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Evaluating Different Fixation Protocols for Spectral Cytopathology, Part 2: Cultured Cells

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Abstract

Spectral cytopathology (SCP) is a robust and reproducible diagnostic technique that employs infrared spectroscopy and multivariate statistical methods, such as principal component analysis to interrogate unstained cellular samples and discriminate changes on the biochemical level. In the past decade, SCP has taken considerable strides in its application for disease diagnosis. Cultured cell lines have proven to be useful model systems to provide detailed biological information to this field; however, the effects of sample fixation and storage of cultured cells are still not entirely understood in SCP. Conventional cytopathology utilizes fixation and staining methods that have been established and widely accepted for nearly a century and are focused on maintaining the morphology of a cell. Conversely, SCP practices must implement fixation protocols that preserve the sample's biochemical composition and maintain its spectral integrity so not to introduce spectral changes that may mask variance significant to disease. It is not only necessary to evaluate the effects on fixed exfoliated cells but also fixed cultured cells because although they are similar systems, they exhibit distinct differences. We report efforts to study the effects of fixation methodologies commonly used in traditional cytopathology and SCP including both fixed and unfixed routines applied to cultured HeLa cells, an adherent cervical cancer cell line. Data suggest parallel results to findings in Part 1 of this series for exfoliated cells, where the exposure time in fixative and duration of sample storage via desiccation contribute to minor spectral changes only. The results presented here reinforce observations from Part 1 indicating that changes induced by disease are much greater than changes observed as a result of alternate fixation methodologies. Principal component analysis of HeLa cells fixed via the same conditions and protocols as exfoliated cells (Part 1) yield nearly identical results. More importantly, the overall conclusion is that it is necessary that all samples subjected to comparative analysis should be prepared identically because although changes are minute, they are present.

Keywords

cytopathology; fixation; infrared (IR) spectroscopy; principal component analysis (PCA); spectral cytopathology (SCP)

INTRODUCTION

For the past decade, infrared (IR) micro-spectroscopy has climbed its way to being considered a competitive alternative to conventional cytopathology practices. Traditional cytopathology includes the inspection of stained cells, visually measuring predetermined parameters, such as nucleus-to-cytoplasm (N/C) ratio, staining patterns, morphology of nuclear membrane, etc., and assigning a diagnosis based on these parameters.^{1–2} IR micro-

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spectroscopy is at the forefront of new methods being developed because it is a label free and reproducible method that evaluates a physical measurement, the biochemical composition, of each unstained cell; the term 'spectral cytopathology (SCP)' has been coined to describe the combination of microscopic infrared data acquisition and analysis of the spectral data via multivariate methods.³⁻⁵ After IR acquisition, samples can then be subjected to traditional staining protocols and evaluated via conventional cytopathology means to compare results from both techniques. Since the early successes of SCP, many groups have begun investigating cultured cell lines to provide additional information regarding disease diagnosis and biological information.⁶⁻⁸

Cultured cells serve several purposes ranging from distinguishing between different cell lines to their behavior in understanding disease and evaluation of drug effects and uptake.⁸⁻¹⁰ Cultured cell lines offer a microscopic model system to explore and probe mechanisms, pathways, drug interactions, etc. Most importantly, diseased cells can be potentially biopsied from a patient's organ and propagated in cell culture conditions to be investigated thoroughly.^{8, 11} Often fixation procedures are applied to preserve cells for extended periods; however, the spectral effects of fixation on cultured cells are not entirely understood.¹² Previous reports claim fixation protocols introduce large spectral changes and obstruct proper analyses, speculating fixation methods as obstacles to be avoided.¹³⁻¹⁵

This is the second paper in a series aimed at addressing the effects of fixation and storage conditions on spectral data of cellular samples. In the first paper, we described the influence of these factors to exfoliated oral (buccal) mucosa cells and demonstrated that exceedingly small variances occurred upon various fixation methods that were negligible in comparison to disease induced changes.¹⁶ In the current study, we applied a similar experimental protocol to cultured HeLa cells to investigate sample fixation and storage induced contributions on cultured cells. Cultured cells provide additional caveats for fixation methods to potentially induce changes as they are grown in-house. Including a cultured cell line in this series provides a more extensive and thorough understanding of fixation effects from different cellular samples.

