Appendix A – Comparison of Water Correction Methods

Introduction

In the main paper, we outline a simple dynamic flow system for the study of living cells using SR-FTIR. A crucial aspect of this work is the use of an in-house procedure to correct for the water contribution to the acquired IR spectra, which is described in greater detail elsewhere. [1]

There is a clear lack of consensus within the field regarding the ideal method of water correction, with a number of methods and experimental protocols proposed. [2] Some crude methods simply ignore the effected region of the spectrum [3], while others simply acquire a background spectra through cell-free aqueous media. [4] Other methods use specific regions of the spectrum as a reference, such as the water combination band [5,6] or the C-H alkyl stretch at 2900 cm^{-1}. [7] These methods have the most in common with the method used in this paper, which uses the Amide I and II bands and a Matrigel reference spectrum to determine the scaling factor for the water spectrum to be removed.

To our knowledge, an assessment of the effects of different water correction procedures on the resulting spectra and subsequent analysis has not been performed. There is much scope for further work in this area, and could account for a separate publication. Here, a relatively straightforward comparison will be performed between spectra from the thermal stress study at 37 °C and 60 °C, corrected using our least-squares fitting procedure and the baseline-flattening method described by Vaccari and colleagues. [5,6] This will also provide an indication of the dependency of the proposed dynamic flow system on a particular water correction methodology.

Methodology

Spectra were acquired using the dynamic flow system described in the main paper. The spectra acquired at sample temperatures of 37 and 60 °C were corrected for their water contribution using both the in-house least squares fit procedure, using the 1500-1700 cm^{-1} wavenumber range for fitting and a Matrigel reference spectrum to determine the fraction of the corresponding water spectrum to be subtracted from each cell spectrum. [1] The same spectra were also separately corrected by simply iteratively subtracting increasing fractions of the water spectrum, until a flat baseline was observed in the 1800-2500 cm^{-1} region which contains the water combination band.

The resulting spectra were then put through the same data processing steps. Spectra from different replicates were combined, and then manually quality controlled using an in-house PCA-based method, with the remaining spectra then vector normalised and converted to the second derivative with a 9 point smoothing filter.

The second derivative spectra were then split into high and low wavenumber sections, from 1150-1580 cm^{-1} and 3800-3000 cm^{-1} respectively. Mean spectra were then computed for each temperature and water correction method using the low wavenumber range, to identify any significant differences based on the water correction method applied. The low wavenumbers were used as this is where the majority of variation in the mean spectra at different temperatures was seen in the main results.

Results and Discussion

Figure A1 shows the second derivative mean of spectra acquired at 37 and 60 °C, corrected using our in-house least squares fit method and the Vaccari method. The spectra are shown overlaid, at 37 and 60 °C respectively, in A) and C), and offset by 0.0001 Second Derivative of Absorbance units in B) and D).

