Effect of pre-analytical variables on Raman and FTIR spectral content of lymphocytes.

Electronic supplementary information (ESI)

Materials and methods

1. Sample preparation

1.1. Blood collection system comparison

Two blood collection systems; Vacuette® blood collection system (Greiner Bio-One, Stonehouse, UK) and S-Monovette® (*SARSTEDT, Germany*) collection system was used to collect peripheral venous blood from volunteers (n=3) to assess the influence on the spectral integrity of lymphocytes. Following volunteer blood collection in Vacuette® lithium heparin (Greiner Bio-One) and S-Monovette® lithium heparin (*SARSTEDT*) collection tubes, the samples were then subjected to the cell culture protocols described in Sections 2.3. and 2.4.

1.2. Needle gauge comparison

Needles with two different gauges were used to assess lymphocyte spectral content obtained from peripheral blood. Blood was collected from volunteers (n=3) using a 21 G needle (Greiner Bio-One) or 23 G needle (Greiner Bio-One) and the samples were subjected to the cell culture protocols described in Sections 2.3. and 2.4.

1.3. Anticoagulant

To investigate the possible impact of anticoagulant on the biomolecular profile of lymphocytes, different anticoagulant blood tubes from the same manufacturer were used. Volunteer peripheral blood (n= 3) was collected in Vacuette® blood collection tubes; lithium heparin (LH) sodium citrate (SC), and ethylenediaminetetraacetic acid (EDTA;Greiner Bio-One). The samples were then subjected to the protocols in Sections 2.3. and 2.4. and analysed.

2. Sample storage

2.1. Freezing samples

Following peripheral blood collection from volunteers (n=3), samples were immediately analysed or frozen for analysis to be performed later. For samples to be processed immediately, blood was subjected to the protocols

for cell culturing; CaF_2 slide preparation is described in Sections 2.3. and 2.4. For samples to be frozen and analysed later, PBMCS were obtained as described in Section 2.3. The PBMC cell pellets were resuspended in 2.7 ml of FBS (Sigma Aldrich LLC, MO, USA) and 300 µl of dimethyl sulfoxide (DMSO;Sigma Aldrich LLC) and frozen in 1 ml aliquots in cryogenic tubes (Thermo Fisher Scientific Inc, MA, US). After storage of the samples in the freezer at -80 ° C for 72 hours, the 1 ml aliquots were allowed to thaw at room temperature for 30 minutes and the corresponding samples were pooled in a 15 ml polypropylene tube. The sample was then centrifuged in a swing bucket motor at 400 g for 4 minutes at 18 ° C with the acceleration set to 9. The supernatant was aspirated off. The washing step was repeated twice more with 5 ml of DPBS (Sigma Aldrich LLC) added to the cell pellet at each wash, bringing the total number of washes to 3. Following the washing steps, the PBMCs were prepared for cell culture and deposited on CaF_2 slides (Sections 2.3. and 2.4).

3. Sample processing

3.1. Inter-operator variability

Volunteers (n=3) donated peripheral blood and two operators prepared separate samples from the same volunteer for cell culture and deposited them on CaF_2 slides (Crystran Ltd, Dorset, UK) following Sections 2.3. and 2.4 to assess variability between operators.

3.2. Sample Storage Time: Time to lymphocyte isolation 1 and 24 hour post-sample collection

Blood samples (n=3) were collected to evaluate the processing times on the spectral content of lymphocytes. Isolation of lymphocytes from peripheral blood was carried out at 1 hour and 24 hours after sample collection following the protocols outlined in Sections 2.3. and 2.4.

