching vibrations can be observed around 3300 cm$^{-1}$ and 2500 cm$^{-1}$, respectively. Although their band shapes are distorted by the signal from the substrate, it can be seen that their intensities increase slightly with the concentration of the corresponding species. In order to remove the Raman signal from silica glass and show the signal from mixtures more clearly, difference spectra are calculated for the five solutions (Figure 6(b)) by subtracting the reference spectrum of the empty channel. The difference spectra exhibit the characteristic band shapes of O-H and O-D stretching modes as in bulk spectra. The intensity change with mixture composition is also confirmed from Figure 6(b). According to the above spectral measurement result, two optical filters were selected for concentration imaging; one is for H$_2$O with transmittance wavelength of 641–658 nm (Semrock Inc., FF01-650/13-25) and the other is for D$_2$O with transmittance wavelength of 603–622 nm (Semrock Inc., FF01-615/20-25). The transmittance wavelength ranges are indicated by arrows and colored areas in Figure 6(b). The figure shows that their transmittance wavelengths correspond to the spectral region of the O-H and O-D stretching Raman bands and there is little overlap between them.

Finally, the integrated intensity of the TIR spectrum in the transmittance range of each filter was calculated for the solutions and plotted against the concentration in Figure 6(c). It is obvious that the integrated intensity for each filter linearly increased with the concentration of corresponding species, which is explained by Eq. (3). These results indicate that concentration measurement of desired species can be realized by TIR Raman imaging by utilizing the appropriately-selected filters.
Fig. 6 (a) Total internal reflection Raman spectra from microchannel filled with H$_2$O/D$_2$O mixtures and ambient air (b) Difference Raman spectra of mixtures calculated by subtracting the TIR spectrum obtained with an empty channel (c) The relationship between the concentration and the integrated Raman intensity in the transmittance ranges of the selected filter.
4. **Calibration Experiment**

4.1 **Experimental Condition**

Utilizing the selected filters, a calibration experiment was carried out under a uniform concentration field in a microchannel. The channel was positioned on the prisms along the direction of laser propagation (as shown in Figure 5). The laser power was 0.43 W at the sample stage with an ellipsoidal spot (major axis: 260 μm, minor axis: 100 μm). The exposure time of the camera was set to be 3 s and 10 images were accumulated for each condition.

4.2 **Calibration Result**

Figure 7 shows the temporally averaged Raman images of each mixture obtained through the H₂O and D₂O filters. These intensity distributions are visualized at a spatial resolution of 1.6 × 1.6 μm² which was calculated based on the pixel size of the camera and the total magnification of the system. It is clearly seen that the intensity detected through the H₂O filter decreases with a decreases in H₂O concentration (Figure 7(a)), whereas that obtained through the D₂O filter increased (Figure 7(b)). This result indicates that these two filters are adequate for concentration imaging of the corresponding species. Although these images were acquired under uniform concentration, they have non-uniform intensity distributions according to the ellipsoidal intensity profile of the evanescent wave spot.

In order to obtain the relationship between the Raman intensity and the concentration of each species, the intensities at arbitrary ten points in the Raman images, which were randomly selected from the region of evanescent wave spot, are plotted against the concentration (in Figure 8). For reducing the influence of the inhomogeneous intensity profile, as indicated in Eq (4), the Raman intensities were normalized by the reference intensity being the one of at 50 mol%. The normalized intensities obtained for the H₂O and D₂O filters linearly increase with H₂O and D₂O concentrations, respectively. Linear least-squares fit provides the solid straight lines in Figure 8, which served as calibration curves for subsequent concentration measurement. The error bars represent the standard deviation of intensity ratios between the ten points. By applying this calibration result, Raman images can be converted into concentration maps of H₂O and D₂O.

![Evanescent wave spot](image)

**Fig. 7** Raman images of each mixture obtained through (a) H₂O filter and (b) D₂O filter
5. Near-wall Concentration Imaging in Mixing Flow

Using the above calibration result, near-wall concentration measurement was performed in the non-uniform mixing flow field in a T-shaped microchannel (Figure 5). Pure H$_2$O and D$_2$O were injected into the channel by pressure-driven flow from Inlets A and B, respectively. The position of the second evanescent wave spot was adjusted to the junction area where the two liquids merged (Figure 9(a)). The conditions for the data acquisition (such as laser poser, exposure time and the frame number of accumulation) were set to be the same as the calibration experiment. Figures 9(b) and (c) give the measurement results for H$_2$O and D$_2$O concentrations, respectively. It should be noted that the visualized concentration maps represent the ‘initial concentration’ without consideration of isotopic exchange reaction (H$_2$O + D$_2$O ↔ 2HOD). The non-uniform concentration distributions were visualized for each species in the evanescent wave spot using the corresponding filter and calibration curve. The concentration gradient can be successfully observed in the X-direction in the central region of both distributions although the concentration around the edge of the spot is considered to suffer from lower signal level. These results clearly demonstrate the availability of TIR Raman imaging for visualization of molecular distributions in the vicinity of the channel wall.

Fig. 9 (a) Measurement area at the junction of the T-shaped microchannel. (b) Two-dimensional distribution of H$_2$O concentration and (c) of D$_2$O concentration.
6. Conclusions

The present study describes the development of a non-intrusive visualization technique for near-wall concentration distribution using total internal reflection Raman imaging. The evanescent wave, which was generated by a two-prism-based illumination system, was used as the excitation light source for Raman process at solid-liquid interface in a microchannel. H₂O/D₂O mixtures were prepared as sample solutions and optical bandpass filters were selected based on their TIR Raman spectra for concentration imaging. The main accomplishments obtained from this work are summarized below.

(1) A calibration experiment was carried out under a uniform concentration field. The Raman scattering intensity obtained through the selected filters linearly increased with the concentration of the corresponding species.

(2) A near-wall concentration distribution measurement was performed in the mixing flow field in a T-shaped microchannel. The non-uniform concentration map in the evanescent wave spot was visualized for each species at a spatial resolution of 1.6 × 1.6 μm² with the penetration depth of around 90 nm.

It can be concluded that the results obtained in this study prove the feasibility of the TIR Raman imaging technique for quantitative visualization of near-wall molecular distributions for analyzing surface-related phenomena.

Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research (A) (No. 23246037), a Grant-in-Aid for JSPS Fellows (No. 24-7201) (for the first author) from the Japan Society for the Promotion of Science and Keio Gijuku Academic Development Funds.

References