We illustrate that fixation methods, whether unfixed (dried) or chemically fixed (SurePath alcohol mixture or phosphate-buffered formalin solution), do produce minor spectral changes. However, results from both exfoliated and cultured cells parallel each other indicating that fixation methods act similarly regardless of cell type or sample origin.

MATERIALS AND METHODS

Cell Culture

HeLa cells, an adherent cervical cancer cell line, (ATCC, Manassas, VA) were cultured onto CaF₂ (25mm × 2 mm; Sigma-Aldrich, St. Louis, MO) windows using cell culture protocols that have been previously reported.⁸ [CaF₂ windows were utilized as cell culture substrates due to the incompatibility between aqueous environments and MirrIR (Kevley, Chesterland, OH) slides.¹⁷] After removing the windows from cell culture medium, supplemented with 10% fetal bovine serum (FBS; ATCC), cells were washed with phosphate buffer solution (PBS; ATCC). Subsequent to cell culture medium removal, unfixed (defined in the section "Sample Preparation Protocols" below) windows were thoroughly washed with PBS and de-ionized water and immediately desiccated. The remaining windows were submerged into 13 mm² sterilized square disposable polystyrene Petri dishes (Fisher Scientific, Pittsburg, PA) filled with fixative solution: SurePath (TriPath, Burlington, NC) or phosphate-buffered formalin (10 % buffered solution; Sigma-Aldrich). After appropriate fixative exposure (Table 1), windows were removed and subjected to a final washing step. The exact fixation protocols are described below in the section "Sample Preparation Protocols". A detailed

description of the fixatives used and their modes of action can be found in Part 1 of this series.¹⁶

Sample Preparation Protocols

Samples referred to in this paper as “unfixed” (dried) were washed with PBS and de-ionized water immediately following removal from cell culture medium, then quickly air-dried under a clean, dry air stream, and immediately desiccated for 24 h before the first data acquisition. The desiccated samples were sufficiently dried so that they did not contribute to the water vapor background of the spectra. Subsequently, these samples (A, E, and I, see Table 1) were stored in a desiccator for 24 h, or 1, 2, or 4 weeks, and rescanned at each time interval. These results elucidate spectral changes as a result of the degradation of unfixed and fixed for 24 h samples over time.

“Fixed” cultured cells were left in their respective fixative solution for various time periods: 24 h, or 1, 2 or 4 weeks. After these exposure times, cells were washed with de-ionized water, dried under a clean, dry air stream, and kept in a desiccator for 24 h until the first data acquisition. These results illustrate spectral changes due to extended exposure in the fixative solutions.

Spectral Data Acquisition and Data Analysis

Data were acquired using three Perkin Elmer (Shelton, CT) Spotlight 400 imaging infrared spectrometers in transmission mode. For each window, a 1 mm × 1 mm area was mapped at 4 cm⁻¹ resolution using a pixel size of 6.25 μm × 6.25 μm (~40 μm²) and with the co-addition of 16 interferograms per pixel. Instrumentation, instrumental parameters and data analysis procedures have been detailed in previous publications.¹⁸ One of these documented methods of analysis, the PapMap algorithm^{18–19}, was used to reconstruct a cellular spectrum from individual pixel spectra that make up a single cell; depending on the cell's size, between 5 and 100 pixels were co-added to reconstruct a cellular spectrum. Cultured HeLa cells typically measure 50 μm × 50 μm in size, but in some instances their pseudopods extend further resulting in the need to include additional pixels. Conversely, cells can be smaller depending on their stage in the cell cycle and the type of cell being investigated. For HeLa, 5 pixels were found to be the minimum required for one cell, whereas 9 was the minimum for exfoliated oral mucosa cells.

