Comparing IHC, IR, and RAMAN Spectroscopy for Classification of Tissue and Cell Lines Containing Different Level of HER2 Protein Expression

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Abstract

Tissue of breast cancer patients is classified based on HER2 protein expression to decide on eligibility of Herceptin treatment. HER2 expression is measured by IHC technology using antibodies, chemistry based detection systems, and scoring with a microscope or using an imaging system for quantification. IR and Raman spectroscopy can identify molecular level changes in protein environment. In recent years, significant efforts have gone into exploring the potential of IR and Raman imaging for biomedical screening as these complementary methods can differentiate abnormal proteins from normal ones. Aforementioned vibrational microspectroscopic methods require minimal sample processing, unlike the traditional methods used today. The goal of this investigation is to determine if the classification based on IHC can be replicated with spectroscopic methods. Serial sections of fixed and FFPE tissues and cell lines are quantitatively analyzed by all three methods.

Introduction

Vibrational spectroscopy methods, including Fourier transform infrared spectroscopy (FT-IR) and Fourier transform Raman spectroscopy, have been used to quantitatively analyze molecular concentrations within liquid-based biological samples (e.g. blood & urine), differentiate pathogenic tissues from normal surrounding tissue, classify subtypes of cancer, and identify specific biomarkers within a tissue (1, 9). In FT-IR, a biological sample absorbs infrared light, causing covalent bonds within said biological sample to vibrate at frequencies related to bond strength within that particular functional group(9). The intensity of observed bands tells us information about the quantity of particular functional groups, and the frequencies correlates to the chemical structure and local environment of that functional group(9). With Raman spectroscopy, biological samples are exposed to monochromatic excitation source (LASER), in near IR to ultraviolet frequency range; upon photon exposure, a bond within the biological sample becomes excited to a virtual state. When it returns to its original energy level, a photon is emitted at a different frequency; this difference in frequency between the excitation and emitted energy of the photon can be measured and is referred to as Raman shift. Measurement of the Raman shift of molecules provides us information about its quantity and structure complementary to IR spectroscopy. Taken together, these methods create a biological "finger print" or spectrum that is unique to each molecule. In so doing, FT-IR and FT-Raman allow us to identify and measure biological molecules without altering their pre-analytical state. The true value of FT-IR and Raman in histopathology comes in to play when we apply them to the problem of standardization of the pre-analytical phase of immunohistochemistry (IHC). It has been well-studied and documented in the literature that an accurate IHC outcome depends upon several pre-analytical inputs (13). One of the inputs that have been proposed for an area of standardization is tissue fixation (13). Not only type of fixative, but also time of exposure and volume ratios are to be controlled & documented (13). Standardization for biospecimen fixation is an important feature for success in IHC. Yet it seems due time to identify a method, or series of methods, that can be used to identify and quantify what is meant by "complete fixation" and further assure us that we can rely upon the final IHC result.

Objective

With accuracy and reliability of immunohistochemical expression of specific biomolecules of histological samples becoming increasingly critical to pathology, it seems appropriate to explore methods to standardize and validate biomarker presence and conformation. Since FTIR and Raman can provide detailed information about biomolecules, including the secondary structure of proteins, these methods appear to lend themselves to defining the pre-analytical phase. This study utilizes FTIR and Raman techniques to characterize the Her-2/*neu* (c-erbB2) tyrosine kinase after fixation with 10% neutral buffered formalin / traditional processing and the Thermo Scientific Richard-Allan Scientific Fast Flex system.

Materials & Methods

Wet tissue samples were comprised of sister sections collected in either Thermo Scientific 10% neutral buffered formalin (NBF) or Fast Flex™ Holding Solution. The tissues fixed in 10% NBF were processed using a traditional set of chemicals and a traditional, overnight, 12 hour processing protocol on the Miles Scientific VIP 1000 tissue processor. The samples collected in Fast Flex Holding Solution were processed using the Fast Flex system and either the Thermo Scientific STP 420ES or Thermo Scientific Pathcentre® tissue processors.

The immunohistochemical reagents internally utilized in this study were Thermo Scientific anti-Her-2/*neu* rabbit monoclonal antibody clone SP3 and Thermo Scientific UltraVision Quanto HRP DAB advanced polymer detection kit.

Thermo Scientific Nicolet iN10MX equipped with a single point mercury cadmium telluride (MCT) detector and Thermo Scientific DXR micro Raman spectrometer equipped with 780 nm LASER were used for the analysis. iN10MX results shown were acquired using ultrafast mapping mode where interferometer scanning velocity is optimized for fast screening. Aperture size, i.e. IR beam size, was 100 x 100 mm with step size of 100 mm. Spectral resolution of 16 cm^{-1} was used.

FIGURE 1. Breast tissue case S09-11 stained with Her-2/*neu* IHC, using traditional IHC methods. 1a Fixation with 10%NBF & traditional processing. 1b. Fixation with Fast Flex Holding Solution & Fast Flex processing system 1c Positive control (1+) from cell culture. 1d Positive control (+2) from cell culture. 1e Positive control (+3) from cell culture.

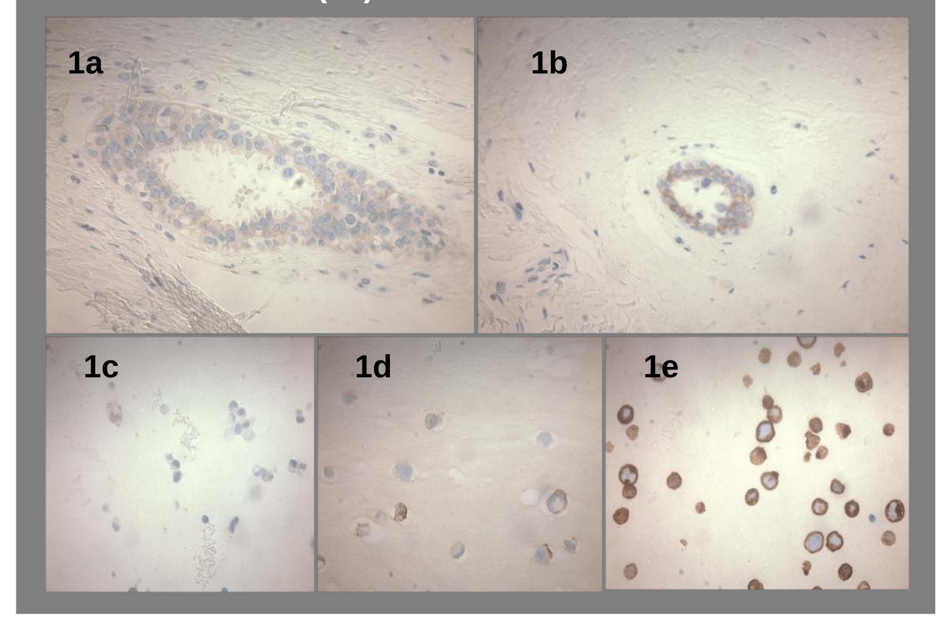


FIGURE 2. Breast tissue case S09-11 stained with Her-2/*neu* IHC, using Thermo Scientific anti-Her-2/*neu* rabbit monoclonal antibody clone SP3 and the UltraVision Quanto HRP DAB detection kit . 2a Fixation with 10%NBF & traditional processing. 2b Fixation with Fast Flex Holding Solution & Fast Flex processing system 2c Positive control (1+) from TMA. 2d Positive control (+2) from TMA. 2e Positive control (+3) from TMA.

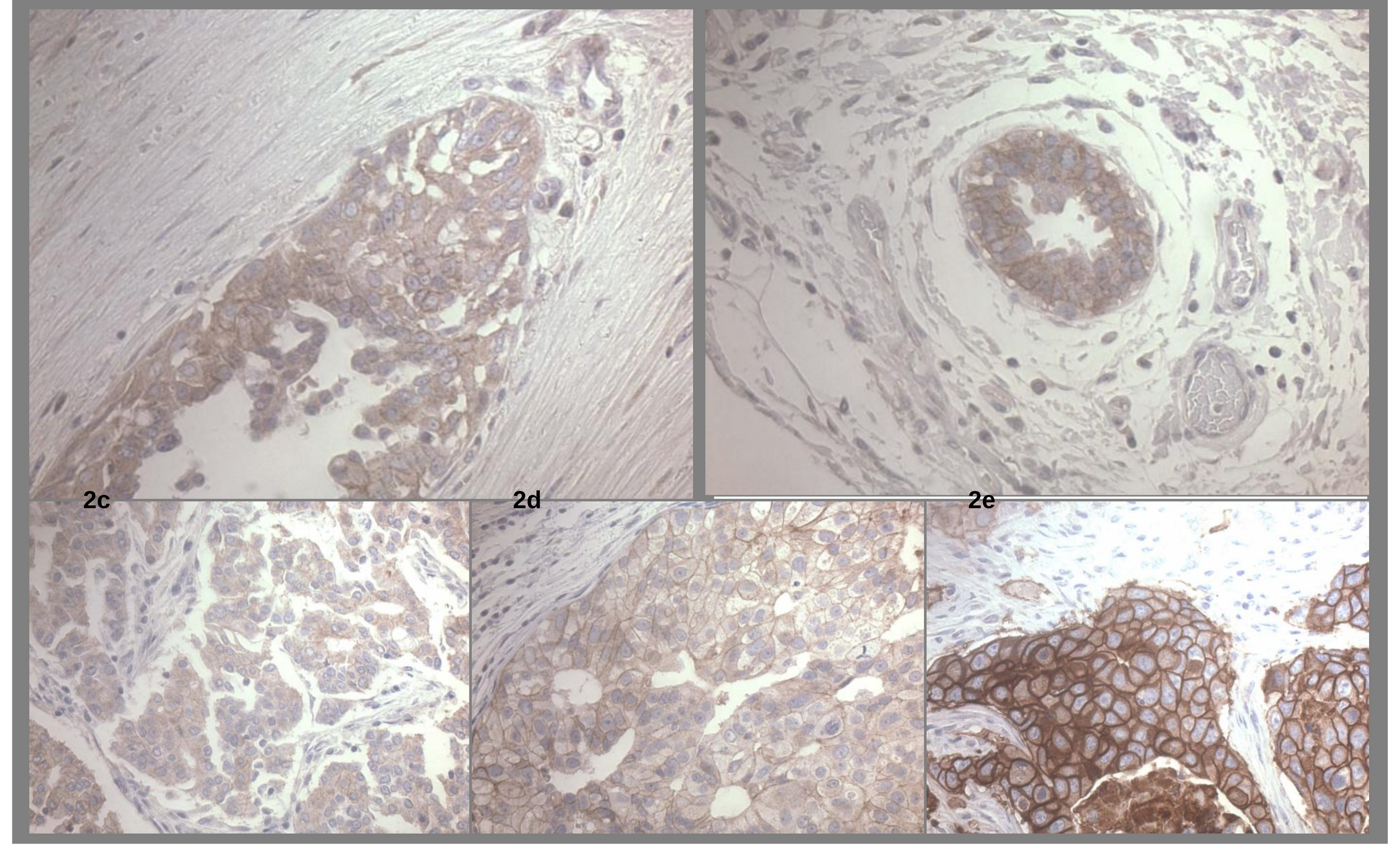


FIGURE 3. FTIR spectroscopy of tissue sample S09-11, fixed with 10% NBF & processed traditionally. 3a1-2. FTIR images showing distributions of protein (amide I) signal in 2 and 3D. 3b. Visible image showing morphology. 3c. IR spectrum of breast tissue with Her-2/*neu* protein. 3d IR spectrum of paraffin

