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. 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.](image)

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
\text{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 \text{cm}^{-1}, and small
changes in intensity and position between 1350 and 1282 \text{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 D₃₁-PAL 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