Electronic Supplementary Material (ESI) for Analyst.

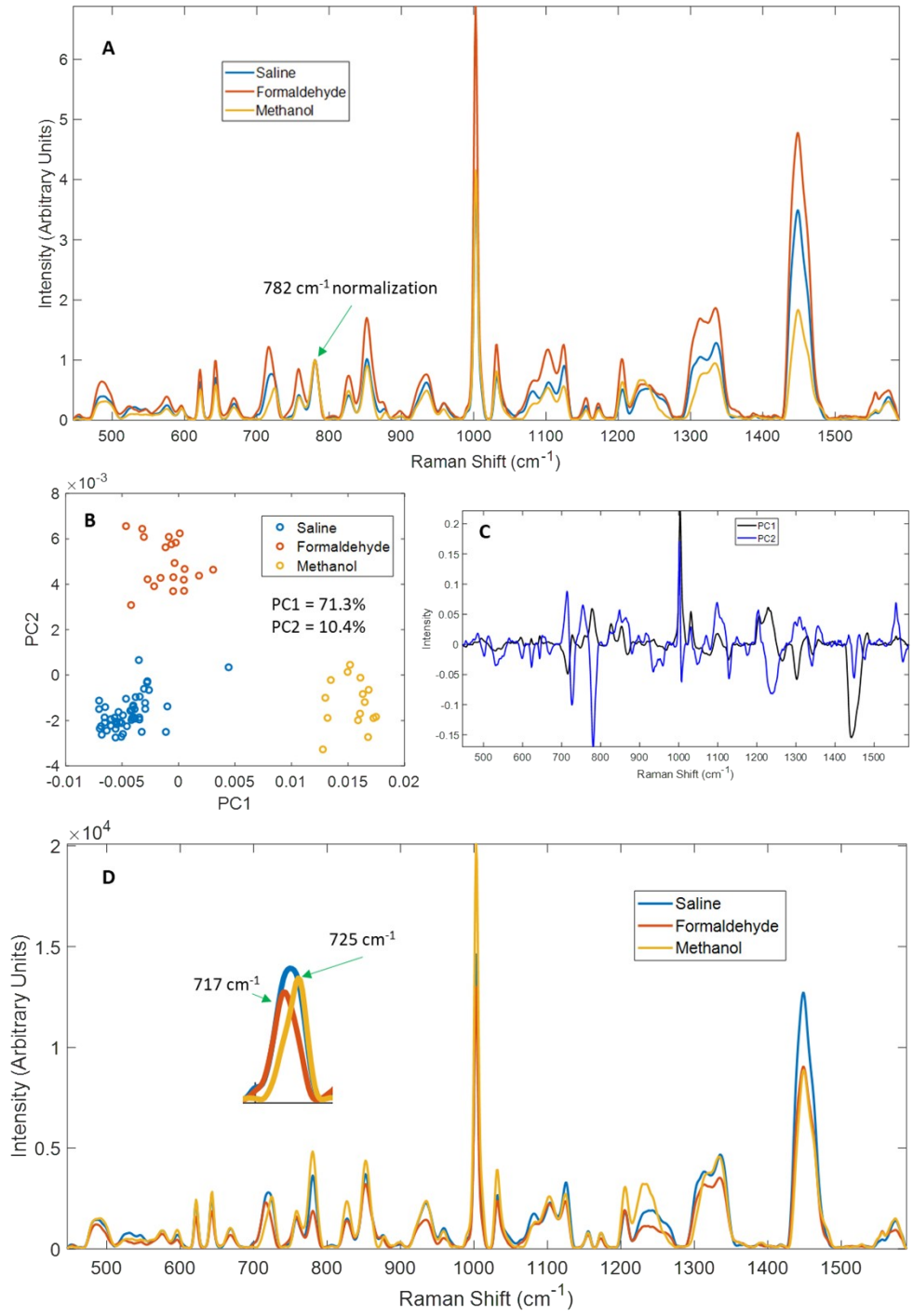


Figure S1. Raman spectra of human embryonic stem cells dry-fixed with saline or fixed with methanol or formaldehyde. **(A)** Normalized mean spectra showed systematic spectral differences between the fixatives. **(B)** Cells fixed with the three fixatives clustered separately in PCA. **(C)** PCA loadings revealed the basis for separation, with methanol-fixed cells separating based on lipid signals, and formaldehyde-fixed cells separating based on protein and nucleic acid signals. **(D)** Unnormalized mean spectra showed certain biomolecular changes more clearly, e.g., loss of lipids at 717 cm^{-1} and loss of adenine at 725 cm^{-1}.

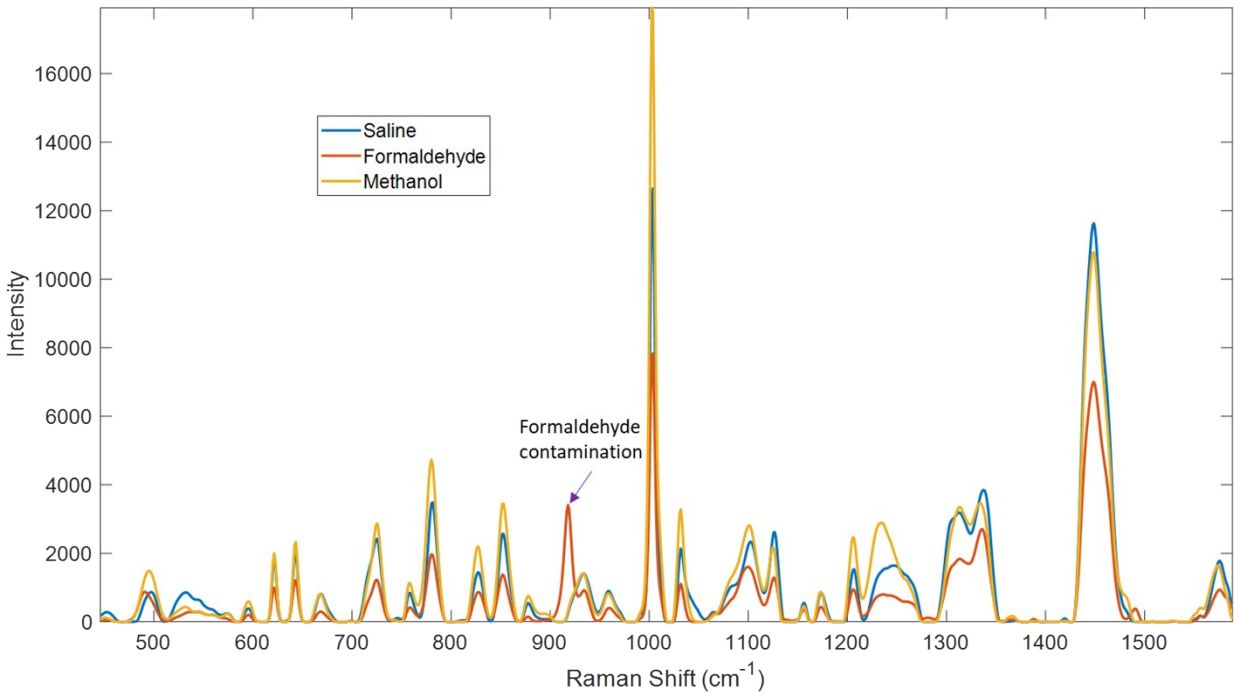


Figure S2. Unnormalized mean spectra of Jurkat cells dry-fixed with saline or fixed with methanol or formaldehyde. The loss of intensity in formaldehyde-fixed cells is evident, along with contamination from formaldehyde, particularly at 915 cm^{-1}. In contrast, methanol-fixed cells generated more intense protein signals – an expected outcome of protein precipitation.

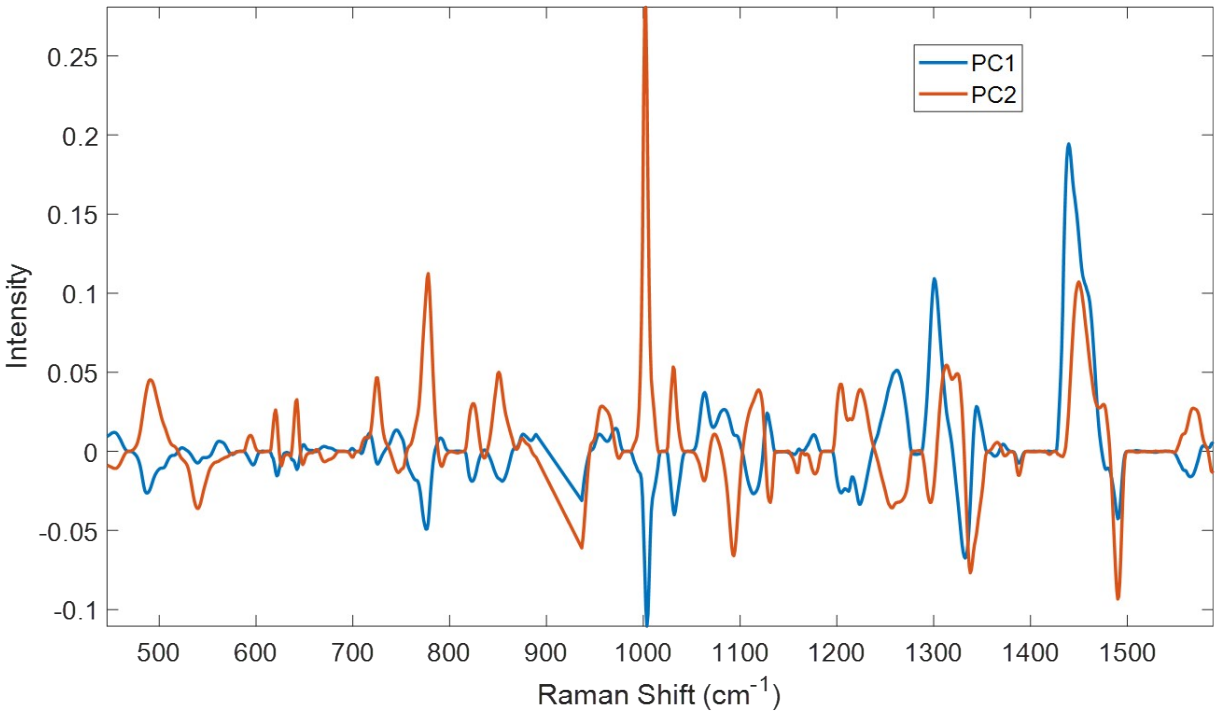


Figure S3. Principal component loadings from 100%, 75%, 50%, and 25% formaldehyde-fixed Jurkat cells. Strong contributions were observed in the first principal component from 1440 cm^{-1}, 1330 cm^{-1}, 1300 cm^{-1} and 1260 cm^{-1}, while the second principal component showed strong contributions from 780 cm^{-1}, 1002 cm^{-1}, 1093 cm^{-1}, 1337 cm^{-1} and 1450 cm^{-1}, indicating that peaks generated by formaldehyde as well as peaks impacted by formaldehyde fixation contribute to the observed separation.

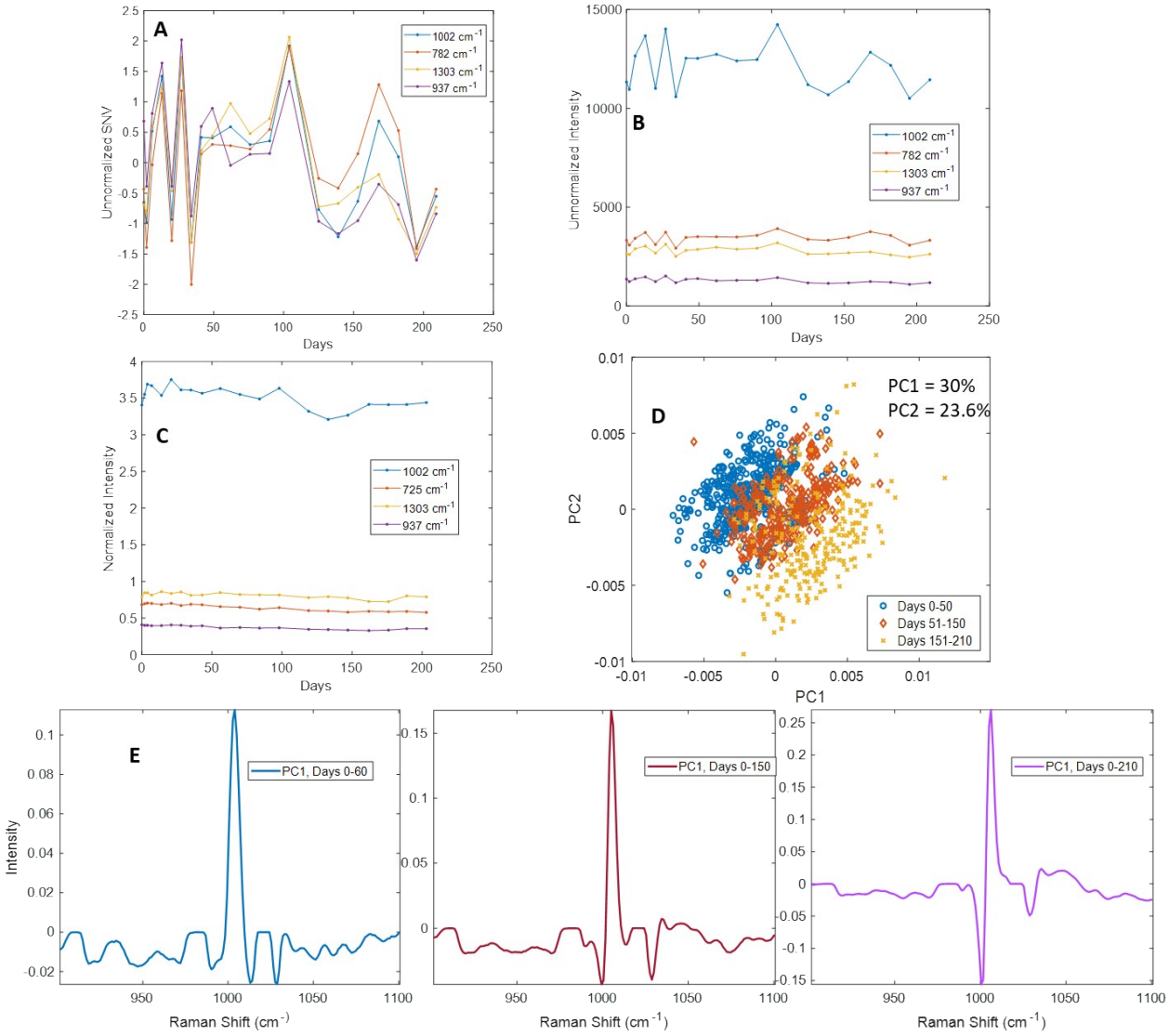


Figure S4. Temporal stability of saline dry-fixed Jurkat cells – replicate 2. **(A)** SNV profiles of peaks representative of different macromolecules over the measurement range and **(B)** unnormalized intensity profiles of the same peaks. **(C)** Normalized intensity profiles of peaks corresponding to different macromolecules showed <10% variation across the measurement range. **(D)** PCA showed two clusters in the first 150 days, with a third cluster observed from days 151-210. **(E)** PC1 loadings showed slight derivative-like features at 1002 cm^{-1} when comparing across days 0-150, which became much more pronounced when comparing across days 0-210, suggesting that the observed variability arose from instrument drift in the over the latter two-thirds of the time course of measurement.

