Combining vibrational and fluorescence microscopies with mass spectrometry imaging for visualization of drug delivery

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ABSTRACT

Recent advances in label-free chemical imaging approaches have yielded new methods for measuring drug distribution in biological tissues. