

Label-free optical sensor based on red blood cells laser tweezers Raman spectroscopy analysis for ABO blood typing

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Abstract: The clinical significance of ABO blood typing extends beyond transfusion medicine and is demonstrated to be associated with susceptibility to various diseases, even including cancer. In this study, a home-made laser tweezers Raman spectroscopy (LTRS) system was applied to detect red blood cells (RBCs) with the aim to develop a label-free, simple and objective blood typing method for the first time. High-quality Raman spectra of RBCs in the fingerprint region of 420-1700 cm^{-1} can be obtained, meanwhile exciting blood typing results can be achieved, especially with an accuracy of 100% for identifying Type AB from other blood types with the use of multivariate statistical analysis based on principal component analysis (PCA) combined with linear discriminant analysis (LDA). This primary work demonstrates that the label-free RBCs LTRS analysis in conjunction with PCA-LDA diagnostic algorithms has great potential as a biosensor for ABO blood typing.

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1. Introduction

The ABO blood group system, firstly discovered by a Viennese pathologist (Karl Landsteiner) in 1901, consists of 4 different categories including A, B, AB, and O types. It is well known that the four phenotypes result from the variable combinations of three main alleles (two co-dominant A and B and one recessive O), which is controlled by a single gene on chromosome 9 (9q34.2). Consequently, different ABO antigens are expressed on the extracellular surface of red blood cells (RBCs), achieving characteristic RBCs in different blood types. For

example, RBCs in Type A and Type B possess an A antigen and a B antigen on their cellular surfaces, respectively, whereas RBCs in Type AB have both A and B on the surface of them [1]. In clinical practice, it is of extremely importance to identify ABO blood types due to the fact that the fatal intravascular transfusion reaction may be caused by erroneous transfusions of ABO-incompatible RBCs [2]. Recently, growing evidences have demonstrated the potential association between ABO blood types and infectious diseases (e.g. plasmodium falciparum malaria, vibrio cholerae infections and echerichia coli infection), cardiovascular disease (e.g. venous thromboembolism, myocardial infarction, ischemic stroke, and peripheral arterial disease), and other diseases (e.g. parkinson's disease and incident cognitive impairment). Especially, other epidemiological studies have discovered that ABO blood types are also associated with the risk of developing certain cancer. For instance, people with blood Type A were found to have an increased risk of gastric cancer, and O blood type has a protective effect against pancreatic cancer [1]. Current standard blood typing methods such as the absorption–elution assay, absorption–inhibition assay and mixed cell agglutination reaction (MCAR), is commonly carried out through murine monoclonal reagents to determinate the presence or absence of ABO antigens on the RBCs. However, those methods suffer the disadvantage of requiring precious exogenous reagents, and subjective determination of the hemagglutination phenomenon [3, 4]. Therefore, development of a label-free, objective, sensitive and convenient detection technique would be of imperative clinical value to identify different blood types via RBCs analysis.

Raman spectroscopy (RS) based on inelastic scattering is a noninvasive and sensitive optical analytical technology, which is capable of providing biochemical “fingerprint” information of biological sample, since the shifted energy of the incident light corresponds to characteristic vibration of different molecular bonds. Recently, RS technology has been widely used to detect biological samples, ranging from macromolecules, cells, body fluid to tissues samples, for biomedical applications. Notably, RS has attracted considerable interest as a powerful tool in the study of living cells, since water exhibits no Raman spectral signals in the “fingerprint” region, making RS extreme suitable for cells detection within liquid environment [5, 6]. Nevertheless, there are also some limitations associated with conventional living cell RS detection. For instance, it is impractical to precisely monitor dynamic characteristics of single living cell over a long period by RS due to the Brownian motion of the cells in an aqueous solution. Immobilization of cells on a substrate by physical or chemical approaches would be an alternative way to solve the limitation, however this procedure may perturb cellular biology, and make it challenging to acquire cell Raman signals above the background signal generated from the surface [7, 8].