Data were pre-processed via a MATLAB based algorithm, noise adjusted principal component reconstruction (NA-PC)^{20–21}, to optimize the signal-to-noise (S/N) ratio of spectral data. This was addressed by calculating both noise and spectral covariance matrices separately and reconstructing the spectra from a reduced number of noise-adjusted principal components (30 PCs used for this experiment).²² In addition, data were adjusted for water vapor contributions using a scheme modeled in MATLAB.²³ Data were then min/max normalized to the amide I band, ca. 1650 cm⁻¹, and the second-derivative spectra were computed using a nine-point Savitzky-Golay smoothing function.²⁴ Principal component analysis (PCA)²⁵ was performed on mean centered data to evaluate the potential fixation-induced changes stated previously.

RESULTS

In this section, we present mean absorbance and second-derivative spectra and PCA scores plots of cultured HeLa cells to illustrate the effects of fixation based on the type of fixative, duration in fixative, and sample storage conditions post fixation. PCA scores plots illustrate variance among data sets where each symbol denotes one cellular spectrum to account for changes within the entire sample. Much like in Part 1 of this series, color legends remain

consistent where red symbols and spectra represent cells at 24 h, green at 1 week, black at 2 weeks, and blue at 4 weeks (a further description of the time points can be found in the section “Sample Preparation Protocols” in the Materials and Methods). In addition, all analyses were performed using the $1800 - 1000 \text{ cm}^{-1}$ spectral region, eliminating the C–H stretching region, to evaluate these results with data from Part 1 (extensively discussed in the section “Exfoliated Oral Mucosa Cells vs. Cultured HeLa Cells” in the Discussion) and previous reports from the laboratory.

Time Evolution of Unfixed Cell Spectra

It is necessary to first understand the time course study for unfixed (dried) cells in order to properly compare and contrast the effects of chemical fixations. As previously mentioned, these cells were cultured for a minimum of 24 h and after removal from cell culture medium, were immediately air dried and subsequently desiccated for an additional 24 h prior to the first data acquisition. The samples were desiccated between collection time points, and the same areas were re-scanned for each time point. The number of individual cells averaged for all four time points was 71 ± 2 . Figure 1A illustrates a superposition of the mean absorbance spectra for the unfixed cells recorded at 24 h, 1 week, 2 weeks, and 4 weeks.

There are no observed differences between the mean absorbance spectra among the four time points. This is indicated by the overlay shown in the inset (Figure 1A), where the spectra are nearly superimposable resulting in a correlation coefficient of 0.9927. This result suggests there are no major biochemical changes for an unfixed (dried) sample stored in a desiccator within 4 weeks of preparation.

As mentioned in Part 1 of this series, second-derivative spectra accentuate small changes, compared to absorbance spectra, where often times minor changes in absorbance spectra are resolved as individual bands in second-derivative representation. Accordingly, Figure 1B demonstrates the second-derivative spectra of the data in Figure 1A, and similarly spectra are nearly identical. A stack plot of the second-derivative data is shown in Figure 1C as well to aid in observing the individual spectra. Since both the mean absorbance and second-derivative spectra are nearly indistinguishable, we postulate that no gross biochemical changes occur within 1 month of preparing an unfixed (dried) sample, if the cellular samples are stored in a clean and dry environment. PCA was used to elucidate any spectral variances that may have been too subtle to observe upon initial visual inspection. The results of the PCA analysis are shown in Figure 2.

Shown in Figure 2A is the PCA scores plot of all second-derivative spectra of the unfixed (dried) cells illustrated in Figure 1, in the $1800 - 1000 \text{ cm}^{-1}$ spectral region. There is a small transition between the data points starting with the earliest time point (24 h) to the latest (4 weeks), differentiated along PC2 (Figure 2C). Inspection of loading vector PC1 (Figure 2B) constitutes the minor changes present in the scores plot to variance in the protein region, specifically the amide I band, ca. 1650 cm^{-1} in addition to a large change in the symmetric carboxylate stretching band, ca. 1400 cm^{-1} and symmetric $-\text{PO}_2^-$ stretching region, 1090 cm^{-1} . This change is also observed in PC2 in addition to contributions at the amide II band, ca. 1550 cm^{-1} . Nonetheless, the trend is minimal, considering that the axes in Figure 2A are scaled by 10^{-3} , indicating that these results are extremely small. This suggests that spectra of an air dried sample kept in a desiccator remain unchanged after 1 month.

Unfixed (dried) cells are typically not interrogated in conventional cytopathology because it is thought that morphological changes occur during the drying period and that the uptake of immunohistochemical stains is poor when cells are dried. Nevertheless, no morphological changes are apparent in the photomicrographs of unstained, dried cells captured at each time point during this experiment, demonstrated in Figure 3. Since these images are of unstained

cells, it is not completely understood how the immunohistochemical stains would react to prolonged dried cells, but these results do not show any morphological or spectral changes to be present in unfixed (dried) cells for 1 month post sample preparation.

Comparison of Fixed and Dried Cells (24 h Fixation)

In this section, we will compare results for unfixed (dried) cells and cells fixed with chemical fixation protocols discussed in the section “Sample Preparation Protocols” of the Materials and Methods. Specifically, we discuss samples that were fixed and unfixed (dried) for 24 h, subsequently desiccated, and monitored for 1 month (A, E, and I; see Table 1). The number of individual cellular spectra for SurePath-fixed and formalin-fixed cells were 24 ± 1 and 85 ± 7 , respectively. Illustrated in Figure 4, is a stack plot of the mean second-derivative spectra for cells subjected to the three different fixation protocols at each time point.

This plot shows two highlighted areas depicting changes that occur between the three methods of fixation, but overall, these spectra are exceedingly similar. The first change is primarily observed for the SurePath-fixed cells and presents as a small shoulder on the amide I band, ca. 1628 cm^{-1} . This band was also seen in the exfoliated oral mucosa cells discussed in Part 1 and is attributed to a minor, fixation-induced spectral change occurring only upon SurePath fixation (see below). Such a change in the amide I profile could be simply due to a conformational change toward higher β -sheet content induced by alcoholic solvents. [The effects of organic solvents on numerous proteins (i.e. myoglobin, apomyoglobin, hemoglobin, lysozyme and ribonuclease) have been studied using infrared spectroscopy indicating that specific solvent effects provoke the β -structures of proteins. The extent of which is dependent upon the hydrocarbon content of the alcohol, specifically: methanol > ethanol > propanol > butanol.²⁶] Alternatively, the 1630 cm^{-1} region in proteins is also indicative of precipitated proteins.¹⁰ The second difference is more prominently observed in the unfixed (dried) cell spectra as a band in the symmetric $\text{-PO}_2\text{-}$ stretching vibration; however, both of these changes remain consistent throughout each time point.

From our present understanding about the modes of action of each fixation method¹⁶, it is surprising how similar these spectra appear. Nonetheless, PCA is able to detect the small spectral changes between these three fixation methods (Figure 5).

Figure 5A illustrates that each fixation method clusters tightly and separately from each other illustrating exceptional inter-class variability and intra-class similarity. The loading vector for PC1 is displayed in Figure 5B and is accountable for the separation of SurePath-fixed cells from the formalin-fixed and unfixed (dried) cells. The strong peak on the low frequency amide I wing at ca. 1630 cm^{-1} demonstrates more clearly the aforementioned spectral changes due to Surepath fixation and indicates clearly the emergence of a new protein secondary structure (precipitated and/or higher in β -sheet contribution). It is interesting that this structure is found neither in the dried nor the formalin-fixed cells. Protein peaks in this wavenumber range have previously been identified in necrosis and apoptosis.¹⁰ PC1 attributes variance mainly to a shift in the amide I band, discussed previously regarding Figure 4. Loading vector PC2, shown in Figure 5C, separates the formalin-fixed and SurePath-fixed cells from the unfixed (dried) cells; the changes are mostly due to a splitting in the amide I peak components, as indicated by two derivative-shaped peaks in the amide I region at 1698 and 1670 cm^{-1} (see arrows), and a shift in the amide II band. Additionally, PC2 shows changes in the lower wavenumber region indicative of changes in the phosphate region and a broad band at ca. 1400 cm^{-1} , associated with the symmetric carboxylate stretching vibration which could indicate a protonation/deprotonation of the acid function amino acid glutamate and aspartate.^{27–28} These changes, although small,

are attributed to the modes of action each fixation method imposes on a cell; however, spectral changes within one method of fixation are minimal, and samples remain stable for the duration of at least 1 month once fixed. For that reason, one fixation protocol should be adopted and maintained for sample preparation protocols, eliminating any influence(s) due to fixation, and the particular fixation protocol suitable for the study must be evaluated prior to data analysis to ensure the appropriateness of the fixative to the type of specimen.

To further illustrate this point, we present one method of fixation, SurePath, monitored for 1 month where each area was rescanned and samples were desiccated between each time point (Figure 6). Similar results were observed for formalin-fixed cells (not shown). The PCA scores plot in Figure 6A depicts that samples do not show any spectral changes after 2 weeks, but when rescanned at 4 weeks, minor changes arise in the amide I and II bands of PC1. Moreover, sharp bands are present in both loading vectors PC1 and PC2 (Figure 6C) between 1600 cm^{-1} and 1400 cm^{-1} , which are indicative of the rotational-vibrational spectral bands of atmospheric water. Since atmospheric water is a main contributor to the variance observed in the scores plot, we postulate that the minor spectral changes are negligible. Furthermore, the photomicrographs illustrated in Figure 6D indicate no morphological changes present over the course of 1 month.

Time Evolution of Cells Treated with Varying Fixation Exposure Times

Presented in this section are results of cells exposed to fixative solution (SurePath and phosphate-buffered formalin) for prolonged periods of time: 24 h, 1 week, 2 weeks, and 4 weeks. The number of individual cellular spectra for each fixation method were 30 ± 7 and 59 ± 10 for SurePath-fixed and formalin-fixed cells (Figures 7 and 8), respectively. Much like the results in Part 1 of this series, both figures depict a change in the background below 1400 cm^{-1} , in addition to a broad, weak peak at 1090 cm^{-1} . These differences disappear in the second-derivative spectra for both fixation methods and are not consistent with a specific collection time; therefore, we postulate this change to be due to a variation in the substrate material in contact with the fixative. Furthermore, both results illustrate very similar absorbance spectra with very little intra- and inter-class variations, indicated by the correlation coefficients, 0.9961 and 0.9934, for SurePath and formalin fixations, respectively.

The main difference between the two fixation methods is shown in the second-derivative spectra of the SurePath-fixed cells. As indicated previously in Figure 4, there is a band in the amide I shoulder, ca. 1628 cm^{-1} (arrow). The band intensity change observed at the 24 h time point demonstrating the greatest absorbance intensity in comparison to 4 weeks having the least. In addition, the intensity altered in the dip between the amide I and amide II bands of the absorbance spectra but is more pronounced in the formalin spectra. These results reinforce those of Part 1 and are consistent with results described previously in this paper.

As previously mentioned, the mean absorbance and second-derivative spectra are very similar among unfixed (dried) cells or chemically fixed cells (SurePath or phosphate-buffered formalin). Differences between each time-point are nearly indistinguishable, indicating that fixation induced changes are minimal regardless of the fixation method or exposure time to fixative. Whether samples are unfixed (dried) or chemically fixed (SurePath or phosphate-buffered formalin), samples remain stable for at least 1 month despite the length of time in fixative or desiccator.

