Raman Tweezers Spectroscopy of Live, Single Red and White Blood Cells

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Abstract

An optical trap has been combined with a Raman spectrometer to make high-resolution measurements of Raman spectra of optically-immobilized, single, live red (RBC) and white blood cells (WBC) under physiological conditions. Tightly-focused, near infrared wavelength light (1064 nm) is utilized for trapping of single cells and 785 nm light is used for Raman excitation at low levels of incident power (few mW). Raman spectra of RBC recorded using this high-sensitivity, dual-wavelength apparatus has enabled identification of several additional lines; the hitherto-unreported lines originate purely from hemoglobin molecules. Raman spectra of single granulocytes and lymphocytes are interpreted on the basis of electrochemical potentials across the cell membrane that, in turn, may give rise to changes in the physiochemical microenvironment and may give rise to changes in spectral features in red and white blood cells.

Introduction

Optical tweezers are proving to be of widespread utility in contemporary research in a number of fields, particularly in the biomedical sciences [1,2]. Optical tweezers use tightly-focused laser light to create a sharp intensity gradient over tiny spatial dimensions such that microscopic dielectric objects floating in liquid media may be trapped by the action of a gradient force [3]. Such trapped objects can then be subjected to spectroscopic investigation [4–22] in such manner that the deleterious effects on spectral quality of the inevitable Brownian motion are circumvented. By combining spectroscopic techniques with optical tweezers, the possibility opens of being able to extract precise information about biochemical changes at a single-cell level under physiological conditions and without the necessity of chemical fixing of cells. Cell immobilization by chemical or physical means alters the physiochemical microenvironment and may give rise to changes in electrochemical potentials across the cell membrane that, in turn, affect cellular functions [5]. In order to facilitate spectroscopic studies of single living cells in a physiological medium, we have developed a high-resolution, dual-wavelength apparatus that combines optical trapping with Raman spectroscopy, utilizing near infrared wavelength light at 1064 nm for trapping and 785 nm light for Raman excitation at very low levels of incident power (<10 mW). We have utilized this apparatus to probe, in sensitive and high-resolution fashion, Raman features in red and white blood cells.

Among several spectroscopy techniques, Raman spectroscopy is a particularly potent tool to probe the biochemical composition of cells; it has proved to have the potential of being able to differentiate between, for example, normal and malignant cells [9–11]. In recent years, micro-Raman spectroscopy has developed into a powerful tool to spectroscopically probe single cells with high spatial resolution, requiring relatively simple sample preparation procedures [23–27]. Resonance Raman spectroscopy has also been utilized to probe DNA bases and aromatic amino acids in proteins, and the technique has been successfully applied to rapidly identify bacteria such as E. coli, P. fluorescens, S. epidemis, B. subtilis and E. cloaca [26,27].

The relatively recent marriage between the techniques of optical trapping and Raman spectroscopy [4], giving rise to Raman Tweezers, has resulted in a number of “offspring” in the form of interesting and important applications in the biomedical sciences [4–22]. This marriage has opened new vistas for biomedical applications that require spectroscopic information with good spatial resolution and identification of intra-cellular components under physiological conditions. The early examples of Raman Tweezers utilized a single, low power laser beam, usually of 785 nm wavelength, that was used for both optical trapping and near-infrared Raman spectroscopy [4]. The advantages of using a dual wavelength set-up for confocal Raman spectroscopy were established by Petrov and coworkers [6]; one of the two laser beams was used for trapping micron-size cells and a second laser beam was used to carry out Raman excitation.

Applications of Raman Tweezers to studies of red blood cells (RBCs) have attracted considerable contemporary attention [16–20]. Raman spectra have been measured of red blood cells in their equilibrium geometry as well as when they are stretched; spectral changes have been interpreted in terms of mechanically induced effects and indications have been obtained that stretched RBCs...
have significantly enhanced oxygen concentration [16]. Raman Tweezers experiments have also been reported that seek to examine the oxygenation capability of β-thalassemic RBCs and to probe the response to photo-induced oxidative stress of diseased cells with respect to normal ones [17]. The effect of alcohol on single RBCs has been reported [18] wherein the viability of cells was characterized by the porphyrin breathing mode in Raman spectra at 752 cm$^{-1}$; the intensity of this band decreases with alcohol concentration [18].

We describe in the following details of a successful coupling in our laboratory of a Raman spectrometer with an optical trap that allows, without physical contact, simultaneous optical trapping, optical manipulation, and recording of Raman spectra of micron-sized objects floating in a medium. The trapping is accomplished using tightly-focused, 1064 nm wavelength laser light while Raman spectra are recorded by exciting the trapped particle by 785 nm laser light. We have recorded Raman spectra of single, living red and white blood cells under physiological conditions, achieving an enhancement of overall spectral quality that has enabled identification of several additional lines in the Raman spectra of trapped cells.

**Methods**

**Ethics Statement**

The experimental work involved extraction of blood samples from healthy volunteers. In the present series of experiments, two of the co-authors (AB and EZ) were such volunteers from whom the blood is drawn. Approval for this was obtained from the University Ethics Committee, Manipal University, on 11 December 2000 (reference UEC/35/2000 and UEC/36/2000) and written, informed consent was obtained from the two co-authors whose blood was taken. All the experimental work reported in this paper relied only on blood samples taken from the co-authors, AB and EZ.

1 ml of whole blood was extracted from co-authors AB and EZ and was centrifuged at 3000 rpm for 5 minutes to remove the serum. The RBCs were diluted in Phosphate Buffered Saline (PBS) to get a suspension of living RBCs. About 10 μl of serum was again added to 1 ml of suspension to ensure that the RBCs remained intact for an extended period of time. For collecting while blood cells (WBC) same centrifugation procedure is followed. About 20 μl of serum near the buffy coat layer was pipetted out and diluted in 1 ml of PBS to obtain a solution of live WBCs.

**Apparatus**

We first discuss a number of considerations that need to be addressed in the process of designing an apparatus that would find practical application in the biomedical sciences. On the trapping of biological matter, an important question relates to potential laser-induced damage within the trapping volume. Such potential hazards are expected to be wavelength and power dependent. For instance, photo-chemically induced alterations in the properties of trapped cells might include damage to DNA, alterations in cell metabolism and chemical effects like oxidation [28]. The tight spatial focusing that is inherent in optical tweezers inevitably leads to localized laser intensities that might easily exceed several MW cm$^{-2}$, leading to localized thermal effects such as the formation of bubbles [29] and acoustic waves [30], leading to photomechanical effects that might prove to be deleterious to trapped biological matter. We refer to a recent, cogent discussion of such experimental considerations by Snook and coworkers [15] in the light of which we have designed and implemented the following experimental scheme.

Figure 1 is a schematic depiction of the Raman Tweezers setup that we have designed and assembled in our laboratory. Our optical tweezers mainly comprises an inverted microscope (Nikon Eclipse Ti-U, Japan) with a high numerical aperture (1.3 NA), 100X oil immersion objective (Nikon, Plan Fluor) to produce a sharp focus to trap micro-particles suspended in solutions in a custom-made sample cell. The sample cell is made by attaching a microscope cover slip on to a metal plate with a 1 mm deep wedge of 0.8 cm width and 1 cm length. An Nd:YAG laser (Laser Quantum, UK) with an output beam of 1064 nm wavelength is used as the trapping laser. Measurements made on E. coli and CHO cells in the earlier reports have indicated that wavelengths in the close vicinity of 1 μm are expected to cause minimal laser-induced cell photodamage [31,32]. In order to get a tightly focused, diffraction-limited spot at the sample, the back aperture of the objective has to be overfilled. We achieved this by expanding the beam from the Nd:YAG laser to nearly 9 mm diameter by means of a manual beam expander (BE1).

The laser beam was amenable to manipulation by a 1:1 telescopic arrangement comprising two convex lenses of equal focal length, f, at a distance of 2f from each other as well as from the back focal plane of the microscope objective. A dichroic mirror (DM$_2$) which has high reflectivity at 1064 nm wavelength directed the sufficiently expanded laser beam to the back aperture of our microscope objective. A CCD camera (Nikon DS-2MBW, Japan) was attached to one of the exit ports of the microscope, enabling visualization and recording of optically trapped micron-sized objects. Manipulation of the trapped objects was achieved simply by means of a controllable x-y translational stage.