The above drawbacks of conventional RS technique can be overcome by the advent of laser tweezers Raman spectroscopy (LTRS) technology, which combines laser trapping and micro Raman spectroscopy to achieve trapping, manipulation, and biomolecular fingerprinting of single cell. The use of LTRS enables an individual cell to be optically immobilized in a laser trap away from any substrates generated by a focused laser beam, and the Raman signals are acquired using the same laser, simultaneously, providing a unique opportunity to achieving label-free, rapid, nondestructive and objective analysis of living cells [8]. For example, Ahlawat et. al reported on the use of LTRS for label free analysis of cell cycle (human colon adenocarcinoma cells synchronized in G0/G1 and G2/M phases) using the DNA Raman band at 783 cm^{-1} as a indicator of the DNA content in the nucleus [9]. Also, the LTRS was successfully used for single cancer cell detection, and the cancer groups could be accurately discriminated from the normal groups based on characteristic Raman signals generated from DNA/RNA and proteins [10, 11]. Beyond that, many studies have demonstrated the applications of LTRS in RBCs detection. Raj et. al presented the Raman spectra of RBC at different stretched states using LTRS, and found that mechanical deformation of the cell mainly affect the hemoglobin [12]. Additionally, a spectral comparison of normal and thalassemic RBCs via LTRS revealed that thalassemic RBCs had a

reduced oxygenation capability of hemoglobin, and the two types of cells also presented different responses to photo-induced oxidative stress [13]. Other investigations on deoxygenation of RBCs induced by optical trap, and the characteristics of *Plasmodium vivax* infected RBCs using LTRS were carried out using LTRS by Gupta's group [14, 15]. However, to the best of our knowledge, the potential of LTRS technique for identification and analysis of RBCs from different ABO blood types has not been assessed for blood type determination.

The aim of this study thus was the first to assess the feasibility of LTRS technique for biomolecular analysis of RBCs samples belonging to Type A, B, AB and O for blood type determination. Multivariate statistical algorithms, including principal component analysis integrated with linear discriminant analysis (PCA-LDA), were used to analyze and differentiate the RBCs spectra obtained from the four groups. This primary study may develop a label-free, rapid and objective blood typing method.

2. Materials and methods

2.1 Preparation of RBCs samples

A total of 120 human blood samples were collected for this study, containing Type A ($n = 30$), Type B ($n = 30$), Type AB ($n = 30$) and Type O ($n = 30$) according to ABO blood system, by clinical confirmation. Each sample was diluted with phosphate-buffered saline, and then underwent centrifugation and wash procedures to remove impurities. Finally, a quartz-bottom culture dish containing 2 mL aliquots of the diluted RBC suspension liquid was placed on the LTRS microscope stage for single-cell analysis. In addition, all these samples were provided by The Second Hospital of Fuzhou, and this study was approved by the ethical committee at that institution.

2.2 Laser tweezers Raman spectroscopy (LTRS) system

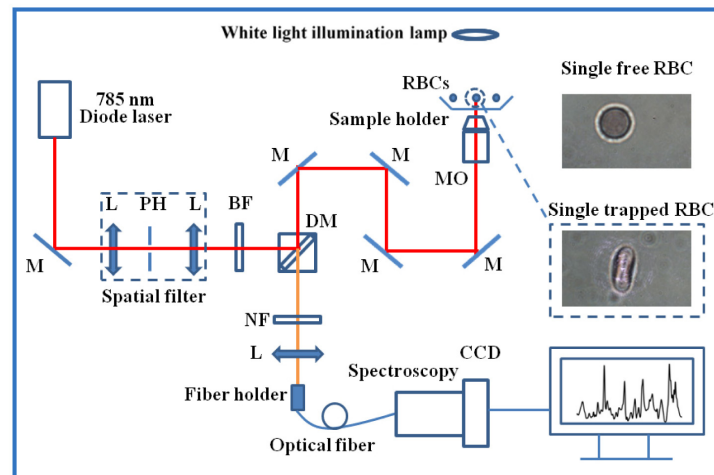


Fig. 1. Schematic of the home-made laser tweezers Raman spectroscopy (LTRS) system. A 785 nm diode laser beam was delivered to an inverted microscope for both trapping RBCs and generating the Raman signals from cells. Backwards Raman scattering light was recorded by a back-illuminated, deep-depletion near-infrared intensified CCD. In this schematic, M: mirror; L: lens; PH: pinhole; BF: band pass filter; DM: dichroic mirror; MO: microscope objective; NF: notch filter.