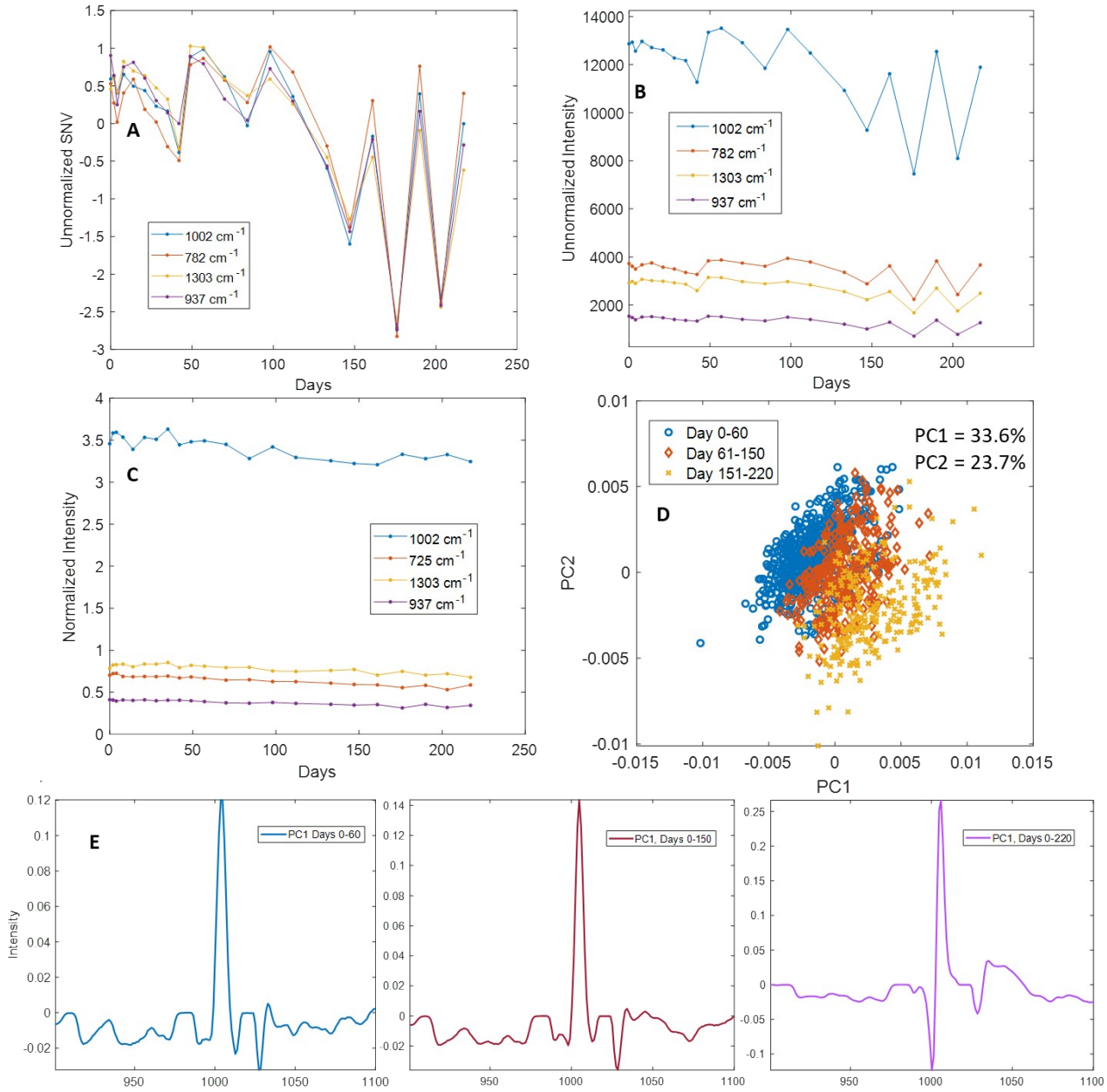


Figure S5. Temporal stability of saline dry-fixed Jurkat cells – replicate 3. **(A)** SNV profiles of peaks representative of different macromolecules over the measurement range and **(B)** unnormalized intensity profiles of the same peaks. **(C)** Progression of selected peaks over time showed good consistency for the first ~150 days of measurement, with subsequent variations not monotonic, suggesting that some observed variability arises from spot-to-spot variability in the sample. **(D)** PCA showed tight clustering for the first ~150 days, with some separation observed from days 151-210. **(E)** PC1 loadings showed derivative-like features at 1002 cm^{-1} when comparing across the entire measurement range, suggesting that some of the observed variability arose from instrument drift in the later part of the time-course of measurement.

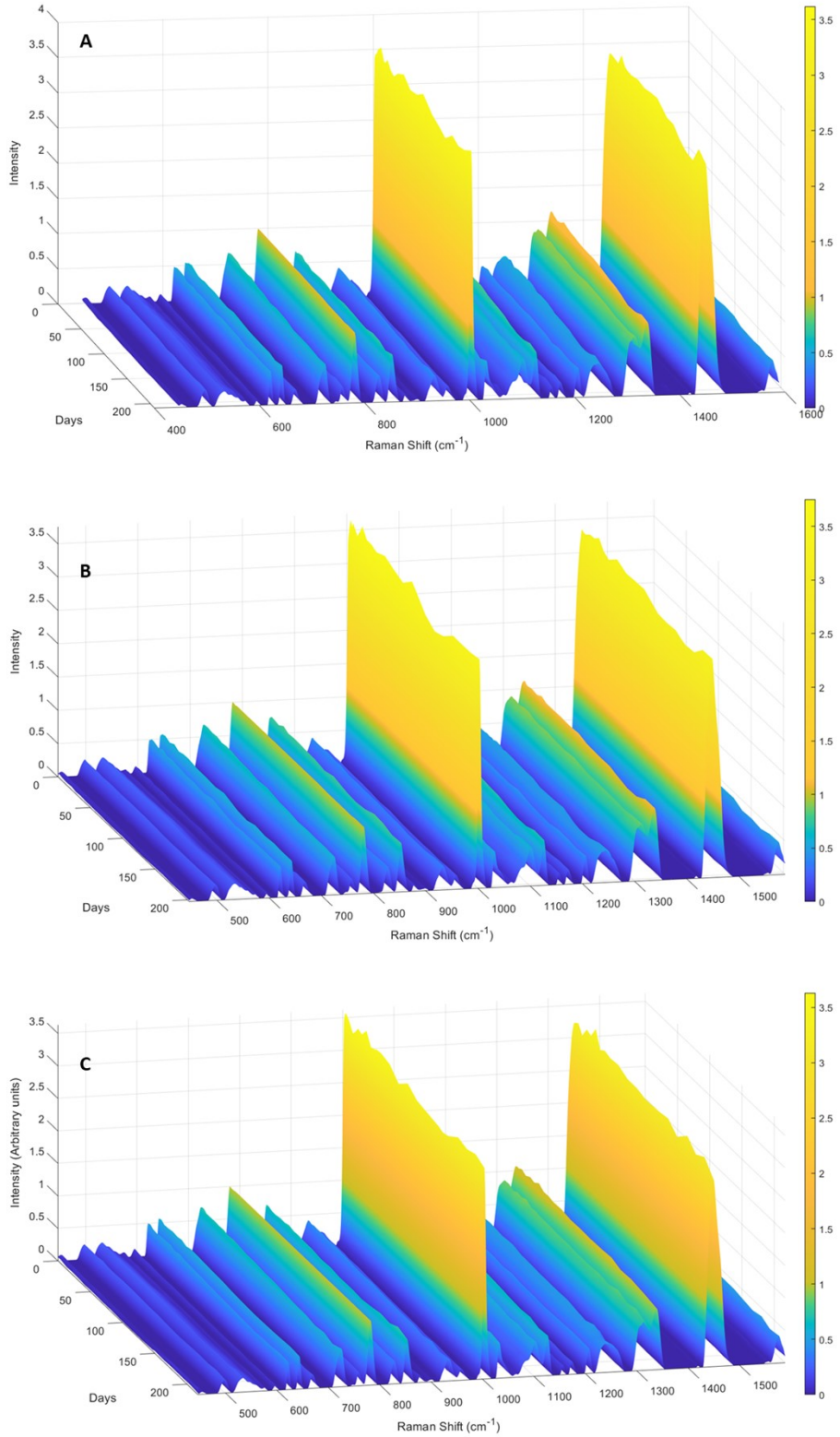


Figure S6. Surface plots of normalized mean spectra over time showed relatively minor variations across ~210 days of measurement. **(A)** Replicate 1, **(B)** Replicate 2, **(C)** Replicate 3.