Raman excitation of trapped particles was achieved in our apparatus by means of a 785 nm wavelength laser beam (Starbright Diode Laser, Torsana Laser Tech, Denmark) that was focused onto the sample by the same microscope objective. The choice of wavelength was dictated by the following considerations. The efficiency of Raman scattering exhibits a $\lambda^{-4}$ wavelength dependence, indicating the desirability of using short wavelengths. However, as indicated by the work of Neuman and coworkers [31], short wavelengths result in increased propensity for laser-induced photodamage. The use of longer wavelengths also offers a potential advantage of reducing fluorescence effects that compete with the weak Raman signals that we anticipate in the type of work that we wish to undertake on biological samples. However, longer wavelengths also lead to inescapable problems that are related to the efficiency of CCD-based photon detectors that are readily available at present. As discussed by Snook and coworkers [15], the best available detection efficiency of ~50% is obtained over the wavelength range 600–800 nm. The corresponding value around 1 μm wavelength is 10% while at 1064 nm it is almost zero. Our choice of 785 nm as the Raman excitation wavelength appears to be the optimum utilitarian compromise.

Might it be possible to utilize the 785 nm light for both trapping and excitation purposes? We have considered this possibility and, in some cases, the answer is in the affirmative. However, cognition has to be taken of the fact that the stiffness of the optical trap strongly depends on the laser beam quality. The diode lasers presently available for Raman excitation have distinctly inferior beam quality to that obtained from a laser like the Nd:YAG laser that we utilize for trapping. Consequently, use of the Raman laser for trapping results in considerably less efficient trapping. However, this can be tolerated under some circum-
stances. For instance, in some preliminary experiments we did use only the 785 nm laser beam for both trapping and excitation of RBCs, albeit less efficiently. This scheme certainly cannot be applied to WBCs whose refractive index (1.360 ± 0.004) is somewhat lower than that of RBCs (1.399 ± 0.006); hence a more efficient trap is required when work is to be conducted on WBCs.

In our experiments with the 785 nm laser we observed that the output beam contained an additional weak line around 790 nm which might interfere with the Raman signals. To circumvent this, we passed the laser beam through a holographic band pass filter BF (Kaiser Optics, USA) that effectively blocked the extraneous 790 nm line. The 785 nm beam was first expanded using a beam expander (BE2) so as to overfill the back aperture of the microscope objective and then reflected into the microscope by the 785 nm dichroic mirror (DM1). The combination of two dichroic mirrors was carefully chosen such that they transmitted Raman signals to the spectrometer and visible light to the CCD camera so as to facilitate visualization of trapped samples. Figure 2 shows the transmission curves that we measured for the two dichroic mirrors.

A 1:1 telescopic arrangement allowed us to exercise proper control over the Raman probe beam. Using the telescopic arrangements both the laser foci was brought to the same point in the sample plane so as to enable simultaneous trapping and excitation. A backscattering configuration was used to collect the scattered signals from the trapped particle, as indicated in Fig. 3. The backscattered light was collected by the same objective and focused onto the spectrograph slit by an f/4 lens, which was coupled to one of the exit ports of our microscope. A 783 nm high pass edge filter (Razor Edge LP02-785RU-25, Semrock, USA) was used to exclude the intense Rayleigh scattering signal that
inevitably accompanies the weak Raman signals. The Raman signals were dispersed using a Horiba Jobin Yvon HR320 spectrograph with 0.6 nm resolution at 435 nm wavelength when using a 1200 grooves/mm grating blazed at 750 nm. Detection was by means of a liquid-nitrogen cooled, charge coupled detector (Symphony CCD-1024×256-OPEN-1LS) with 1024×256 pixels, operating at 140 K.

Results and Discussion

Alignment and calibration of our set-up was accomplished using polystyrene beads of average diameter 3 \( \mu \text{m} \) (Sigma Aldrich, USA). A bead suspended in deionised water was trapped, typically using a trapping power of \( \sim 5 \text{ mW} \), and the Raman spectrum was recorded by exciting it with 785 nm laser light. Raman spectrometer optics were aligned for maximum signal-to-noise ratio in measured spectra. Figure 4 shows typical Raman spectra of a trapped polystyrene bead with integration times of 2 s and 10 s. These spectra are consistent with those reported in the literature [4]. Typically, up to 20 spectra were recorded using 10 mW excitation power, and all of them are more or less identical to those shown in Fig. 4, with reproducibility on the wavelength axis being \( \pm 1 \text{ cm}^{-1} \). Values of laser power used to trap cells and to record Raman spectra were measured by locating an integrating sphere just after the microscope objective in our set-up (Fig. 1). The spectral resolution of our system was determined to be \( \sim 5.7 \text{ cm}^{-1} \) (with the spectrometer slit width kept at 100 \( \mu \text{m} \)) by measuring the FWHM of the 997 cm\(^{-1}\) Raman band of the polystyrene bead spectrum. The inset to Fig. 4 shows an image of the trapped and untrapped polystyrene bead.