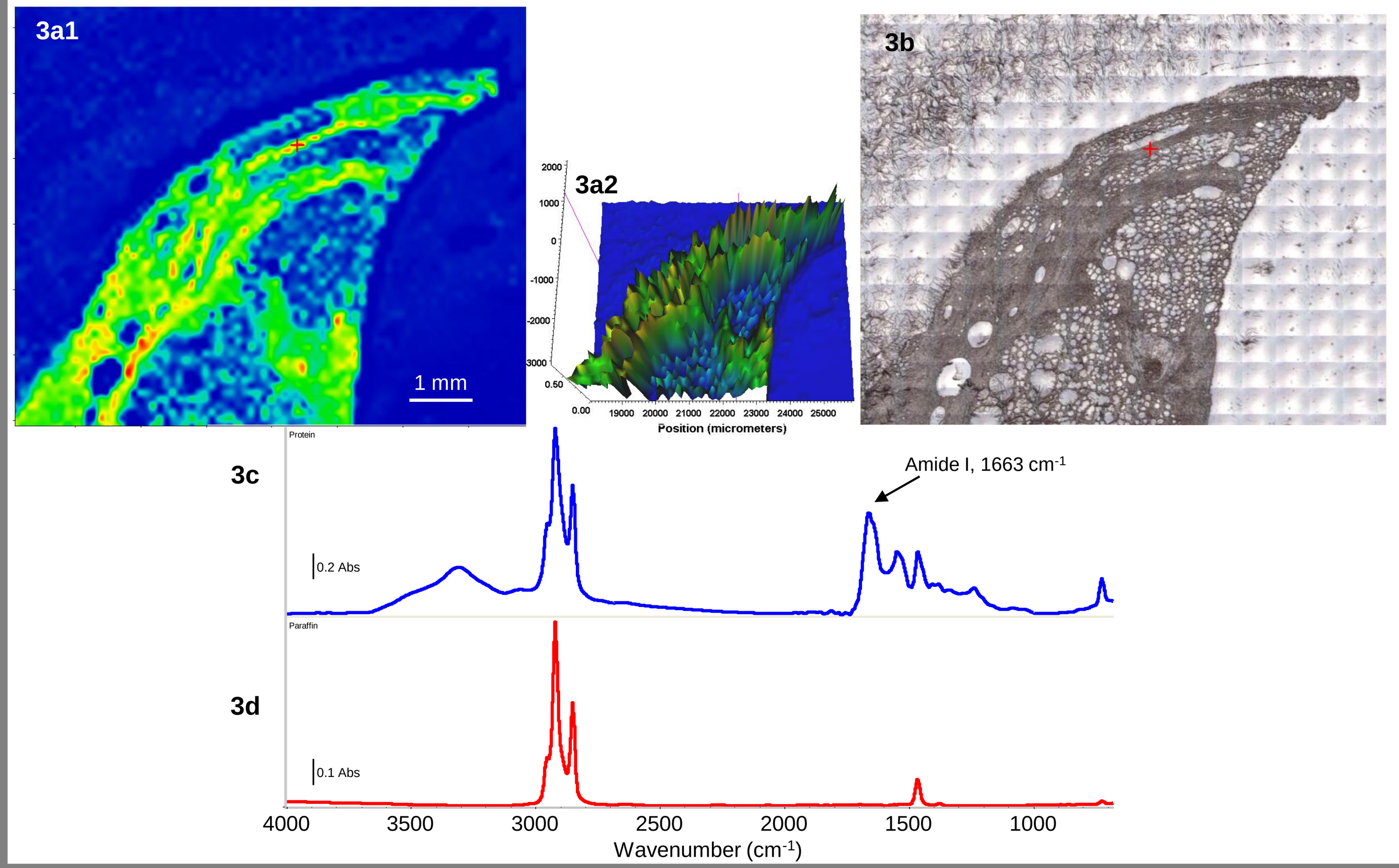


FIGURE 4. FTIR spectroscopy of tissue sample S09-11, fixed with Fast Flex Holding Solution and processed using the Fast Flex system. 4a1-2. FT-IR images showing protein distribution in 2 and 3D. 4b. Visible image showing morphology. 4c. FTIR spectrum of tissue with Her-2/*neu* protein. 4d FTIR spectrum of paraffin fingerprint alone.