DISCUSSION

Exfoliated Oral Mucosa Cells vs Cultured HeLa Cells

Given that cultured cells exhibit weaker spectra, which are subject to slight changes with the cells' status in the cell cycle, we find that their response to fixation protocols is very similar to the response exhibited by exfoliated cells. Band shifts, peak positions, and PCA loading contributions in some cases are identical and in others exceedingly similar. The PCA scores plot depicted in Figure 9A illustrates the fixative data of exfoliated oral mucosa cells (Part 1, circles) compared to the fixative data presented thus far (squares), where red denotes unfixed, green: SurePath-, and blue: formalin-fixed cell spectra. In contrast to Figure 5, all cells from each fixation length are included in Figure 9 whereas Figure 5 only includes cells fixed for 24 h but monitored for the entire month. Loading vector PC1 (Figure 9B) differentiates each cell type based on the contribution from the amide I band with exfoliated cells laying to the left and cultured HeLa cells to the right of the ordinate. Interestingly, loading vector PC2 (Figure 9C) discriminates the SurePath-fixed exfoliated cells from the other fixation methods for exfoliated cells, but for cultured cells, PC2 describes mainly the variance amongst the amide I shoulder as described previously, ca. 1628 cm^{-1} . For the cultured cells, both SurePath- and formalin-fixed cells occur on both sides of the ordinate y-axis in the scores plot; however, this is not due to the presence of the exfoliated cells. This same separation was demonstrated when all cultured cells were subjected to PCA alone, without the presence of the exfoliated cells (not shown). In this plot, a similar separation was noted where the SurePath- and formalin-fixed cells appeared on both sides of the ordinate y-axis. The separation was due to fixation length where cells fixed for 24 h clustered with the unfixed cells, and the cells from the remaining fixative time points clustered away from the unfixed cells. After a detailed inspection of both SurePath- and formalin-fixed cultured cells individually using PCA, the differences noted were also scaled to 10^{-3} as previous plots and were mainly attributed to water vapor contributions. Therefore, since this same pattern was seen when exfoliated cells were included, PC2 in Figure 9C distinguishes cultured cells fixed with both SurePath and formalin for longer than 24 h as indicated in Figure 9A; however, these changes are due to smaller differences not demonstrated in the loading vector.

These observations truly evaluate the biochemical composition of the cells and changes therein rather than morphological features between each cell type. This plot confirms that fixation-induced changes are extremely minimal, and if one method of fixation is maintained throughout a study, these changes will not introduce additional variance. Previous discussions attributing fixation protocols as the cause of large spectral changes¹³ were, in all likelihood, due to morphological changes and/or scattering effects that often lead to major spectral changes and mask true spectral profiles.

CONCLUSIONS

Fixation protocols, for both unfixed (dried) and chemically fixed cells, have been shown to maintain the morphology and spectral integrity of both exfoliated and cultured cells, where small variances were observed depending on the duration in fixative and/or desiccator. However, the changes observed were truly variance within the biochemical composition of the cells. One method should be maintained throughout an entire study and samples must be kept in a dry environment between collection times to avoid major changes. Ultimately, fixation-induced changes are negligible when compared to disease-induced effects (Part 1) or different cell types (Part 2), and spectral characteristics are comparable for both exfoliated and cultured cell samples.