It is important to note here that laser power levels that were incident on the trapped cell were kept low enough to ensure that cell viability was likely to be maintained. In experiments whose results we report in the following, we typically made measurements on a single trapped cell for periods as long as \( \sim 1 \text{ hour} \); in the course of this time we recorded Raman spectra every 5–10 minutes. The fact that recorded spectra was consistent during the entire stretch of the experiment helped allay concerns regarding cell viability. Moreover, other optical trap work conducted in our laboratory over several years [33–35] has provided evidence that optically-induced damage to RBCs and WBCs is unlikely to occur at incident laser power levels that are less than \( \sim 30 \text{ mW} \) in our system provided that the irradiated cells are kept in a physiologically relevant medium, which they were. The lowest possible incident power levels were utilized to obtain spectra presented in the following sections.

Raman spectroscopy of trapped RBCs

Raman spectra of optically trapped single RBCs and WBCs were measured with our apparatus using trapping and excitation power levels that are significantly less than earlier work [4]. The glass coverslip induced band in the region 1000–2000 cm\(^{-1}\) was overcome by using a fused silica coverslip which leaves only a peak at 798 cm\(^{-1}\) in the Raman spectrum. We first focus attention on spectra of trapped red blood cells (RBCs) of which typical spectrum is presented in Fig. 5. A single living RBC was trapped, typically using \( \sim 5 \text{ mW} \) power, and Raman excited using typical power levels of \( \sim 5 \text{ mW} \). Our recorded spectrum tends to cover a significantly larger region than that of earlier micro-Raman work [36–38] and several additional lines are observed in our measurements. Assignment of the vibrational bands observed in our spectra relied on the pioneering work on RBCs carried out by Wood and coworkers [36–38] and on Raman transitions in hemoglobin studies carried out by Hu and coworkers [39]. The complete assignment of spectra recorded by us in the present measurements is presented in Table 1.
The spectra contain a mixture of bands from the heme group and various protein components. The protein bands include the amide at 1649 cm\(^{-1}\) and the CH\(_2/CH_3\) deformation modes primarily from amino acid side chains at 1445 cm\(^{-1}\). The Raman spectrum of RBC seems to be dominated by peaks corresponding to various vibrational modes of heme. The bands obtained here

![Figure 5. Raman spectrum of a trapped single RBC over the wavelength range 400–1750 cm\(^{-1}\). Laser power: \(-5\) mW, acquisition time: 60 s, average of 5 accumulations. The inset shows the image of trapped and untrapped RBCs. doi:10.1371/journal.pone.0010427.g005](#)