Figure 1 illustrates the schematic of the home-made laser tweezers Raman spectroscopy (LTRS) system used in this study. Briefly, a 785 nm diode laser beam was firstly collimated with a spatial filter to achieve a beam diameter of approximately 6 mm. Then, the laser beam was spectrally filtered using a band pass filter and finally delivered into an inverted

microscope (IX71; Olympus, Center Valley, PA, USA) equipped with a $100\times$, 1.3 N.A. oil immersion objective through a dichroic mirror. The output beam was focused to generate a single beam optical trap, through a sample holder with a quartz bottom of thickness 80 μm , which placed on the 3D scan stage. A RBC in the culture could be selected and trapped within the laser focus at 20 μm height above the quartz glass. In the meantime, the same laser beam was employed to excite Raman scattering for the trapped cell with the focus size of 1 μm . Back scattered Raman signal from the cell was collected by the same objective and then it was directed to reach a transmissive holographic (Holospec-f/2.2-NIR) coupled to a back-illuminated, deep-depletion near-infrared (NIR) intensified CCD detector (Princeton Instruments), which was cooled to -120°C in order to reduce the dark current, through the dichroic mirror, a notch filter, lens and a single fiber. The single fiber with 50 microns core diameter could collect and transmit Raman signal to the spectrometer. At the same time, the end face of the fiber was the equal of 50 microns pinhole, which enabled the optical path of the LTRS system to have confocal configuration and eliminate the stray light with a great extent. The LTRS system acquired Raman spectra in the wavelength region of 420–1700 cm^{-1} within 40 s integration time and 2 mW laser power. The software package WinSpec32 (Princeton Instruments, Trenton, NJ, USA) was employed for spectral acquisition and analysis. A total of 81 RBCs, 99 RBCs, 87 RBCs and 88 RBCs, belonging to Type A, Type B, Type AB and Type O, respectively, were detected by LTRS. Three measurements were carried out for each RBC, and an average spectrum of them was used for representing the Raman characteristic of each RBC. In addition, the RBCs were illuminated by a white light illumination lamp above the sample holder and the images of the trapped cell (inserted pictures in Fig. 1) were constantly monitored through a video camera system.

2.3 Data processing and multivariate statistical analysis

The raw Raman spectrum of single living RBC represented a combination of cell Raman, autofluorescence and noise signals. To extract the pure Raman signals, a Vancouver Raman Algorithm based on fifth-order polynomial fitting method developed by Huang et al. [16] was performed for the raw spectral preprocessing. Then, the background-subtracted Raman spectrum was normalized to the area under curve in the spectral range 420–1700 cm^{-1} of each spectrum to reduce the influence of spectral intensity variability generated by possible laser power fluctuations, enabling a better comparison of spectral characteristic between different RBCs samples.

Multivariate statistical analysis based on principal component analysis (PCA) combined with linear discriminant analysis (LDA) was used for analyze and differentiate the Raman spectra obtained from RBCs belonging to different blood types. In brief, PCA, an unsupervised manner, was employed to reduce the high dimension of the spectral space (each Raman spectrum of single RBC with a set of 635 intensity variables) to a few principal components (PCs), which were linear combination of the original variables and retained the most diagnostically significant information for cell differentiation. Then, the obtained PCs were analyzed by an independent sample T-test to determinate diagnostically significant PC scores ($p < 0.05$). The collected PCs were lastly retained and fed into an LDA model for correctly predicting the RBCs samples from the four blood types. Here, LDA was carried out in a supervised manner, and integrated with the leave-one-out and cross-validation methods on all spectral data. Finally, receiver operating characteristic (ROC) curves were used for illustrating the performance of LTRS integrated with PCA-LDA method for ABO blood typing.