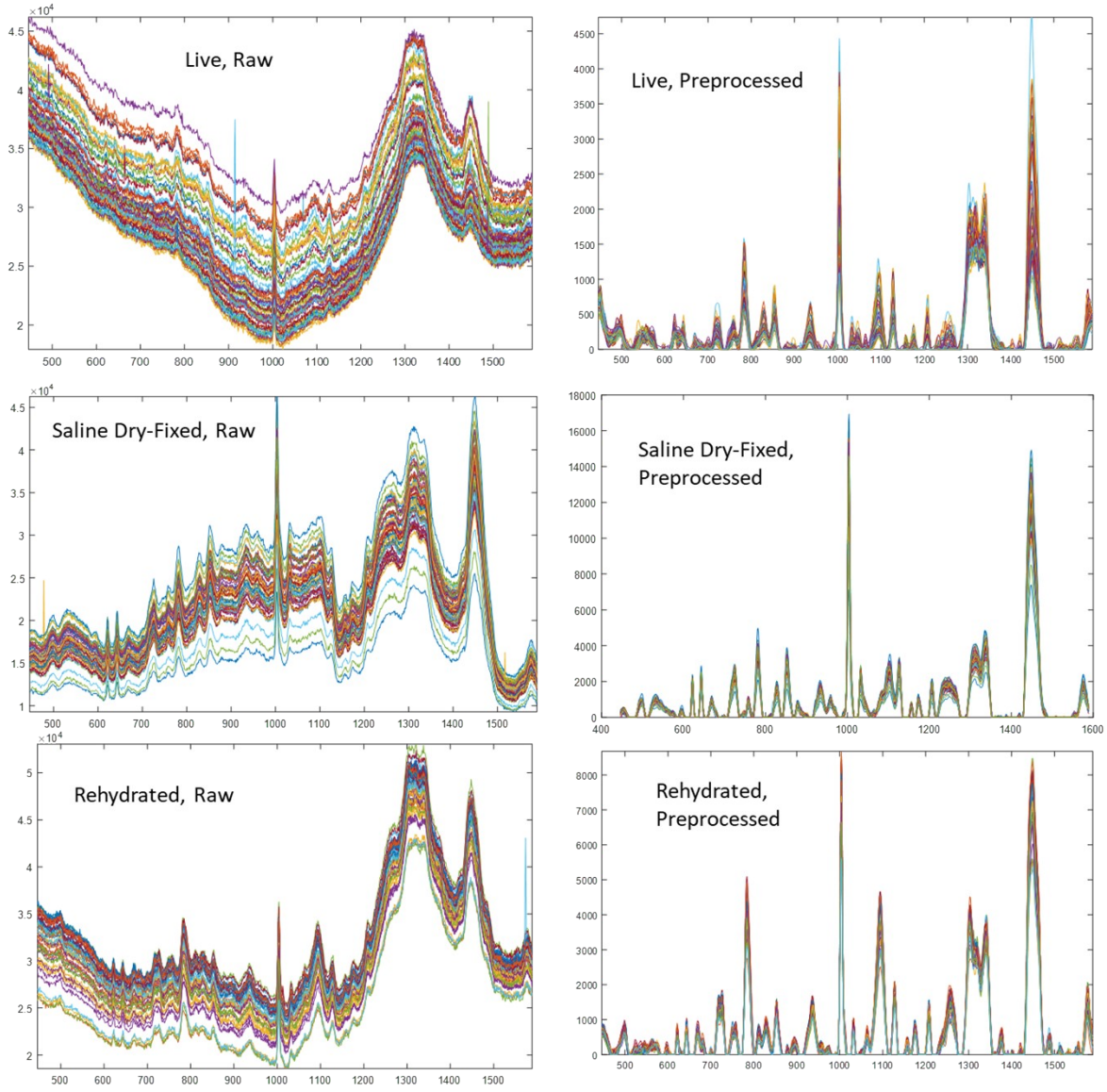


Figure S7. Raw and preprocessed spectra from live, saline dry-fixed, and rehydrated samples showed that live cells produce substantially more variable spectra than saline dry-fixed samples. Rehydrated samples also showed more variability than dried samples, suggesting that some of the observed difference arises sample hydration.

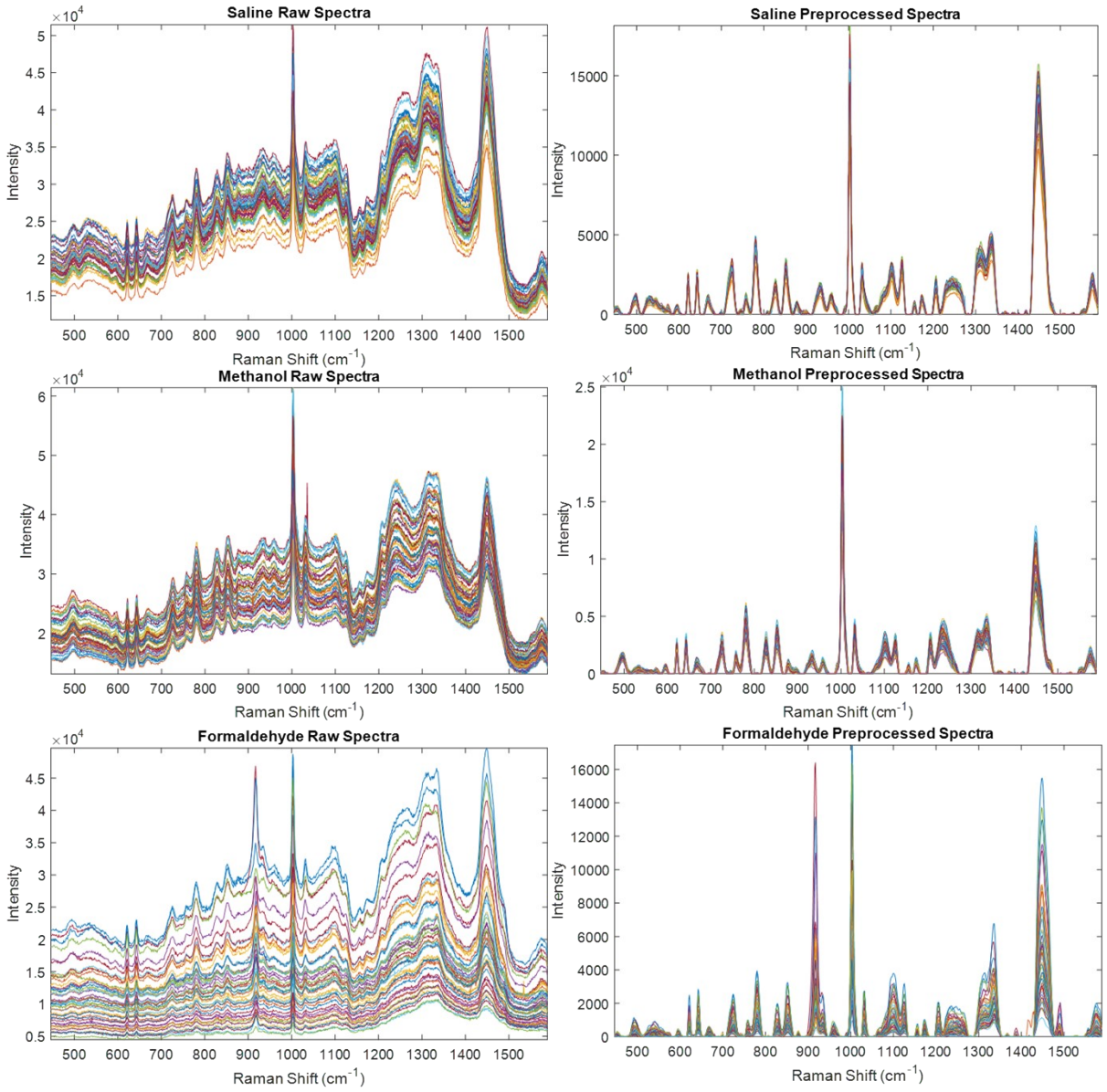


Figure S8. Raw and preprocessed spectra from saline dry-fixed, methanol-fixed, and formaldehyde-fixed Jurkat cells showed that cells fixed with formaldehyde were much more variable than the other two methods. The observed variability arises from both biomolecular signals as well as signals contributed by formaldehyde itself.