**Table 1.** Observed Raman frequencies with corresponding band assignments for RBCs and whole blood-Serum.

| Raman Band Positions in single RBC (in cm\(^{-1}\)) | Raman Band Positions in whole blood (in cm\(^{-1}\)) | Band Assignments | Raman Band Positions in single RBC (in cm\(^{-1}\)) | Raman Band Positions in whole blood (in cm\(^{-1}\)) | Band Assignments |
|-----------------------------------------------|-----------------------------------------------|------------------|-----------------------------------------------|-----------------------------------------------|------------------|
| Wood et al | Present work | Wood et al | Present work | Wood et al | Present work | Wood et al | Present work |
| ----- | 473 | 478 | \(\nu_{33}\) | ----- | 1138 | ----- | \(\nu_{43}\) |
| ----- | 490 | ----- | p: S-S str | 1156 | 1155 | 1157 | \(\nu_{44}\) |
| ----- | 517 | 517 | p: S-S str | 1174 | 1171 | 1170 | \(\nu_{40}\) |
| ----- | 545 | 547 | \(\tilde{\nu}_{21}, \tilde{\nu}_{20}, p: S-S\) str | 1212 | 1209 | 1212 | \(\nu_{34}, \nu_{18}\) |
| 567 | 567 | 570 | \(\nu(Fe=O)\) | 1226 | 1223 | 1224 | \(\nu_{13}\) or \(\nu_{12}\) |
| ----- | 589 | 584 | \(\nu_{48}\) | 1306 | 1301 | 1310 | \(\nu_{21}\) |
| ----- | 620 | 621 | \(\tilde{\gamma}_{12}, Phe-C-C\) twist | 1332 | 1321 | ----- | \(p:CH\_twist\) |
| ----- | 635 | 643 | pC=C str | 1337 | 1339 | 1340 | \(\nu_{41}\) |
| 676 | 672 | 673 | \(\gamma_2\) | 1371 | 1376 | 1376 | \(\nu_4\) |
| 754 | 751 | 752 | \(\gamma_{15}, \gamma_1\) | 1397 | 1399 | 1398 | \(\nu_{20}\) |
| 789 | 789 | 789 | \(\gamma_6\) | 1430 | 1423 | ----- | \(\nu_{28}\) |
| 827 | 820 | 824 | \(\gamma_{10}\) | 1448 | 1445 | 1450 | \(\delta(CH\_2/CH_3)\) |
| ----- | 860 | 855 | \(\gamma_{10}\) | 1526 | 1523 | 1525 | \(\nu_{36}\) |
| ----- | 898 | 898 | pC=C skeletal | 1547 | 1544 | 1546 | \(\nu_{11}\) |
| ----- | 936 | 927 | \(\nu_{46}\) | 1566 | 1562 | 1562 | \(\nu_2\) |
| ----- | 962 | 971 | p: Skeletal vib | 1582 | 1579 | 1583 | \(\nu_{37}\) |
| 974 | 989 | 989 | \(\gamma(C\_H=)\) | 1604 | 1602 | 1603 | \(\nu_{10}\) or \(\nu(C=\equiv C)\) \_aryl |
| 993 | 998 | 997 | \(\nu_{45}, Phe\) | 1620 | 1617 | 1617 | \(\nu_{10}\) or \(\nu(C=\equiv C)\) \_aryl |
| 1031 | 1025 | 1026 | \(\delta(C\_H_2)_{asym}\) | 1639 | 1631 | 1635 | \(\nu_{10}\) |
| 1047 | 1049 | 1050 | \(\nu(O=O), \delta(C\_H_2)_{asym}\) | 1653 | 1649 | 1649 | Amide I |
| 1074 | 1079 | 1066 | \(\delta(C\_H_2)_{asym}\) | ----- | 1667 | ----- | Amide I |
| 1127 | 1119 | 1129 | \(\nu_5\) |

Abbreviations: - \(\nu\) & \(\tilde{\nu}\): in-plane modes, \(\gamma\): Out-of-plane modes, sym: symmetric, asym: asymmetric, Str: stretching, p: protein, Phe: Phenylalanine.

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are well resolved and the apparatus that we have set up is clearly sensitive to even faint peaks. The richness of the spectrum brings to the fore the fact that Raman studies of live cells in suspension are more informative as far as molecular concentration and conformation are concerned than micro-Raman studies where the cells are chemically bound to a glass coverslip.

A comparison of our spectra with those reported by Wood and coworkers [36] shows that several new peaks are observed in our experiments; these are located at 1138 cm\(^{-1}\), 962 cm\(^{-1}\), 936 cm\(^{-1}\), 898 cm\(^{-1}\), 860 cm\(^{-1}\), 635 cm\(^{-1}\), 620 cm\(^{-1}\), 589 cm\(^{-1}\), 545 cm\(^{-1}\), 517 cm\(^{-1}\), 490 cm\(^{-1}\) and 473 cm\(^{-1}\). Most of these peaks are amenable to assignment on the basis of the conventional Raman measurements on hemoglobin conducted by Hu and coworkers [39]. The peaks 962 cm\(^{-1}\), 898 cm\(^{-1}\), 635 cm\(^{-1}\), 517 cm\(^{-1}\) and 490 cm\(^{-1}\) were not assigned in the studies of Hu and coworkers, though these lines may be contributed by the hemoglobin itself. We have assigned all these peaks in the present study using standard literature on vibrational spectroscopy of proteins [40] and we are able to conclude that the hitherto-unreported lines in RBC spectra obtained in the present study originate purely from hemoglobin molecules.

The presence of all these peaks in the present measurements on trapped RBCs may be a reflection of the relatively larger sample volume that is probed in our apparatus in comparison to that in the case of micro-Raman studies. Furthermore, the enhanced resolution in the present set of measurements may also be a contributory factor.