3. Results and discussion

3.1 Characteristics of RBCs Raman spectra

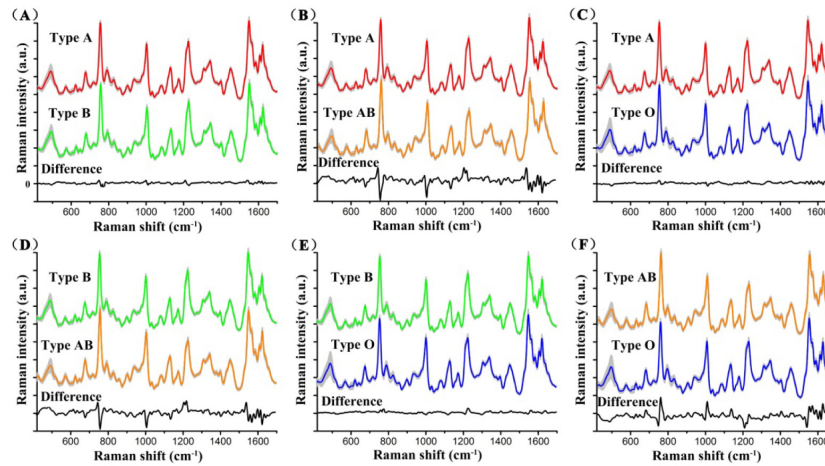


Fig. 2. Pair-comparison of normalized mean Raman spectra from Type A, Type B, Type AB and Type O RBCs samples. The shaded areas represent the standard deviations of the means. Also shown at the bottom is the difference spectrum.

High-quality Raman spectra of human RBCs in the “fingerprint” region ranging from 420 to 1700 cm^{-1} can be obtained using the home-made LTRS system. It is well known RBC has a simple architecture containing a lipid bilayer with many transmembrane proteins and the underlying cytoskeleton without nucleus and intra-membrane organelles, and it consists mainly of hemoglobin (Hb), a globular protein with an embedded porphyrin (heme group) [12, 17]. Therefore, the obtained Raman spectrum of RBC was primarily dominated by porphyrin macrocycle and various proteins.

Figure 2 illustrated the pair-comparison of normalized mean Raman spectra among RBCs samples from different ABO blood types, including Type A, Type B, Type AB and Type O. The standard deviations (SD), overlying as shaded color fill in Fig. 2, for each group demonstrated the good spectral reproducibility within each group, which guaranteed a better comparison of the spectral characteristics among different groups for both peaks assignment analysis and multivariate statistical analysis. Prominent Raman peaks located at around 490, 567, 621, 676, 753, 791, 827, 857, 898, 937, 1003, 1031, 1082, 1128, 1173, 1224, 1308, 1337, 1397, 1447, 1547, 1583, 1606 and 1620 cm^{-1} could be clearly consistently observed in RBC samples from different blood types, with the strongest signals at 490, 676, 753, 1003, 1128, 1224, 1397, 1447, 1547 and 1620 cm^{-1} . Table 1 listed tentative assignments for the observed Raman bands, according to the previous reports [12, 17–22]. Although there was a close consistency between the spectral patterns of these four groups, small spectral relative intensities changes were detectable for some Raman peaks, especially between Type A and Type AB, Type B and Type AB, and Type O and Type AB. The differences could be viewed more clearly in the difference spectrum at the bottom of each figure in Fig. 2. For example, the relative peak intensities at 753 cm^{-1} , originating from the C–N–C breathing stretch vibrations in the porphyrin ring, exhibited a higher signal in Type AB than in other three blood groups, indicating an increase in the percentage of this kind of porphyrin contents relative to the total Raman active components in RBC. The distinct spectral change at 753 cm^{-1} was demonstrated to be associated with the deformations of RBC, which was observed by Raj et al [17]. Besides, The 753 cm^{-1} Raman band was previously used as a direct measure of the heme groups of the hemoglobins to study the response of RBCs to exogenous substance, such as alcohol [23]. Similarly, the relative peak intensities at 1003 cm^{-1} , tentatively assigned to the symmetric ring breathing of phenylalanine, appeared to be higher