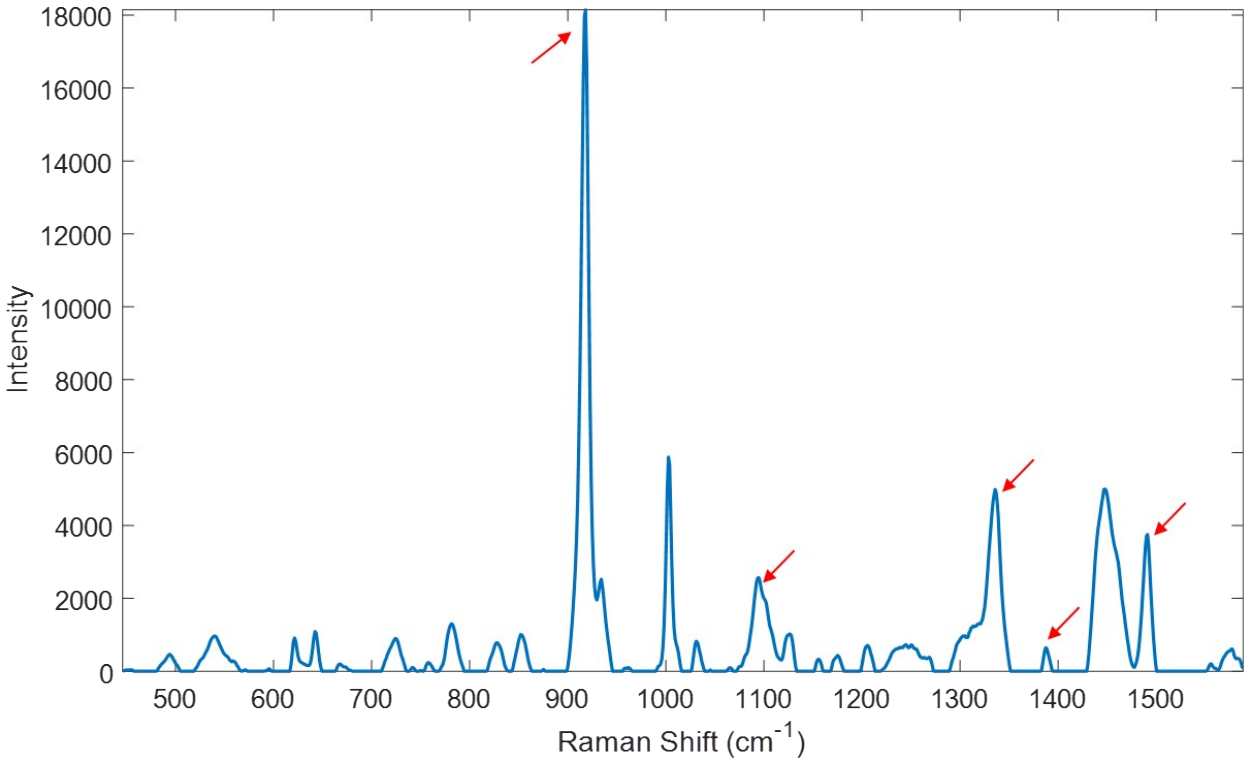


Figure S9. Mean spectrum from improperly washed formaldehyde-fixed Jurkat cells showed very strong peaks arising from formaldehyde, including 917 cm^{-1}, 1094 cm^{-1}, 1335 cm^{-1}, 1387 cm^{-1} and 1490 cm^{-1} (indicated by red arrows).

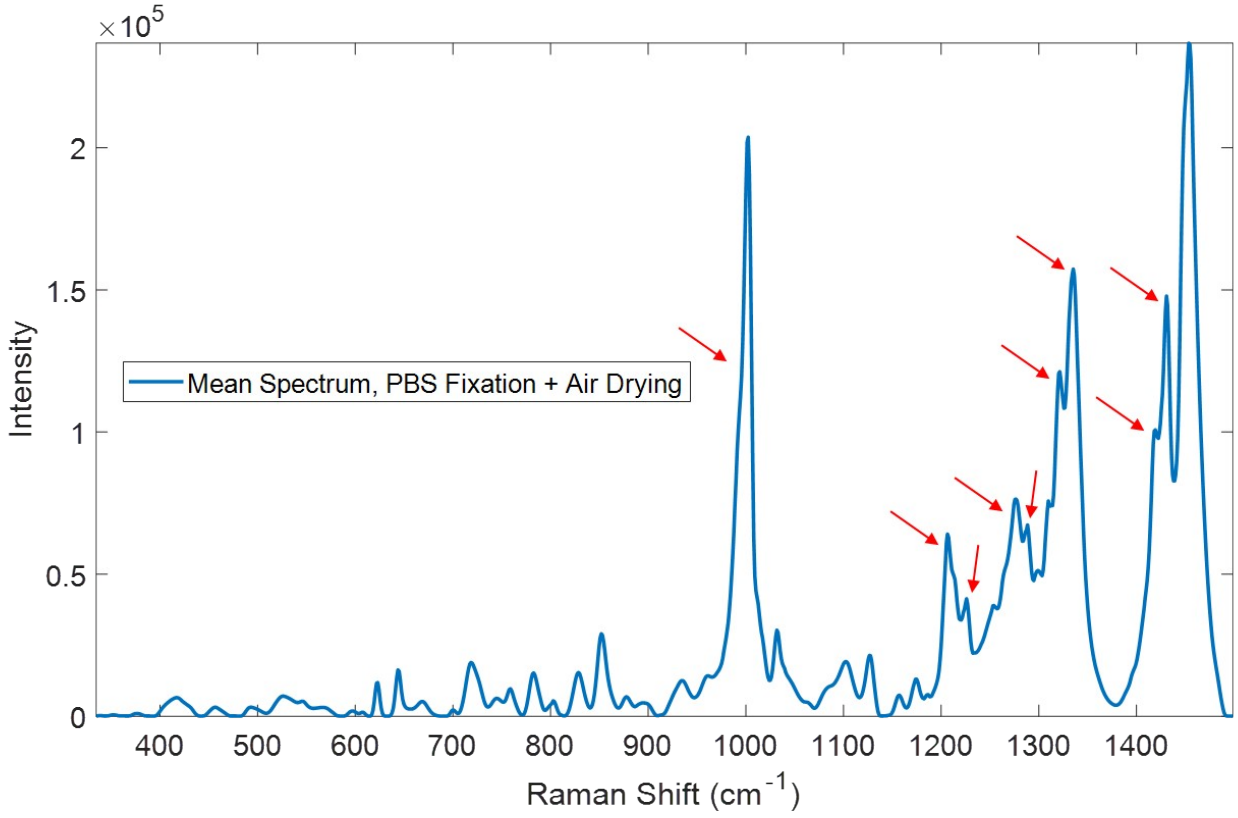


Figure S10. Mean spectrum from Jurkat cells resuspended in PBS and air-dried. Distortions in many peaks are observable (indicated by red arrows), attributable to the presence of phosphate crystals.