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REFERENCES

1. Demay, RM. The Pap Test. Chicago: ASCP (American Society for Clinical Pathology) Press; 2005.
2. Ludlow, EB.; Ashfaq, R.; Hoda, RS.; Kaminsky, DB.; Lightfoot, SA.; Linder, J.; McKee, GT.; Pereira, TC.; Pisharodi, L.; Silverman, JF.; Smith, RA.; Wang, HH.; Weir, MM. ThinPrep® Non-Gyn Morphology Reference Atlas. Marlborough, MA: Cytoc Corp.; 2005.
3. Papamarkakis K, Bird B, Schubert JM, Miljkovic M, Wein R, Bedrossian K, Laver N, Diem M. Lab. Invest. 2010; 90(4):589–598. [PubMed: 20142808]
4. Schubert JM, Bird B, Papamarkakis K, Miljkovic M, Bedrossian K, Laver N, Diem M. Lab. Invest. 2010; 90(7):1068–1077. [PubMed: 20368702]
5. Romeo M, Mohlenhoff B, Jennings M, Diem M. Biochim. Biophys. Acta. 2006; 1758(7):915–922. [PubMed: 16797481]
6. Boydston-White S, Romeo M, Chernenko T, Regina A, Miljkovic M, Diem M. Biochim. Biophys. Acta. 2006; 1758(7):908–914. [PubMed: 16792999]
7. Pacifico A, Chiriboga LA, Lasch P, Diem M. Vib. Spectrosc. 2003; 32:107–115.
8. Marcsisin EJ, Uttero CM, Miljkovic M, Diem M. Analyst. 2010; 135(12):3227–3232. [PubMed: 20981390]
9. Boydston-White S, Chernenko T, Regina A, Miljkovic M, Matthaus C, Diem M. Vib. Spectrosc. 2005; 38(1–2):169–177.
10. Jamin N, Miller L, Moncuit J, Fridman WH, Dumas P, Teillaud JL. Biopolymers. 2003; 72(5): 366–373. [PubMed: 12949827]
11. Moss DA, Keese M, Pepperkok R. Vib. Spectrosc. 2005; 38(1–2):185–191.
12. Krishna CM, Sockalingum GD, Vadhira BM, Maheedhar K, Rao AC, Rao L, Venteo L, Pluot M, Fernandes DJ, Vidyasagar MS, Kartha VB, Manfait M. Biopolymers. 2007; 85(3):214–221. [PubMed: 17103423]
13. Mantsch H, Jackson M. J. Mol. Struct. 1995; 347:187–206.
14. Mariani MM, Lampen P, Popp J, Wood BR, Deckert V. Analyst. 2009; 134(6):1154–1161. [PubMed: 19475142]
15. Gazi E, Dwyer J, Lockyer NP, Miyan J, Gardner P, Hart C, Brown M, Clarke NW. Biopolymers. 2005; 77(1):18–30. [PubMed: 15558657]
16. Mazur AI, Marcsisin EJ, Bird B, Miljkovic M, Diem M. Anal. Chem. 2012; 84(3):1259–1266. [PubMed: 22103764]
17. Marcsisin, EJ. Infrared Spectroscopy to Monitor Drug Response of Individual Live Cells. Boston: Northeastern University; 2012.
18. Schubert JM, Mazur AI, Bird B, Miljkovic M, Diem M. J. Biophotonics. 2010; 3(8–9):588–596. [PubMed: 20449833]
19. Diem, M.; Miljkovic, M.; Romeo, M.; Bird, B.; Schubert, JM. Method of Reconstituting Cellular Spectra from Spectral Mapping Data. U. S. Patent. 20110142324. 2009 May 29.
20. Green AA, Berman M, Switzer P, Craig MD. IEEE Trans. Geosci. Remote Sens. 1988; 26(1):65–74.
21. Reddy RK, Bhargava R. Analyst. 2010; 135:2818–2825. [PubMed: 20830324]
22. Marcsisin EJ, Uttero CM, Mazur AI, Miljkovic M, Bird B, Diem M. Analyst. 2012; 137:2958–2964. [PubMed: 22377958]
23. Bruun SW, Kohler A, Adt I, Sockalingum GD, Manfait M, Martens H. Appl. Spectrosc. 2006; 60(9):1029–1039. [PubMed: 17002829]
24. Savitzky A, Golay MJE. Anal. Chem. 1964; 36(8):1627–1639.
25. Adams, MJ. Chemometrics in Analytical Spectroscopy. 2nd ed.. Cambridge U.K.: Royal Society of Chemistry; 2004.