4. Pre-processing of Lymphocyte Spectra

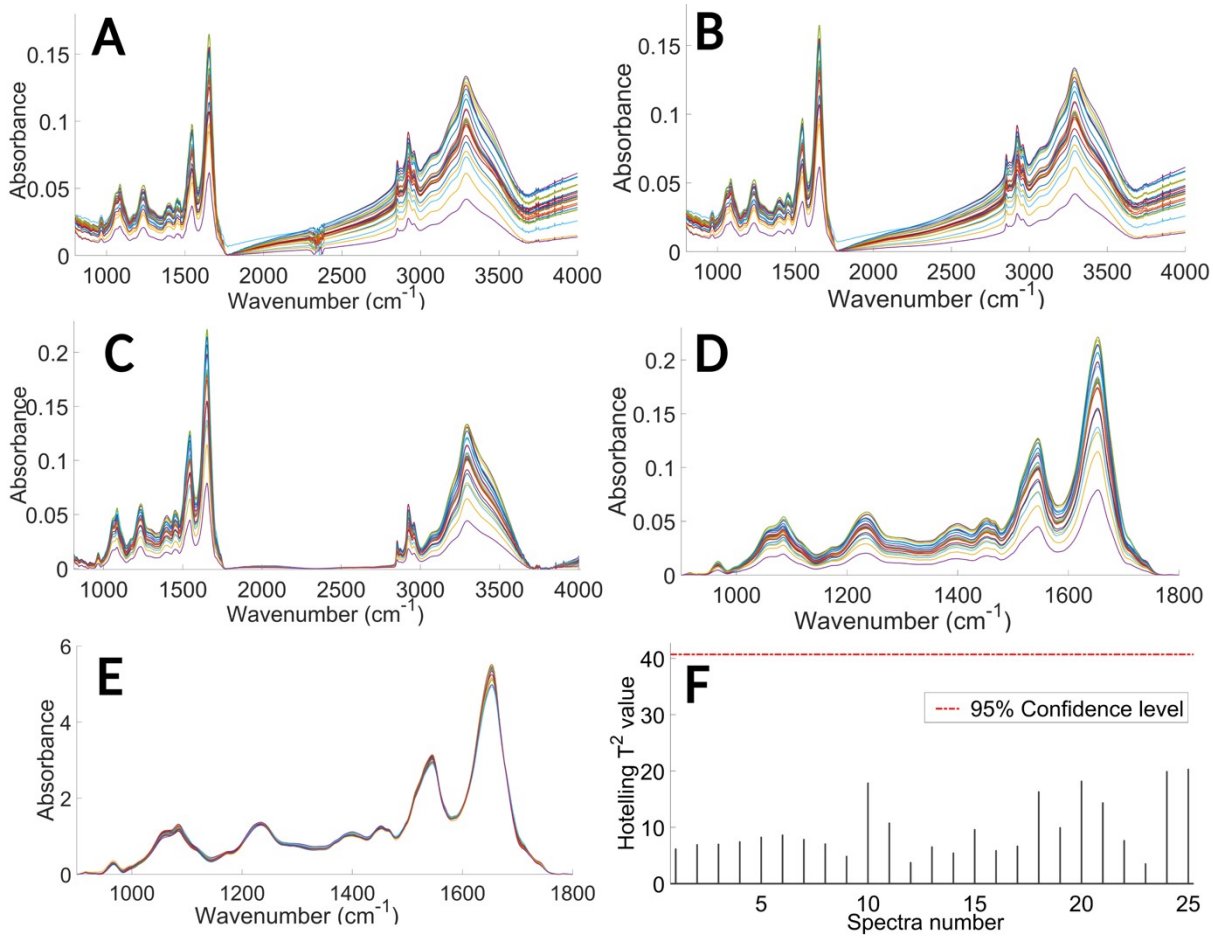

Fig. ESI1: Pre-processing steps carried out on raw lymphocyte FTIR spectra. Lymphocytes from one volunteer within the frozen dataset are shown here for demonstrative purposes.

(A) Raw lymphocyte FTIR spectra. (B) Atmospheric correction of FTIR spectra. (C) Mie scattering correction of FTIR spectra. (D) Rubberband baseline correction of FTIR spectra. (E) Vector normalisation and (F) Hotelling's T^2 test for outlier removal. A 95% confidence level was selected for outlier removal and any spectra that fell outside this range are considered outliers. In this case, no outliers were identified.