in Type AB than that in other groups. Such a considerable difference in intensity could be attributed to an increase in the relative amount of phenylalanines in symmetric ring breathing conformation within the RBCs of Type AB. Phenylalanine, an essential amino acid, is present not only in hemoglobin but also in various membrane proteins, such as ankyrin, band proteins and spectrin [17]. It is regarded as an important biomarker for indicating the variations of proteins in various biological samples. For example, with the use of LTRS, Xie et al. observed a significant increase in the intensity of the 1003 cm^{-1} band (phenylalanine) in yeast cells as a result of increasing temperatures, which could be attributed to the fact that relevant proteins unfolded during this heat-denaturation process [24]. Besides, the phenylalanine exhibited a prominent and stable Raman signal at 1003 cm^{-1} in human blood and tissue samples, acting as a promising biomarker for classification between normal and cancer groups [25, 26]. In contrary to the peaks 753 and 1003 cm^{-1} , a decrease in Raman signals at 1224 cm^{-1} was found in Type AB samples as compared to other groups, indicating a decrease in the percentage of ν_{13} or ν_{42} relative to the total Raman-active constituents in RBC. Similar study on RBC using LTRS showed that this peak was associated with a transitioned from an oxygenated to a more deoxygenated state [27]. In addition, there were also obvious differences between Type AB and other Types in Raman shapes in the spectral ranges of $1500\text{--}1700\text{ cm}^{-1}$, containing the information of some vibration bonds, such as ν_{11} , ν_{37} , Phe/Tyr $\nu(\text{C}=\text{C})$ and $\nu(\text{C}=\text{C})_{\text{vinyl}}$, shown in Fig. 2. These results indicated that there were changes in the percentage of biomolecules in different blood groups, suggesting a potential role of RBCs LTRS technology for ABO blood typing. However, it can be seen that the spectral differences among Type A, Type B and Type AB are relative slight, making it great challenging to comprehensively achieve ABO blood typing by LTRS. Additionally, it should be noted that above simplistic peak intensities analysis only used limited Raman peak information, and some peaks dominated by important biomarkers might overlap, thus possibly resulting in the lost of diagnostic information only with visual observation method.

Table 1. The Raman peak positions and tentative assignment of major vibrational bands observed in RBCs.

Peak positions (cm^{-1})	Vibrational mode and major assignment ^a
490	p: S-S str
567	$\nu(\text{Fe}-\text{O}_2)$
621	Phe: C-C twist
676	ν_7
753	$\nu(\text{C}-\text{N}-\text{C})$
791	ν_6
827	γ_{10}
857	γ_{10}
898	Phe: C-C skeletal
937	ν_{46}
1003	Phe: Sym ring breath
1031	Phe: $\nu(\text{C}-\text{H})$ or $\delta(\text{C}_6\text{H}_2)_{\text{asym}}$
1082	$\delta(\text{C}_6\text{H}_2)_4$
1128	ν_5
1173	ν_{30}
1224	ν_{13} or ν_{42}
1308	ν_{21}
1337	ν_{40}
1397	ν_{20}
1447	p: C-H ₂ def
1547	ν_{11}
1583	ν_{37}
1606	Phe/Tyr $\nu(\text{C}=\text{C})$
1620	$\nu(\text{C}=\text{C})$

^aAbbreviations: ν & δ : In-plane modes, γ : Out-of-plane modes, Sym: symmetric, asym: asymmetric, str: stretching, def: deformation, p: protein, Phe: Phenylalanine, Tyr: Tyrosine.

3.2 Classification of different blood types based on multivariate statistical analysis

A multivariate diagnostic algorithm based on PCA-LDA was thus utilized to improve the efficiency for ABO blood typing by incorporating the entire spectrum and automatically determining the most diagnostically significant characteristics. As a powerful spectral analytical method, this algorithm has been widely investigated for enhancing the efficiency of RS technology for the biomedical diagnostic applications [28–32]. In present work, PCA was first to reduce 635 intensity variables within raw Raman spectrum of single RBC to a few PCs, followed by T-test. Results showed that three PCs (PC2, PC3 and PC5) of the variances were diagnostically significant ($p < 0.05$) for discriminating Type A samples from Type B samples. Similarly, PC 1, 3 and 4, PC 3, 4 and 5, PC 1, 3 and 4, PC 2, 4 and 5, and PC 1, 2, 3 were determined as the most diagnostically significant for Type A vs. Type AB, Type A vs. Type O, Type B vs. Type AB, Type B vs. Type O and Type AB vs. Type O discrimination, respectively. Then the selected PCs were loaded into LDA model for discriminant classification with leave-one-out and cross-validation methods.