In the micro-Raman study, the Raman probe beam is inevitably perpendicular to the plane of RBCs whereas in the case of trapped RBCs the orientation is parallel, and the probe beam passes through larger cell volume than is the case in micro-Raman measurements. In other words, the larger optical density in our experimental arrangement might contribute to higher signal sensitivity coupled with enhanced resolution that is achieved in the present experiments.

The concentration of hemoglobin in normal red blood cell is typically 32% to 36%; the domination of hemoglobin in IR spectra has been noted before and it has been postulated that this could be ascribed to excitonic coupling between aligned porphyrins in the highly aggregated heme environment of the RBC [31, and references therein]. Further work needs to be undertaken to gain a deeper insight.

A comparative analysis was also carried out by recording Raman spectra of blood samples after removal of serum as shown in Fig. 6. We measured a conventional Raman spectrum over the range 350 cm\(^{-1}\) to 1800 cm\(^{-1}\) and the peaks that we observed, along with their assignments, are presented in Table 2. Raman spectra of trapped RBCs closely resemble conventional Raman spectra of whole blood except that lines at 1423 cm\(^{-1}\), 1321 cm\(^{-1}\), 1138 cm\(^{-1}\) and 489 cm\(^{-1}\) are missing in whole blood spectra. These “missing lines” in whole blood spectra are most likely a consequence of the fact that the whole blood Raman spectrum is the ensemble average of many randomly oriented RBCs within the focused laser spot size (30 µm diameter in these measurements). However, further work needs to be undertaken to verify this conjecture of ours.

Raman spectroscopy of trapped WBCs

White blood cells were also probed in the present series of experiments. Relatively little work has been reported [41,42] on WBCs which are the mobile units of the immune system; they form about 1% of the blood of a healthy adult and serve to defend the body against infectious diseases and foreign materials [43]. WBCs consist of granulocytes, monocytes and lymphocytes. The granulocytes are produced in bone marrow and are characterized by abundant granules in the cytoplasm which represent packages of enzymes involved in the killing of ingested microbes and in digestion of phagocytosed material. Lymphocytes are produced in lymph nodes and spleen, are characterized by a large nuclear to cytoplasmic ratio, and have several roles in the immune system, including the production of antibodies and other substances that fight infection and diseases [43,44].

Granulocyte and lymphocytes were trapped using typical power levels of ~5 mW. Some un-trapped and trapped cells are shown in the inset to Figs. 7 and 8. Raman spectra of trapped granulocyte and lymphocyte measured using ~10 mW power of Raman excitation are shown in Figs. 7 and 8 respectively. Raman spectra of trapped granulocyte and lymphocytes were also recorded at larger excitation power (30 mW) to test the quality of the spectra and stability of the cells and are shown in Fig. 9. The exposure of the WBCs to such power did not show any visible damage to the

Figure 6. Raman spectrum of whole blood without serum over the range 350–1700 cm\(^{-1}\). Laser power: 20 mW, Acquisition time: 120 s, average of 5 accumulations. doi:10.1371/journal.pone.0010427.g006
Table 2. Observed Raman frequencies with corresponding band assignments for WBC (Granulocytes).