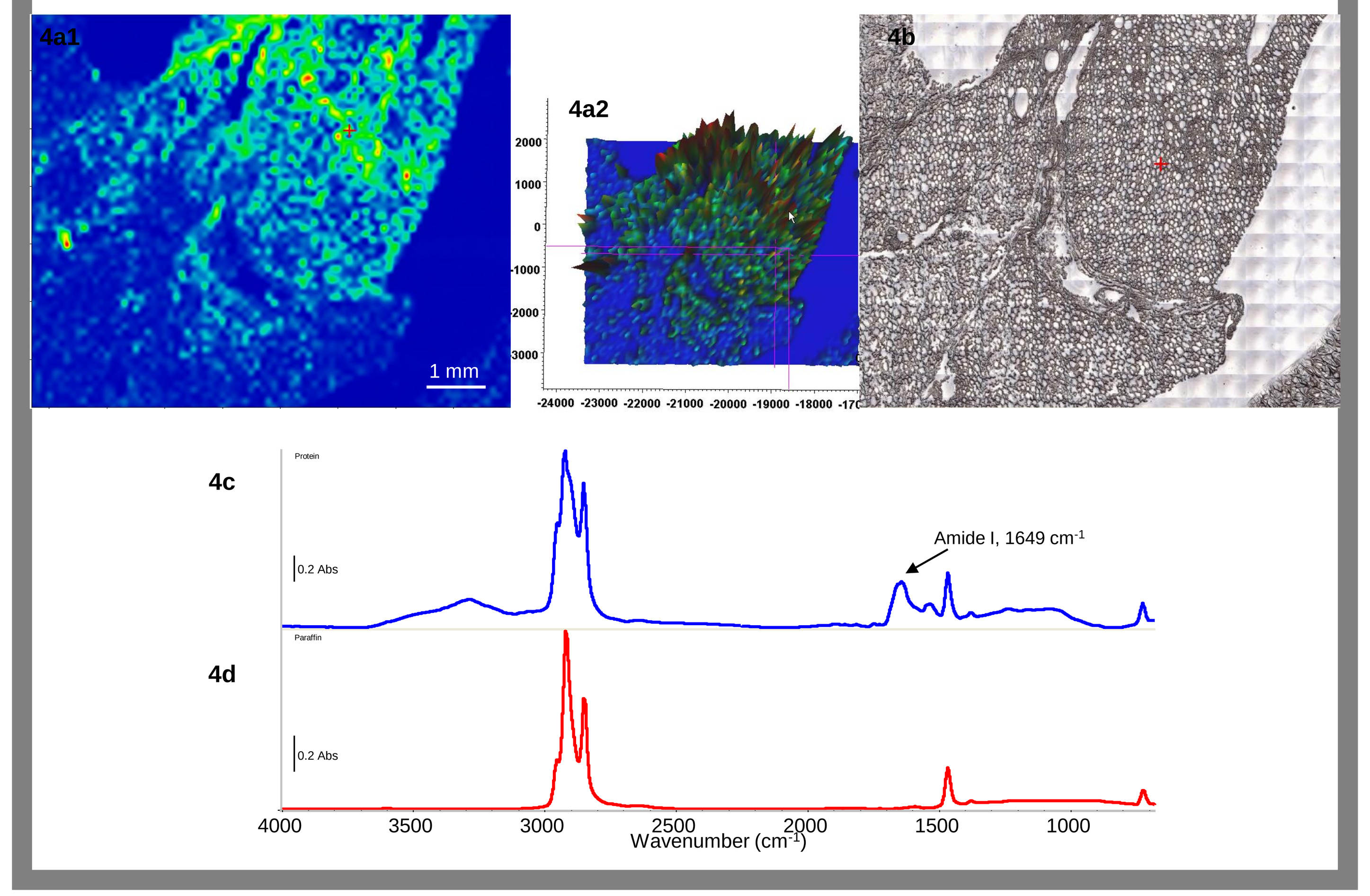