26. Jacobson AL, Krueger P. J. Biochim. Biophys. Acta. 1975; 393(2):274–283.
27. Barth A. Biochim. Biophys. Acta. 2007; 1767(9):1073–1101. [PubMed: 17692815]
28. Dioumaev AK. Biochemistry-Moscow+. 2001; 66(11):1269–1276. [PubMed: 11743871]

Figure 1. (A) Stacked mean absorbance spectra (overlay shown in the inset), (B) overlay mean second-derivative spectra (nine-point Savitzky-Golay smoothing window), and (C) stacked second-derivative spectra of unfixed (dried) cells 24 h (red), 1 week (green), 2 weeks (black), and 4 weeks (blue) after sample preparation.

Figure 2.

(A) PCA scores plot of unfixed (dried) cells 24 h (red), 1 week (green), 2 weeks (black), and 4 weeks (blue) after sample preparation. (B) Loading vectors PC1 and (C) PC2.

Figure 3. Photomicrographs of (top) unfixed (dried), (middle) SurePath- and (bottom) formalin-fixed cells 24 h (red), 1 week (green), 2 weeks (black), and 4 weeks (blue) after sample preparation.

Figure 4. Mean second-derivative spectra (nine-point Savitzky-Golay second-derivative window) for cells fixed and unfixed 24 h (red), 1 week (green), 2 weeks (black), and 4 weeks (blue) after sample fixation of 24 h.

Figure 5.

(A) PCA scores plot of cells fixed (24 h) by three different methods and monitored for 1 month: unfixed (dried; red), SurePath- (green) and formalin-fixed (blue). Corresponding loading vectors (B) PC1 and (C) PC2.

Figure 6.

(A) PCA scores plot of cultured HeLa cells fixed for 24 h in SurePath and desiccated between each time point (24 h, 1 week, 2 weeks, and 4 weeks). (B, C) Corresponding loading vectors PC1 and PC2, respectively. (D) Photomicrographs of one cell monitored at each time point chronologically from top to bottom.

Figure 7. Mean (A) absorbance (overlay shown in the inset) and (B) second-derivative spectra of cultured HeLa cells fixed in SurePath for 24 h (red), 1 week (green), 2 weeks (black), and 4 weeks (blue).

Figure 8. Mean (A) absorbance (overlay shown in the inset) and (B) second-derivative spectra of cultured HeLa cells fixed in formalin for 24 h (red), 1 week (green), 2 weeks (black), and 4 weeks (blue).

Figure 9.

(A) PCA scores plot and loading vectors (B) PC1 and (C) PC2 of exfoliated oral mucosa cells discussed in Part 1 (circles) and cultured HeLa cells discussed thus far (squares) fixed by each method: unfixed (red symbols), SurePath (green), and formalin (blue).

Table 1Experimental Design Including Fixatives and Exposure Times for Cultured Cellular Specimens^a

fixative	HeLa cells (adherent cervical cancer cell line), cultured			
	24 h exposure time	1 week exposure time	2 week exposure time	4 week exposure time
SurePath	A	B	C	D
formalin	E	F	G	H
unfixed	I			

^aFixed samples: HeLa cells were cultured onto CaF₂ windows, then removed from cell culture medium, rinsed with de-ionized water, and then submerged in the appropriate fixative for their allowed exposure time. At the end of each exposure time, windows with cultured cells were washed with de-ionized water, air-dried under a clean, dry air stream, and then desiccated for 24 h before data acquisition. Unfixed sample (I): The CaF₂ window was removed from cell culture medium, washed with PBS and de-ionized water, air-dried, and then desiccated. Windows exposed to fixative for 24 h (A, E), including the unfixed sample (I), were desiccated after the first data collection (24 h), and data were re-collected for each time point thereafter.