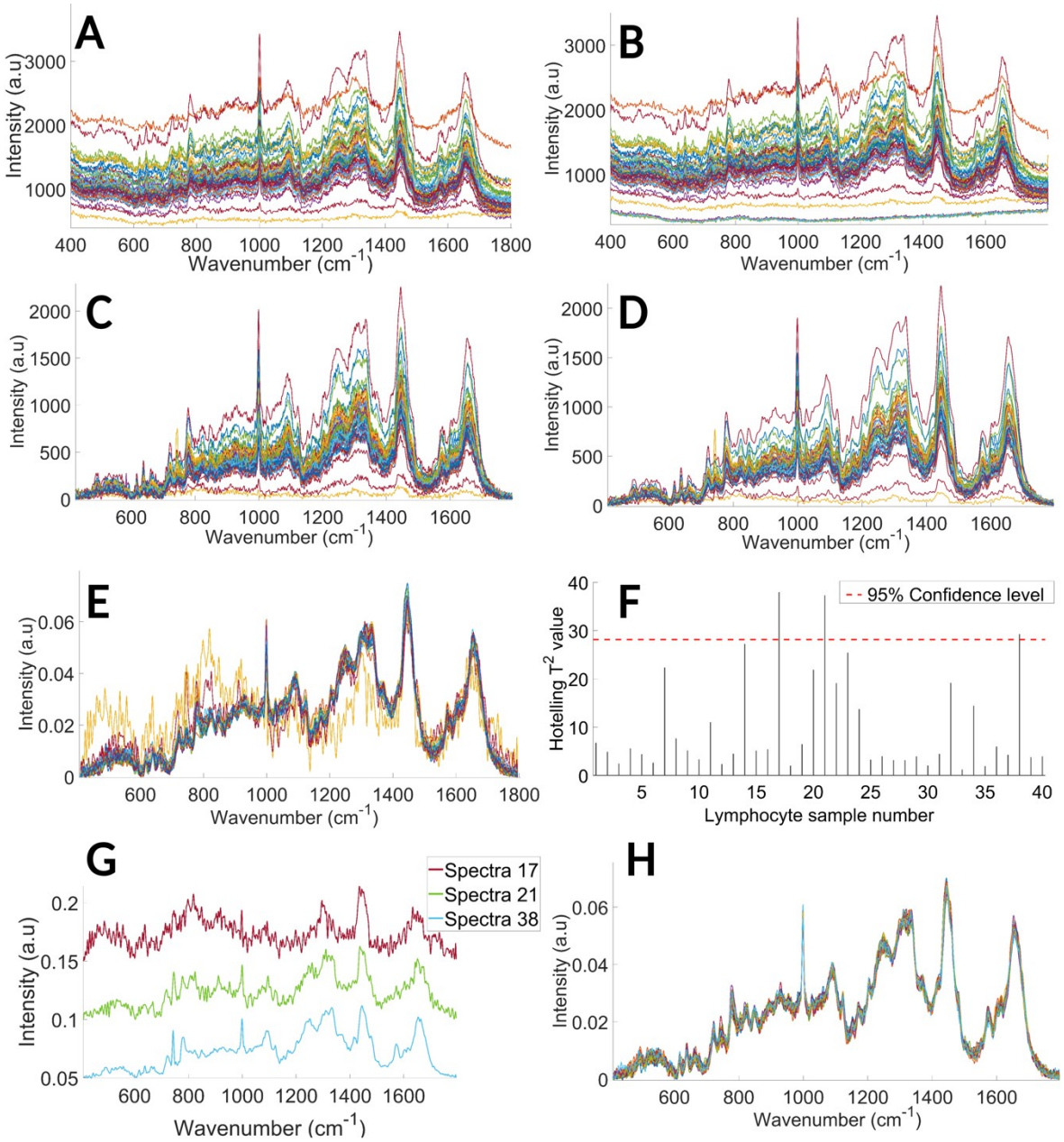

Fig. ESI2: Pre-processing steps carried out on raw lymphocyte Raman spectra. Lymphocytes from one volunteer within the frozen dataset are shown here for demonstrative purposes.

(A) Raw lymphocyte Raman spectra. (B) Wavenumber corrected Raman spectra using 1-4 Bis. (C) Rubberband baseline correction of Raman spectra. (D) Smoothed Raman spectra using a Savitzky-Golay filter. (E) Vector normalisation. (F) Hotelling's T^2 test for outlier removal. A 95% confidence level was selected for outlier removal and any spectra that fell outside this range are considered outliers. (G) Individual outlier spectra. (H) Outlying spectra removed from data set.