| Raman Band Positions in single Granulocyte (in cm$^{-1}$) | Assignments | Raman Band Positions in single Granulocyte (in cm$^{-1}$) | Assignments |
|----------------------------------------------------------|-------------|----------------------------------------------------------|-------------|
| Pupples et al. | Parker | present Work | Pupples et al. | Parker | present Work | |
| 503 | 489 | p: S-S str | ---- | 1100–1110 | 1104 | p: C-N str |
| 519 | 514 | p: S-S str | 1126 | 1128 | 1120 | p: C-N str |
| 543 | 544 | p: S-S str | 1145 | 1144 | 1142 | DNA:RP(deoxyribose phosphate) |
| 624 | 619 | Phe:C-C twist | ---- | 1159 | 1156 | p: C-C, C-N str |
| 645 | 641 | p: C-S str, Tyr: C-C twist | 1175 | 1177 | 1179 | Tyr,Phe,p:C-H bend |
| 669 | 665 | G,T,Tyr,G bk in RNA | 1209 | 1210 | 1206 | Tyr, Phe, A, T, p: Amide III |
| 683 | 685 | G | 1232 | 1235 | 1234 | p: Amide III |
| 710 | 699 | C | 1240 | 1248 | 1249 | A, T, p:Amide III |
| 729 | 723 | A | 1255 | 1260 | 1268 | p: Amide III, C, A |
| 750 | 754 | T,Trp | 1305 | 1304 | 1301 | A, p: Amide III,Phospholipid:CH$_2$ |
| 774 | 772 | C,T | 1339 | 1340 | 1338 | A, G, p: C-H def |
| 783 | 782 | C,T,U, Lip,O-P-O diester sym str | ---- | 1358 | 1359 | Trp |
| 833 | 829 | O-P-O assym. str, Tyr | 1375 | 1378 | 1372 | T,A,G |
| 853 | 850 | Tyr | 1400 | 1400 | 1389 | U, A, IgG: COO$^-$ sym str |
| 868 | 870 | Ribose | 1422 | 1423 | 1418 | G,A |
| 880 | 877 | Trp | 1449 | 1449 | 1445 | p:C-H$_2$ def |
| 898 | 900 | DNA: bk, p: C-C skeletal | ---- | 1458 | 1453 | p:(C-H$_3$ def,C-H$_2$ def),Rib:CH def |
| 936 | 940 | 938 | p: bk C-C str | 1487 | 1491 | 1488 | G,A |
| 963 | 962 | p: skeletal vibration | ---- | 1552 | 1547 | Trp, p: Amide II |
| 975 | 974 | deoxyribose | 1578 | 1580 | 1575 | G,A |
| 1004 | 1004 | 998 | Phe: C-C skeletal | 1606 | 1607 | 1604 | Phe, Tyr, p: C=C |
| 1032 | 1026 | 1026 | Phe, p:C=$N$ str | 1617 | 1614 | 1618 | Tyr, Trp, p: C=C |
| 1048 | 1040 | 1040 | Ribose Phosphate: CO str, p: C-N str | 1659 | 1657 | 1660 | p: Amide I |
| 1064 | 1059 | 1059 | Phospholipids: C-C str | 1670 | 1672 | 1668 | T(C = O),p:Amide I |
| 1094 | 1083 | 1083 | DNA:bk,O-P-O sym.str, p:C=N str | |

Abbreviations: -def: deformation, bk: vibration of DNA backbone, U,C,T,A,G:ring breathing modes of the DNA/RNA bases, Tyr: Tyrosine, Trp: Tryptophan, Rib: ribose, NA: nucleic acids.
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Figure 7. Raman spectrum of a trapped granulocyte over the wavelength range 400–1750 cm$^{-1}$. Laser power: ~10 mW, acquisition time: 120 s, average of 5 accumulations. The insets show the image of a granulocyte before and after trapping.
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cells. The WBCs used here were collected from freshly drawn blood (within 10 minutes).

The vibrational spectra of lymphocyte and granulocytes are mainly dominated by the vibration due to proteins, lipids and nucleic acid bases, as expected from the Raman spectra of eukaryotic cells. The assignments of the Raman spectra of trapped granulocytes and lymphocytes are summarized in Tables 2 and 3, respectively. In comparing our data with the earlier work [41] on the granulocyte nucleus, we note that all previously reported Raman peaks are reproduced in our experiments. Additionally, we have discovered several new peaks that we assign on the basis of information available on standard proteins and nucleic acids [40]. Raman assignment of lymphocyte spectra was also made using available literature [40]. The Raman spectrum we obtained from trapped granulocyte consists of sharp peaks from proteins compared to the somewhat broader features in the spectra of trapped lymphocytes. This is likely a consequence of the dense granulated cytoplasm in the former. The peaks at 1445 cm$^{-1}$,
Table 3. Observed Raman frequencies with corresponding band assignments for WBC (Lymphocytes).