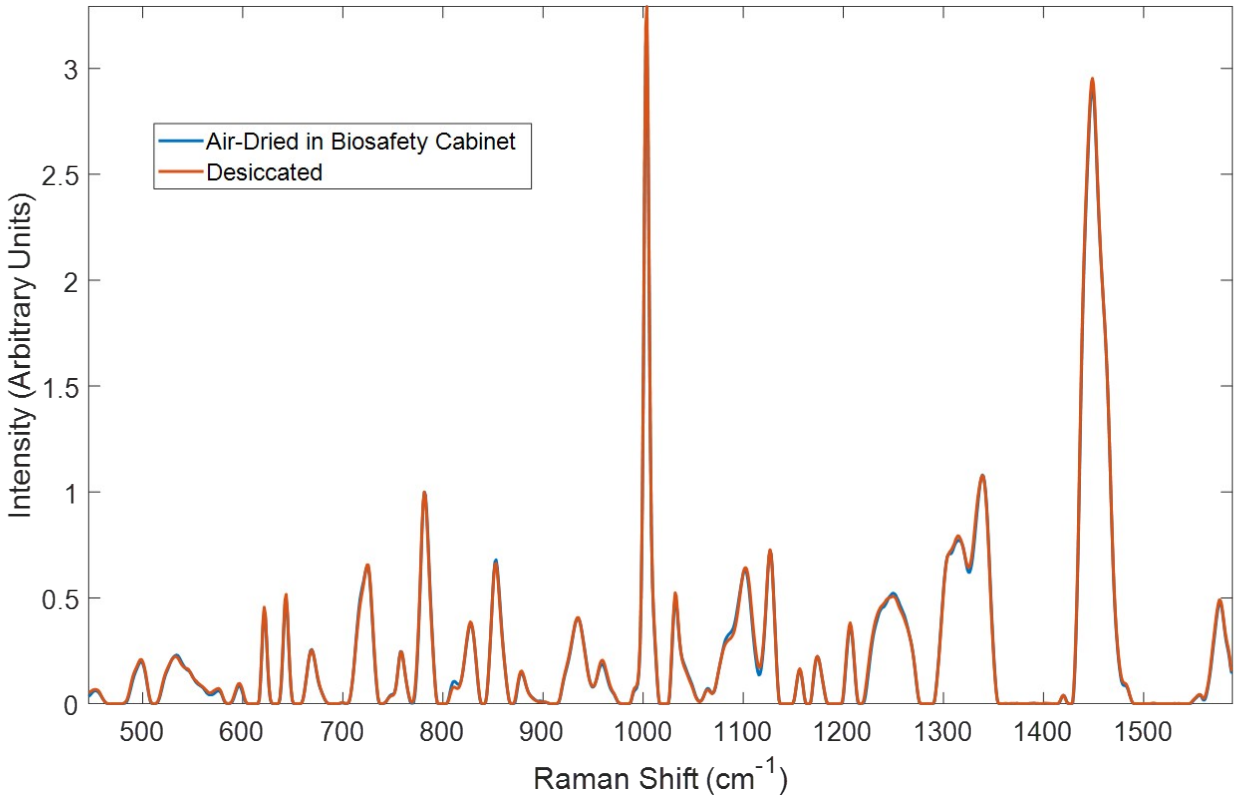


Figure S11. Mean spectra from Jurkat cells resuspended in saline and air-dried or desiccated. No significant differences were observed in Raman spectra collected from either sample.

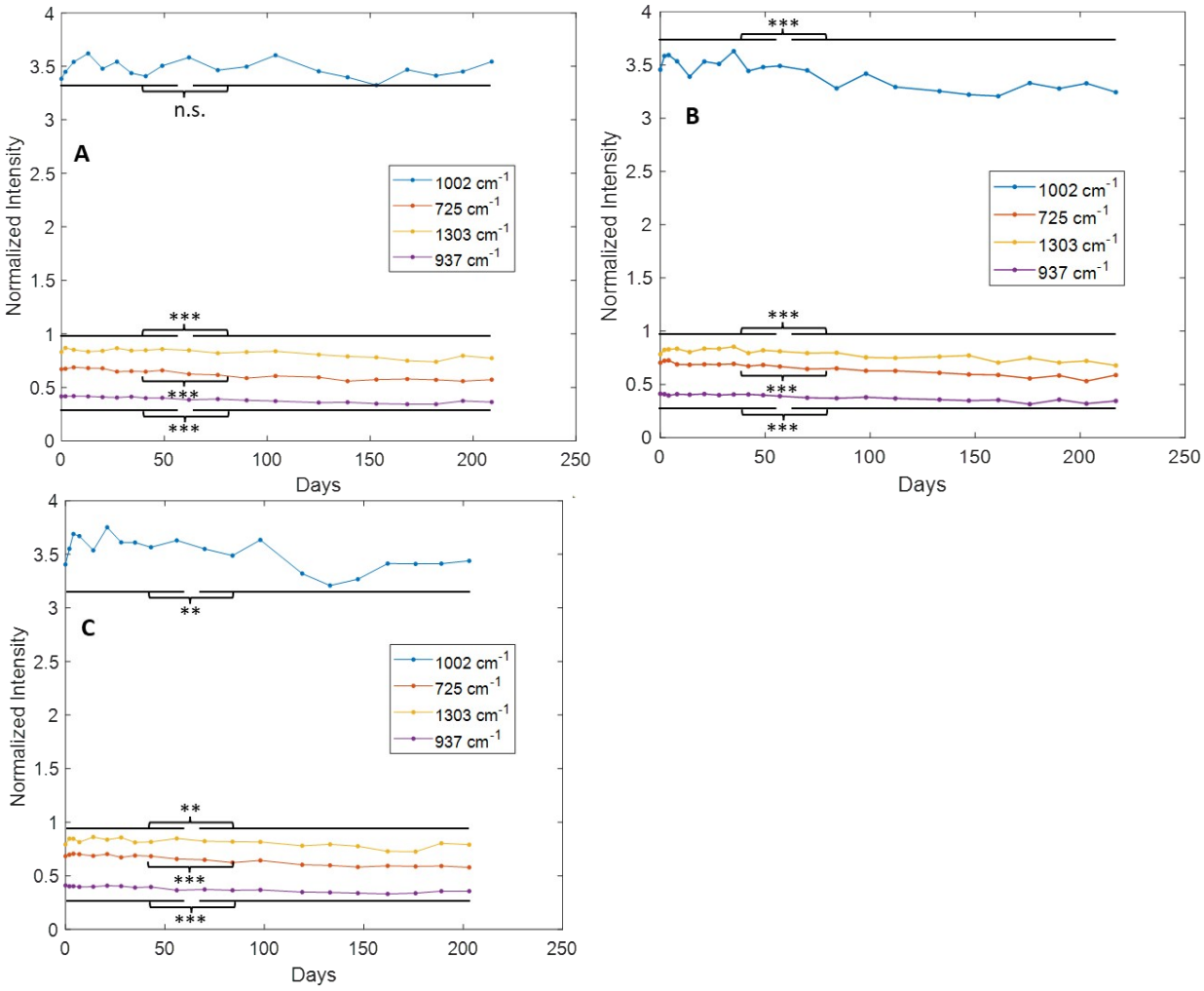


Figure S12. Paired t-tests performed on selected peaks between days 0-49 and days 50-220 of the time course of measurement of saline-dry fixed samples, where **(A)** corresponds to data in Figure 3, **(B)** corresponds to data in Figure S5, and **(C)** corresponds to data in Figure S6. Most pairs of data tested were statistically significant. ** - $p < 0.01$; *** - $p < 0.001$, n.s. – not significant

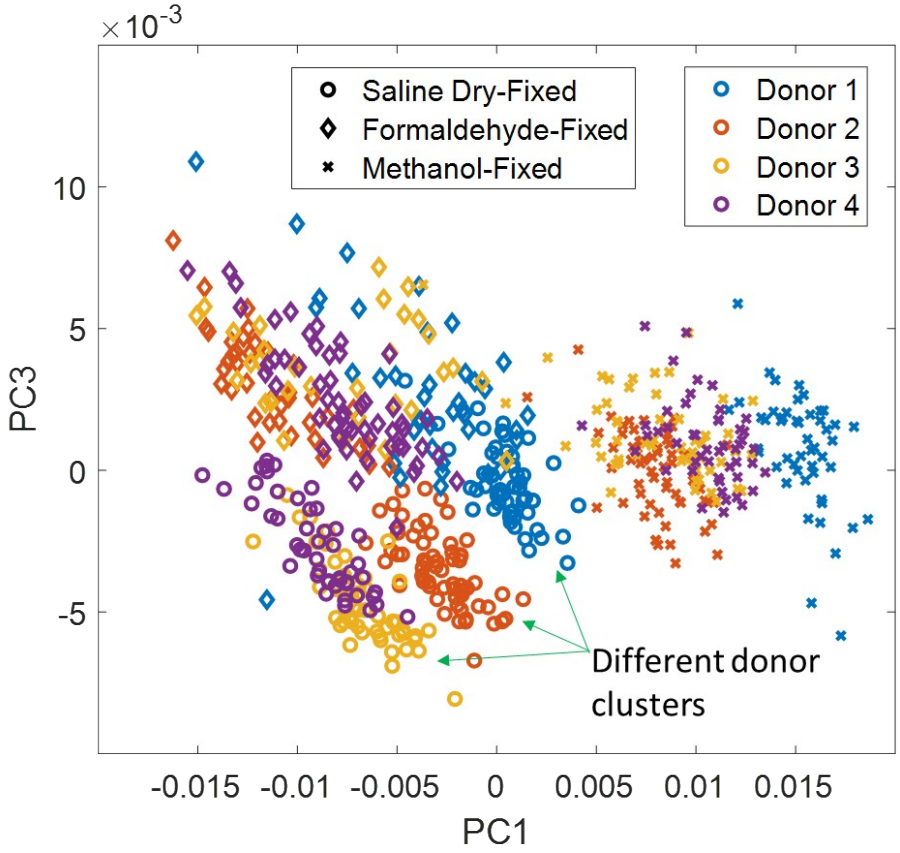


Figure S13. PCA of $CD3^+$ primary T-cell samples shown in Figure 2C. Each color represents data from one donor, and each symbol represents one fixation method. Saline dry-fixation showed the best preservation of donor-to-donor variability.