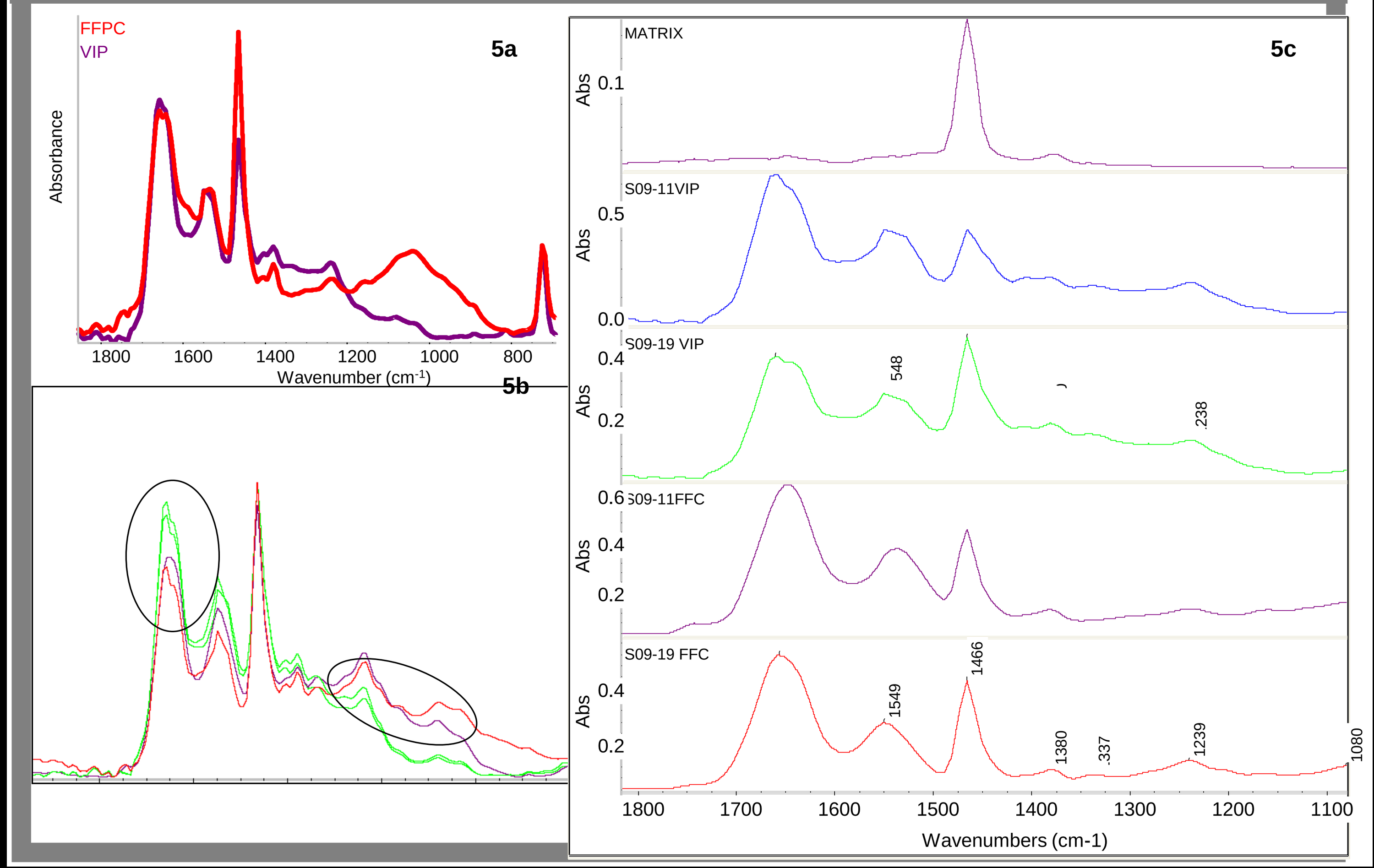