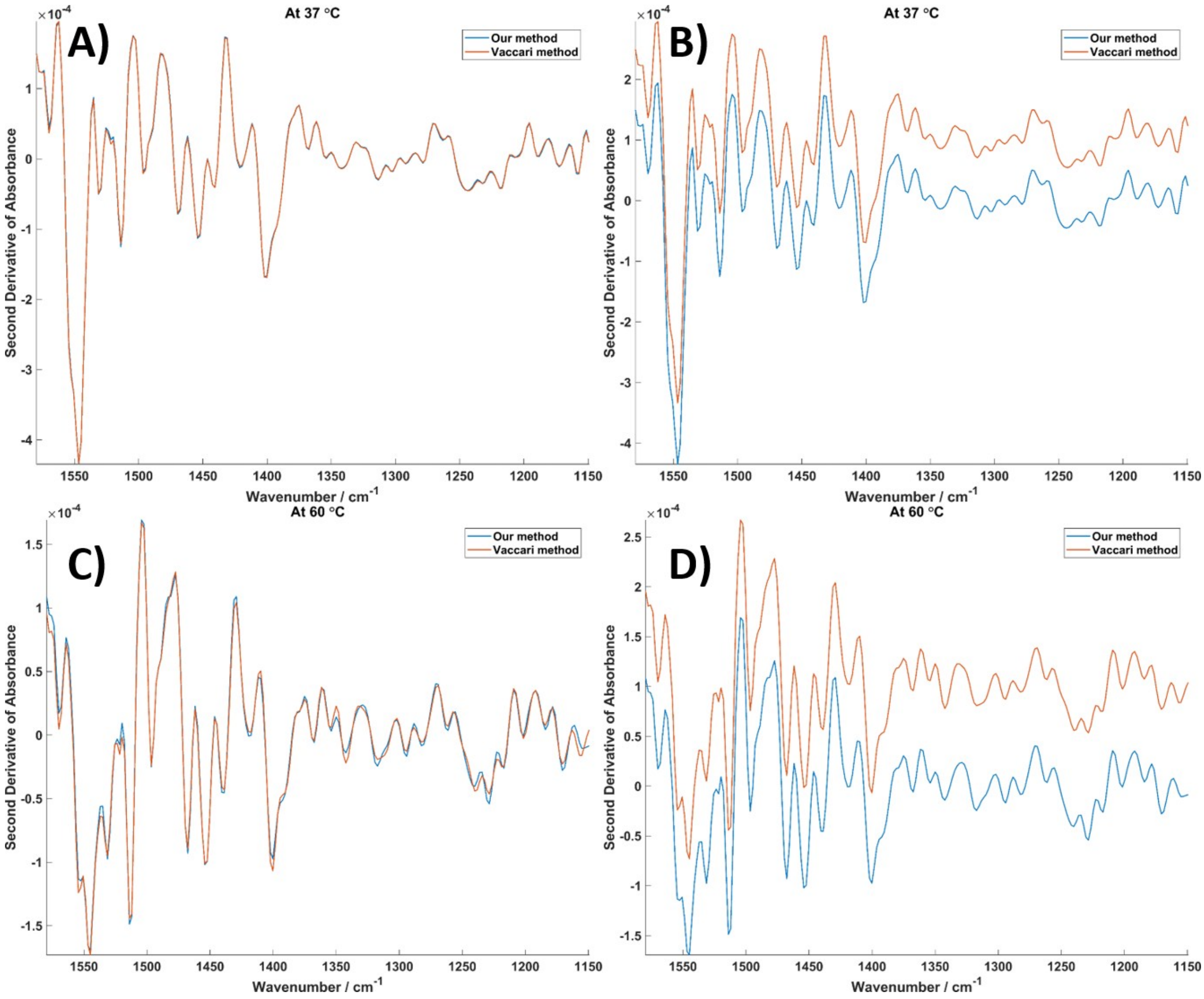

Figure A1 - Comparison of second derivative mean spectra acquired at 37 °C and 60 °C, corrected with the in-house water correction procedure outlined in this paper, and the combination band-flattening method by Vaccari *et al.*[6] A) The spectra at 37 °C, overlaid. B) The 37 °C spectra, offset. C) The 60 °C spectra, overlaid. D) The 60 °C spectra, offset.

Examination of Figure A1 A) and B) shows very little difference between the second derivative mean spectra at 37 °C based on the water correction method used. Very close inspection of the spectra reveals subtle changes in peak shape and position at 1520 and 1514 cm^{-1}, but no other noticeable changes in the spectra across lower wavenumber range.

Figures C) and D) show slightly more variation across the low wavenumber range between the two water correction methods, but the two mean spectra shown still track closely together and the variation seen is minimal. The most noticeable changes occur between 1241 and 1228 cm^{-1}, where there is a slight shift to the higher wavenumbers in the Vaccari method-corrected spectrum, differences in second-derivative peak intensity at 1410 and 1429 cm^{-1}, and small changes in intensity and position between 1350 and 1282 cm^{-1}.

The changes seen between the spectra are minimal, although greater variation is clearly seen between the spectra at 60 °C than at 37 °C. The changes appear relatively minor, but the retention of subtle variation between spectra for further analysis is a crucial measure of a water correction technique, and therefore they cannot be discounted.

Comparison of the regions of variation between the water correction methods with the DFA loadings plot shown in Figure 7 of the main paper shows little correlation between spectral changes based on water correction method, and those highlighted as important to the separation between temperature points. This suggests that, despite the increased variation at higher temperatures, this would not have significantly impacted the results obtained.

The increased variation seen at 60 °C is perhaps unsurprising, given that there is likely to be more variety within the spectra at the higher temperatures due to differences in the response to thermal stress by the cells. Figure 6 in the main paper showed significant spread in DFA space of the 60 °C spectra, suggesting a high level of intra-sample variation at the higher temperatures.

While the subtle variations based on water correction method cannot entirely be ignored, the spectra are broadly similar through the spectral range crucial for this study. This is an important validation of both the in-house water correction method – demonstrating similar results to an established published method – and of the dynamic flow system outlined in the main paper.

Conclusions

The closeness of the second derivative mean spectra, following their processing using two different water correction methods, suggests that the dynamic flow system proposed in the main paper has a relatively low dependency on the correction method used. This is promising for the development and implementation of this methodology, as it can potentially be used alongside existing alternative water correction procedures.

Subtle variations between spectra based on the water correction method employed have been noted, and observed to me more numerous at 60 °C than at 37 °C. However, the regions where the most variation is seen were not key to the analysis of the thermal stress study, and therefore the methodology does not appear to be particularly correction method-dependent.

As has been alluded to previously, a detailed analysis of the effects of different water correction procedures on various regions of the spectrum is an area ripe for significant further work. However, this brief comparison suggests that the dynamic flow system described in this work is suitable for implementation using different data collection and processing methodologies.

The similarity in the results using two different water correction methods does present the question of whether the development of a new, in-house water correction technique is necessary. While it is true that the results are highly similar using both correction methods, the flattening of the water combination band could not have been used to correct the spectra treated with D_{31}-PA for the palmitic acid uptake study, due to the overlapping of the deuterated palmitic acid bands with the water combination band. The in-house least squares fitting method provides a comparable water correction, but is potentially applicable to a greater range of experiments.

References

1. Doherty, J., et al., *Increased optical pathlength through aqueous media for the infrared microanalysis of live cells.* Analytical and bioanalytical chemistry, 2018: p. 1-11.
2. Doherty, J., G. Cinque, and P. Gardner, *Single-cell analysis using Fourier transform infrared microspectroscopy.* Applied Spectroscopy Reviews, 2017. **52**(6): p. 560-587.
3. Mourant, J.R, et al., *Methods for measuring the infrared spectra of biological cells.* Physics in medicine and biology, 2003. **48**(2): p. 243.
4. Quaroni, L., et al., *Infrared imaging of small molecules in living cells: from in vitro metabolic analysis to cytopathology.* Faraday discussions, 2016.
5. Birarda, G., et al., *Infrared microspectroscopy of biochemical response of living cells in microfabricated devices.* Vibrational spectroscopy, ,2010. **53**(1): p. 6-11.
6. Vaccari, L., et al., *Infrared Microspectroscopy of Live Cells in Microfluidic Devices (MD-IRMS): Toward a Powerful Label-Free Cell-Based Assay.* Analytical Chemistry, 2012. **84**(11): p. 4768-4775.
7. Gelfand, P., et al., *Characterization of Protein Structural Changes in Living Cells using Time-Lapsed FTIR Imaging.* Analytical Chemistry, 2015.