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5. Results and Discussion

5.2. Needle gauge comparison

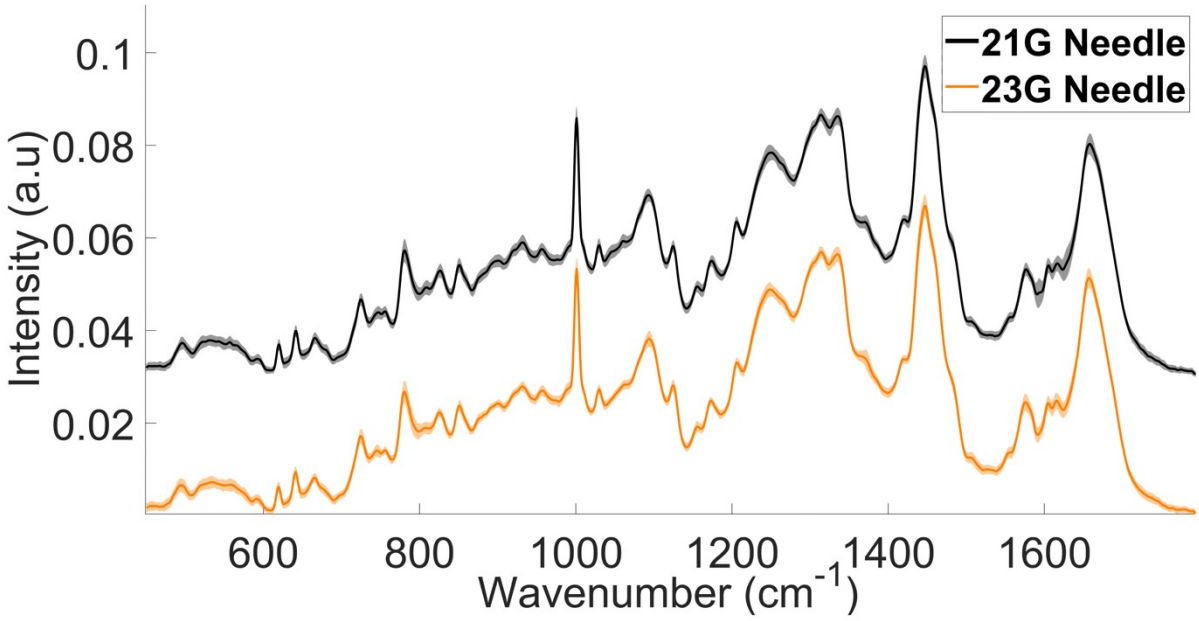

Fig. ESI3 Mean Raman spectra of lymphocytes collected with a 21 G or 23 G needle

5.3. Anticoagulant: EDTA, lithium heparin, and sodium citrate

The mean FTIR spectra (Fig. ESI4A) of the three anticoagulants displayed variance over the wavenumber range of 1000-1140 cm^{-1}, which consists of contributions from carbohydrates, glycogen, C-O-C stretching (nucleic acids and phospholipids), phosphate and oligosaccharides. For LH and SC however, variance was observed for FTIR signatures tentatively assigned to C-O stretching mode of C-OH groups of serine, threosine, and tyrosine of proteins), amide III, phosphate stretching bands from phosphodiester groups of cellular nucleic acids, collagen, symmetric stretching vibration of COO^- group of fatty acids and amino acids (1141-1430 cm^{-1}). The mean Raman spectra (Fig. ESI4B) of lymphocytes displayed minimal variance for the three anticoagulant data classes with the exception of Raman signatures in the EDTA data class tentatively assigned to amide III, CH_2 deformation lipids, adenine and cytosine (1301-1341 cm^{-1}).

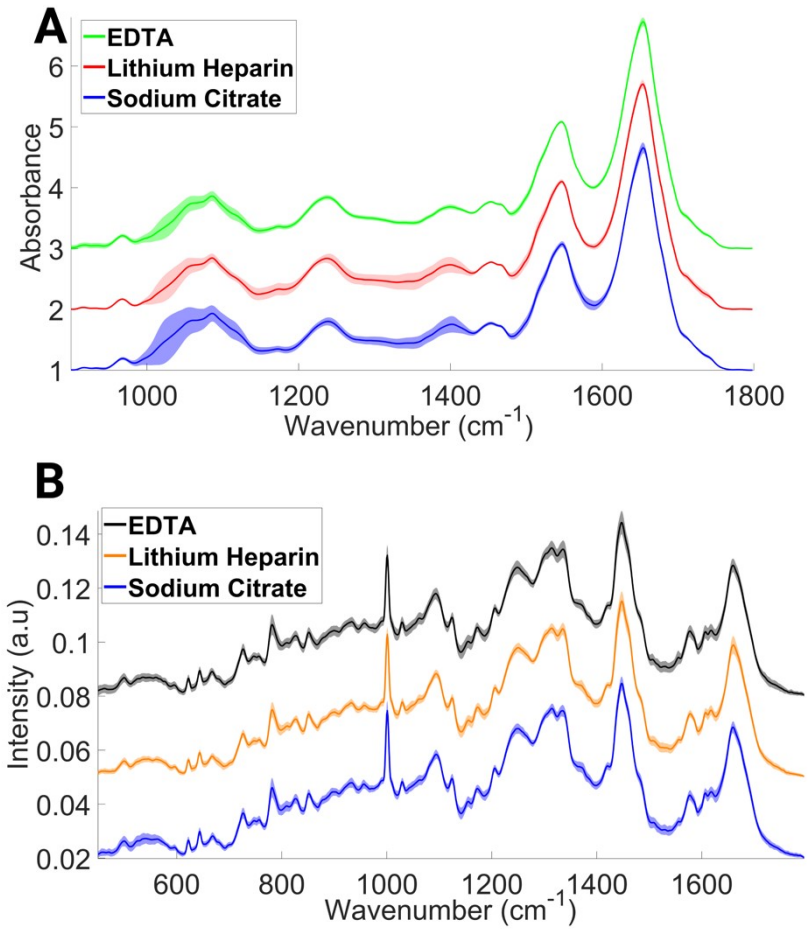

Fig. ESI4 Mean FTIR (A) and Raman (B) spectra of lymphocytes collected with EDTA, SC or LH anticoagulant blood collection tubes.