Figure 3 displayed the posterior probabilities belonging to the Type A, Type B, Type AB and Type O groups as calculated in the LDA model. Using discrimination thresholds of 0.484, 0.5, 0.395, 0.5, 0.442 and 0.061, the diagnostic sensitivities for identifying Type A vs. Type B, Type A vs. Type AB, Type A vs. Type O, Type B vs. Type AB, Type B vs. Type O and Type AB vs. Type O were 84%, 100%, 92.6%, 100%, 100% and 100%, respectively. The corresponding diagnostic specificities for each combination were 83.8%, 100%, 87.5%, 100%, 97.7% and 100%, respectively. These classification results were summarized in Table 2.

Table 2. Classification results of the four blood types using RBCs LTRS combined with PCA-LDA algorithm.

Classification combinations			Predicted parameters		
			Sensitivity	Specificity	Accuracy
Type A	vs.	Type B	84% (68/81)	83.8% (83/99)	83.9% (151/180)
Type A	vs.	Type AB	100% (81/81)	100% (87/87)	100% (168/168)
Type A	vs.	Type O	92.6% (75/81)	87.5% (77/88)	89.9% (152/169)
Type B	vs.	Type AB	100% (99/99)	100% (87/87)	100% (186/186)
Type B	vs.	Type O	100% (99/99)	97.7% (86/88)	98.9% (185/187)
Type AB	vs.	Type O	100% (87/87)	100% (88/88)	100% (175/175)

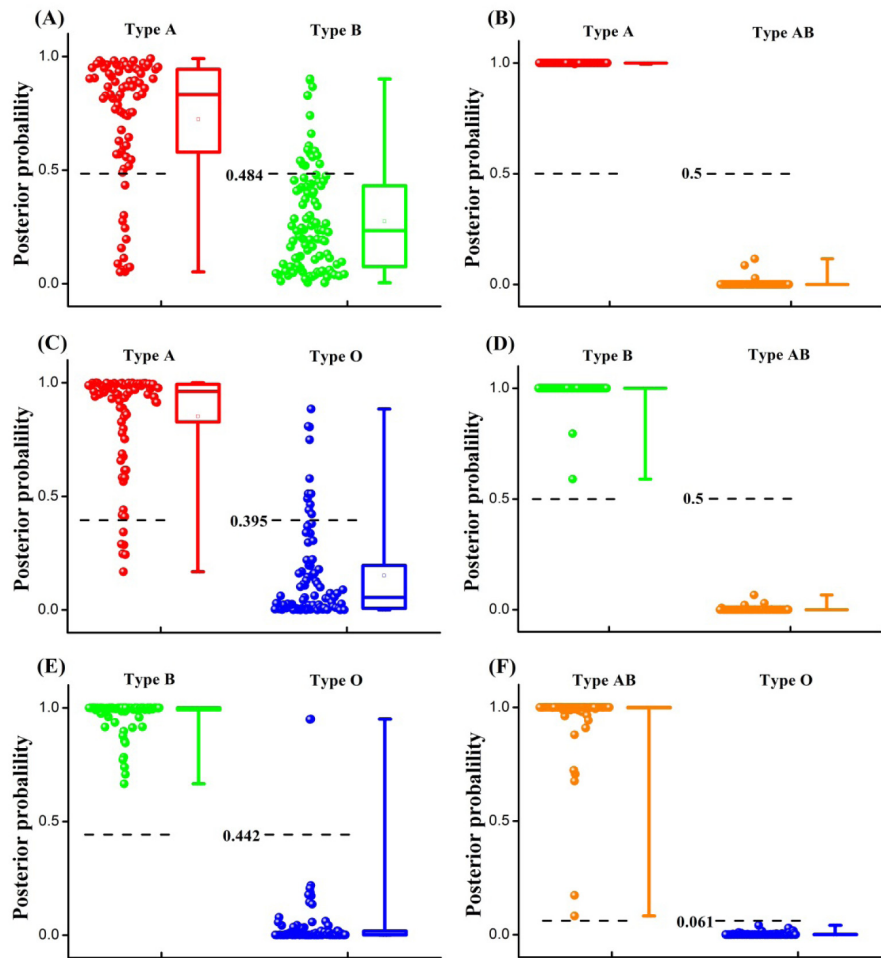


Fig. 3. Scatter plots of the posterior probabilities belonging to the ABO blood groups using the PCA-LDA together with leave-one-out and cross-validation method. Box represents the range of 25%-75%; Whisker represents the range of minimum to maximum. The diagnostic thresholds (dotted lines) are 0.484, 0.5, 0.395, 0.5, 0.442 and 0.061 for each combination.