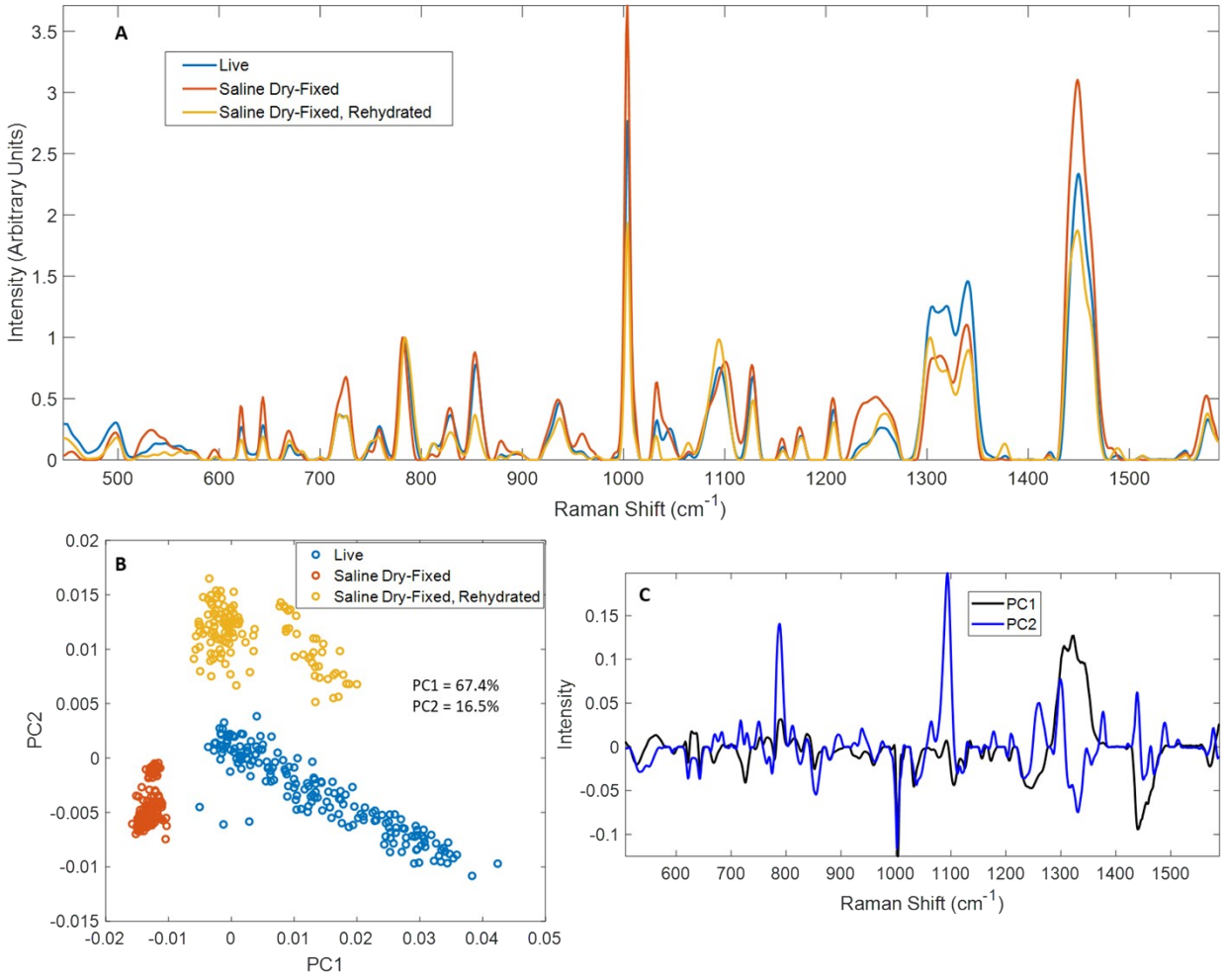


Figure S14. Normalized mean spectra from live, saline dry-fixed, and dry-fixed rehydrated Jurkat cells. **(A)** Mean spectra (n = 4 biological replicates) showed many different features between live and saline dry-fixed cells, both in peak shape and intensity, but most of these were recovered upon hydration. **(B)** The 3 samples clustered separately in PCA, with saline dry-fixed cells showing the least variability and live cells showing the most variability. **(C)** PC loadings show the basis of separation, with differences in the 1300-1340 cm^{-1} region dominating the separation of live cells along PC1, and differences in nucleic acids and 1100 cm^{-1} dominating the separation of rehydrated cells along PC2.