5.4. Freezing samples

Fig. ESI5A depicts the mean FTIR spectra of non-frozen lymphocytes, i.e. processed immediately after the drawing of blood and frozen lymphocytes, i.e. a single freeze-thaw cycle. Non-frozen lymphocyte spectra are highly reproducible, with frozen lymphocytes showing minor variance in spectral regions with contributions from biomolecules such as DNA, glycogen, collagen, phosphate & oligosaccharides (986-1120 cm^{-1}), vC-O of proteins and carbohydrates, collagen, amide III, PO2- of nucleic acids (1126-1362 cm^{-1}), amide II (1478-1501 cm^{-1}), amide I and nucleic acids (1648-1656 cm^{-1}), C=O stretching (lipids) and C=O of guanine and thymine (1706-1742 cm^{-1}).

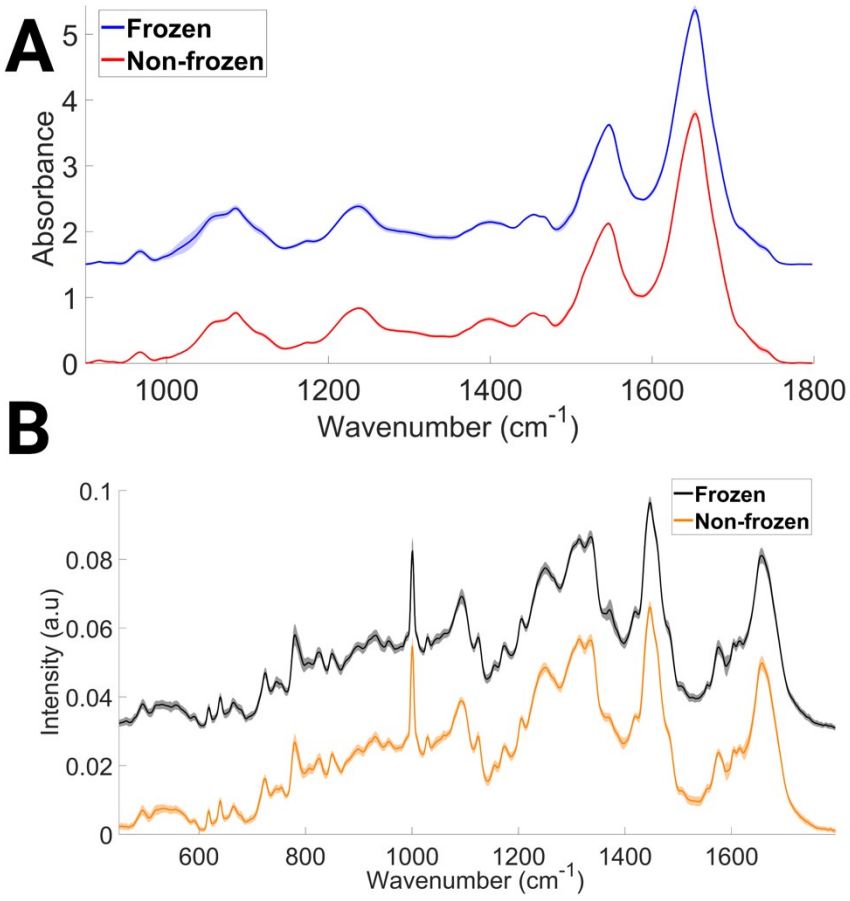

Fig. ESI5 FTIR (A) and Raman (B) spectra of non-frozen (processed immediately) and frozen lymphocytes (single freeze thaw cycle).

Analysis of non-frozen lymphocytes using RS revealed no real variance between the individual samples, with the resultant spectra exhibiting a high degree of uniformity in the spectral signals (Fig. ESI5B). There was notable variance present within the frozen lymphocyte spectral data set for Raman signatures tentatively assigned to tryptophan and DNA (1361-1374 cm^{-1}), CH deformation of DNA, lipids, and carbohydrates, (1414-1427 cm^{-1}), and guanine and adenine, (1476-1482 cm^{-1}).