To further evaluate and the performances of the RBCs LTRS together with PCA-LDA for ABO blood typing, ROC curves were generated in Fig. 4 at different threshold levels. The integration area under the ROC curve (AUC) is a quantitative indicator to represent classifier performance. The larger AUC value suggests the greater forecast accuracy. The AUCs for each combination classification were 0.875, 1, 0.973, 1, 0.997 and 1. These results revealed that different blood types could be identified with ultra-high accuracy by RBCs LTRS method. Interestingly, the tiny difference existed between the spectra belonging to Type B and Type O [Fig. 2(E)], whereas the two groups could be classified with an accuracy of 98.9% by PCA-LDA. The analogous situations appeared in other two combinations (Type A vs. Type B; Type A vs. Type O). It can be concluded that some peaks dominated by vital biomarkers for blood typing were overlapped or masked by other peaks without diagnostic value, however, the diagnostic information contributed by those biomarkers could be fully extracted and used by PCA-LDA for ideally blood typing. Also, spectral comparison and analysis are necessary, because it has ability to provide the detailed information of changes in biomolecular construct and content, which is associated with different biological processes. Additionally, distinct spectral differences between Type AB and other types in Fig. 2 were

correlated with corresponding high classification efficiencies in Fig. 3. Thus, a combination of spectral analysis and PCA-LDA is optimal option for ABO blood typing.

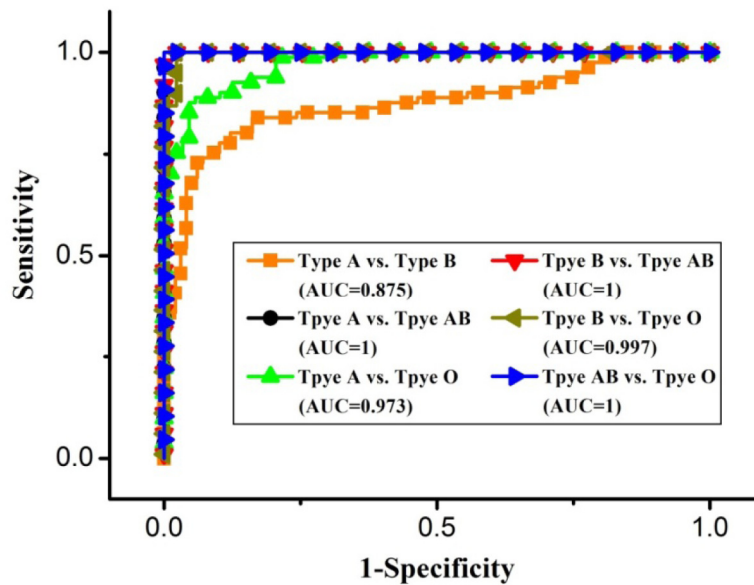


Fig. 4. Receiver operating characteristic (ROC) curves of classification results for ABO blood typing. AUC: the integration areas under the ROC curves.

4. Conclusion

A laser tweezers Raman spectroscopy (LTRS) system was built to detect red blood cells (RBCs) from different ABO blood samples for ABO blood typing, for the first time. High-quality and distinct spectral features of RBCs mainly dominated by proteins (phenylalanine at 1003 cm^{-1} and porphyrin at $753, 1224\text{ cm}^{-1}$) existed among different blood groups, and great classification results could be achieved by PCA-LDA diagnostic algorithm, especially for the identification of Type AB from other blood group, with an accuracy of 100%. The results demonstrated great potential of LTRS combined with PCA-LDA technique to be a label-free and objective optical sensor for blood typing. Next step, we will collect more RBCs samples to verify the reliability of this potential ABO blood typing method, and more technologies, such as surface enhanced Raman spectroscopy, multi-trapping and microfluidics, will be integrated with this LTRS system to further improve its efficiency in ABO blood typing.

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