FIGURE 5 Finger print regions of FTIR spectra 5a comparing between 10% NBF / traditional processing (VIP, purple line) and Fast Flex (Pathcentre, red line). 5b comparisons of four tissues S09-11 & S09-19 VIP (green) and S09-11 & S09-19 Fast Flex (red / purple). 5c FTIR comparisons, separated by tissue / process.



Results

Figure 1 illustrates the Her-2/*neu* expression of sister sections from sample S09-11 after being fixed with 10% NBF and processed traditionally (1a) and after being fixed & processed using Fast Flex (1b). Micrographs 1c through 1e illustrate the positive cell culture controls 1+, 2+, and 3+ respectively. In both cases the IHC protocol was performed by an outside reference laboratory, using standard & industry-accepted methods. The expression of section 1a, fixed with 10% NBF & processed traditionally, more closely resembles a 1+ whereas the expression seen in its sister section, processed by Fast Flex, more closely resembles a score of 2+. Figure 2 illustrates the Her-2/*neu* expression of sister sections from the same sample after being fixed with 10% NBF (2a) and after being fixed & processed with Fast Flex (2b). Micrographs 2c through 2e illustrate the positive tissue microarray (TMA) controls 1+, 2+, and 3+ respectively. In both cases, the IHC protocol was performed in-house using anti-Her-2/*neu* rabbit monoclonal antibody clone SP3 and the UltraVision Quanto HRP DAB detection system. The expression of sections 2a and 2b are comparable now, both appearing to closely represent the staining of the 2+ TMA control (2d).

The FTIR fingerprint of S09-11 fixed in 10% NBF and processed traditionally can be seen in figure 3. The FTIR fingerprint of S09-11 fixed in Fast Flex Holding Solution and processed using Fast Flex reagents is illustrated in figure 4. Note the fingerprints of the paraffin wax, 3d and 4d, have not been subtracted from the tissue fingerprints, 3c and 4c respectively. The fingerprints are juxtaposed and compared in Figure 5. There is a small difference in the FTIR finger print as well as relative area of νOH band between Fast Flex processing and 10% NBF/traditional (VIP) processing. A broad band at ~1000 cm^{-1} is present for samples fixed by Fast Flex. A small feature at 1407 cm^{-1} is present for samples fixed by VIP. This appears to be related to greater dehydration of the samples processed via Fast Flex.

The Raman spectroscopic data could not be collected due to interference with the embedding medium. Additional studies must be performed to optimize the sample preparation techniques prior to Raman microspectroscopy.

Discussion

Although there is a small difference in the FTIR fingerprint of between the fixative/processing techniques, it does not appear to translate into a difference in protein expression via IHC. This points to the sensitivity of FTIR to molecular changes. While the subtle changes between the two processing methods may or may not be detected using IHC, FTIR picks it up repeatedly and reliably. These studies point to FTIR as a potential method for 1) providing quantifiable information about the fixation and processing quality of tissue samples 2) detecting the smallest of changes in biomolecules due to disease and 3) detecting the smallest of changes in biomolecules due to different forms of fixation.

Conclusions

Her 2 *neu* expression by IHC is improved with Fast Flex when using traditional IHC staining methodology, and is comparable between Fast Flex Processing & 10% NBF / traditional processing when using anti-Her-2/neu rabbit monoclonal antibody clone SP3 and the UltraVision Quanto HRP DAB detection system. There is a small difference in the FTIR finger print as well as relative area of νOH band between Fast Flex processing and 10% NBF/traditional (VIP) processing. A broad band at ~1000 cm^{-1} is present for samples fixed by Fast Flex. A small feature at 1407 cm^{-1} is present for samples fixed by VIP. The difference in finger print does not appear to directly correspond to signal sensitivity between fixation/processing types, as the IHC expression appears to be the same. It would be advantageous to conduct additional studies to include more tissues, as well as a method for understanding the FTIR fingerprint of Her-2/*neu* as a native protein, and the FTIR fingerprint of tissues after epitope retrieval methods.

References

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