| Raman Band Positions in single Lymphocyte (in cm\(^{-1}\)) | Assignments | Raman Band Positions in single Lymphocyte (in cm\(^{-1}\)) | Assignments |
|----------------------------------------------------------|-------------|----------------------------------------------------------|-------------|
| Parker present Work                                      |             | Parker present Work                                      |             |
| 503                                                      | p: S-S str  | 1064                                                    | Phospholipid: C-C str |
| 540                                                      | p: S-S str  | 1094                                                    | DNA:bkO-P-O sym str, p:C-N str |
| 624                                                      | Phe: C-C twist | 1100–1110                                              | p: C-N str  |
| 635                                                      | A,U,G,C     | 1128                                                    | p: C-N str  |
| 670                                                      | G,T,Tyr,G bk in RNA | 1159                                                   | p: C-C, C-N str |
| 710                                                      | C           | 1177                                                    | Tyr, Phe, p:C-H bend |
| 720                                                      | Phospholipid: Choline | 1208                                                   | Tyr, Phe, A, T, p: Amide III |
| 752                                                      | T,Trp       | 1265                                                    | p: Amide II CH bend |
| 767                                                      | C,T,U,Lip-O-P-O diester sym str | 1304                                      | A, p: Amide III |
| 835                                                      | DNA:RP(deoxyribose phosphate) | 1320                                             | G,p:C-H2 twist |
| 850                                                      | Tyr         | 1340                                                    | A, G, p: C-H def |
| 867                                                      | Ribose      | 1400                                                    | U, A, IgG, COO- sym str |
| 880                                                      | Trp         | 1423                                                    | A,G          |
| 900                                                      | DNA: bk, p: C-C skeletal | 1448                                      | Deoxyribose, p:C-H2 def |
| 938                                                      | p: bk C-C str | 1462                                                    | Deoxyribose, p:C-H3 def, C-H2 def |
| 963                                                      | p: skeletal vibration | 1514                                    | A             |
| 975                                                      | Deoxyribose | 1580                                                    | G,A          |
| 1004                                                     | Phe: C-C skeletal | 1607                                               | Phe, Tyr, p: C = C |
| 1032                                                     | Phe, p: C-N str | 1650–1670                                         | p: Amide I   |
| 1049                                                     | Ribose Phosphate: CO str, p: C-N str | 1672                                      | T(C = O), p: Amide I |
| 1055                                                     | Na: C-O str, p:C-N str | 1674                                      |              |

Figure 10. Raman spectra of trapped RBCs at a laser power of ~5 mW. Acquisition time: 60 s, average of 5 accumulations. Spectra “a”–“d” are from different individual RBCs trapped at different times. Reproducibility of spectral features is determined to be better than ±1 cm\(^{-1}\) along the wavelength axis.

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1128 cm\(^{-1}\), 998 cm\(^{-1}\), 853 cm\(^{-1}\), 755 cm\(^{-1}\) and 513 cm\(^{-1}\) corresponding to proteins and amino acids are more intense in the granulocyte spectrum than the lymphocyte spectrum. On the other hand, the features at 1486 cm\(^{-1}\), 1007 cm\(^{-1}\), 783 cm\(^{-1}\), 725 cm\(^{-1}\) and 683 cm\(^{-1}\) corresponding to the vibrations of nucleic acid bases and DNA backbone stretching are more prominent in the lymphocyte spectrum. This may be because of the increased nucleus-to-cytoplasm ratio in lymphocytes. However, the Raman line at 975 cm\(^{-1}\) assigned to deoxyribose is very intense in granulocyte spectra (in comparison with lymphocyte
spectra). The reason for these remains unclear at present. The above results seem to be more prominent in the Raman spectra recorded with 30 mW power (Fig. 9). Raman spectra of a few (different) RBCs are shown in Fig. 10. A comparison between these spectra shows a high level of reproducibility. Typically, reproducibility of spectral features depicted in Fig. 9 over the wavelength axis was better than $\pm 1$ cm$^{-1}$, lending credence to the robustness of our methodology and the reliability of our spectroscopic information in Table 1, Table 2 and Table 3.

**Summary**

A dual beam Raman Tweezers apparatus with superior performance has been assembled that involves a marriage of optical trapping methods with Raman spectroscopy. The utility of such a device for biomedical studies is illustrated by measuring Raman spectra of single red and white blood cells kept under physiological conditions. Raman spectra were recorded using sufficiently low power levels to ensure no photo-damage. Several hitherto-unreported Raman transitions were obtained in the present study and have been assigned. We believe that the work reported here reinforces the utility of and opens new vistas for Raman Tweezers to studies that aim to obtain molecular information from single living cells. The method can have wide applications in understanding the basic biochemistry and biophysics of RBC related diseases (Malaria, Sickle Cell Anemia, Thalassemia), cell-drug interactions, cell under stress, radiation induced cell damage, cell undergoing malignant conditions and many more. Work on cells under stress and malaria-infected RBCs is currently underway in our laboratory.

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**Author Contributions**

Conceived and designed the experiments: SC DM. Performed the experiments: AB EZ. Analyzed the data: AB EZ SC. Contributed reagents/materials/analysis tools: MV. Wrote the paper: SC DM.

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