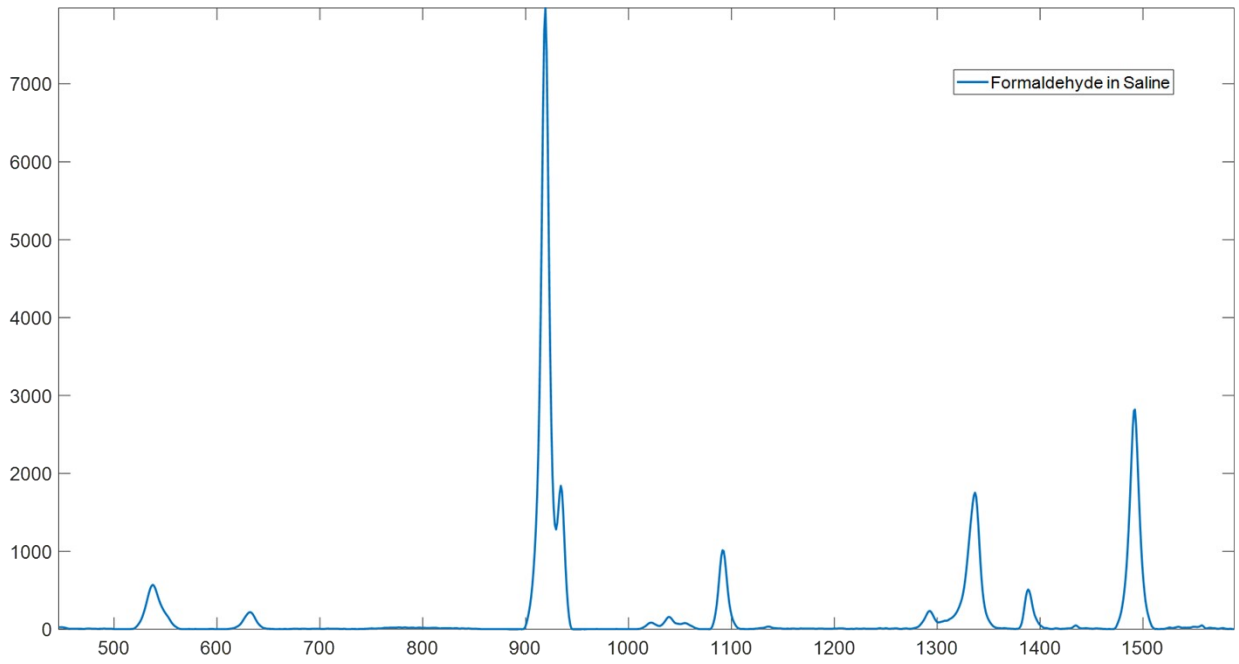
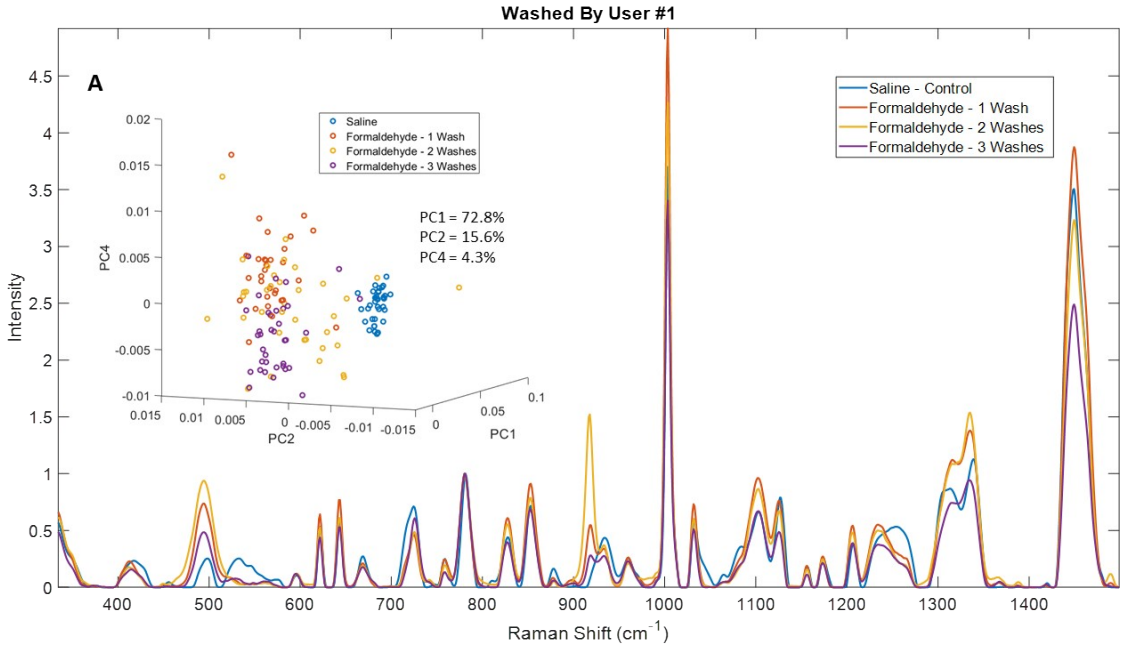
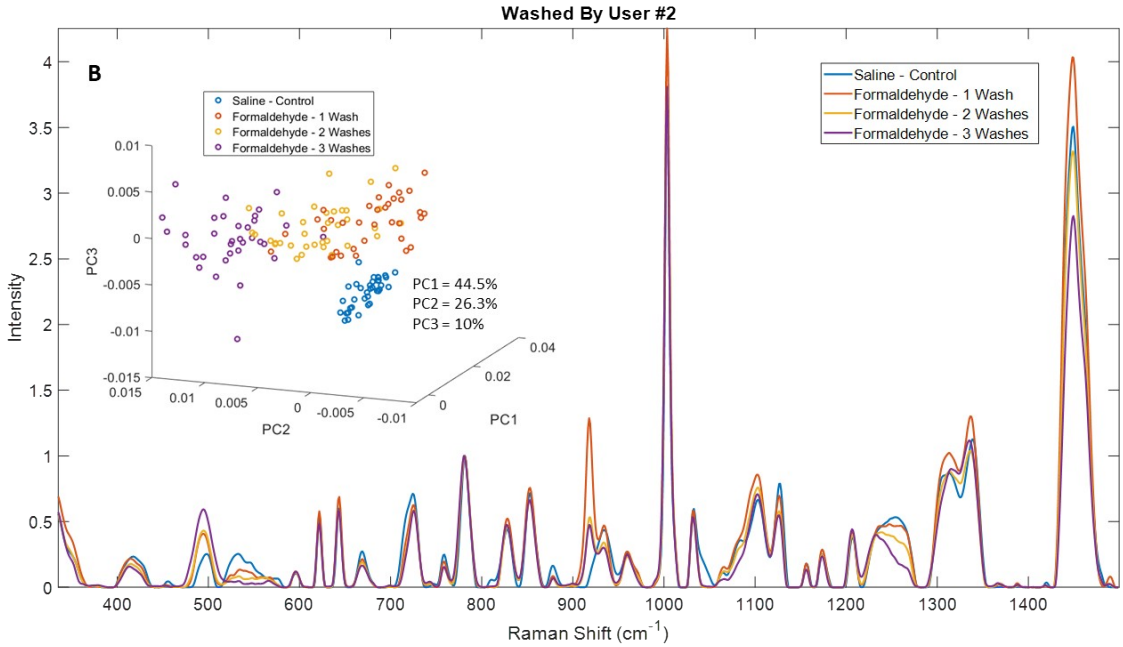
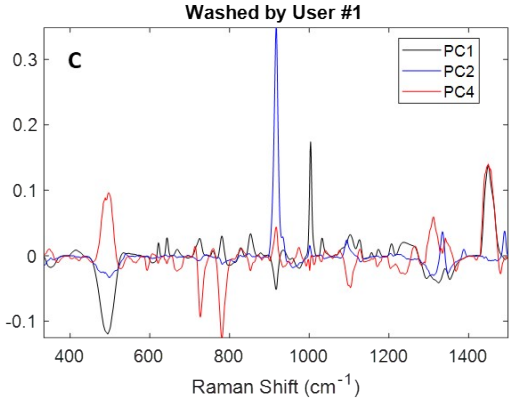


Figure S15. Mean spectrum of formaldehyde dissolved in saline and dried. The main signal contribution from formaldehyde occurs at 919 cm^{-1}, with smaller contributions observable at 538, 632, 934, 1040, 1091, 1292, 1335, 1388, and 1490 cm^{-1}.


Washed By User #1
A
Saline - Control
Formaldehyde - 1 Wash
Formaldehyde - 2 Washes
Formaldehyde - 3 Washes
Saline
Formaldehyde - 1 Wash
Formaldehyde - 2 Washes
Formaldehyde - 3 Washes
PC1 = 72.8%
PC2 = 15.6%
PC4 = 4.3%
PC4
PC2
PC1
Intensity
Raman Shift (cm-1)


Washed By User #2
B
Saline - Control
Formaldehyde - 1 Wash
Formaldehyde - 2 Washes
Formaldehyde - 3 Washes
Saline - Control
Formaldehyde - 1 Wash
Formaldehyde - 2 Washes
Formaldehyde - 3 Washes
PC1 = 44.5%
PC2 = 26.3%
PC3 = 10%
PC3
PC2
PC1
Intensity
Raman Shift (cm-1)


Washed by User #1
C
PC1
PC2
PC4
Raman Shift (cm-1)

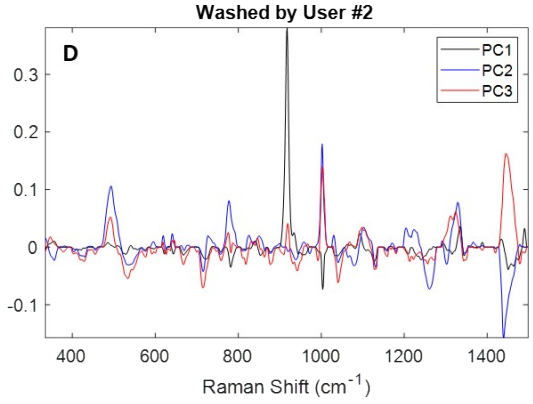

Washed by User #2
D
PC1
PC2
PC3
Raman Shift (cm-1)

Figure S16. Effects of multiple washing steps on formaldehyde-fixed spectra. Mean spectra of formaldehyde-fixed cells prepared by user #1 (**A**) or user #2 (**B**) both show differences from the saline control, with formaldehyde signals persisting throughout 3 washes. Multiple washes also display diminishing lipid peaks and intensity in the 515-540 cm^{-1} region, with the maximum loss of signal observed after 3 washes. Inset PCA score plots show separation from the saline control, as well as clustering of each formaldehyde wash step. Principal component loadings for user #1 (**C**) and user #2 (**D**) both show strong contributions from formaldehyde signals, notably 918 cm^{-1}, as well as contributions from lipid and protein signals.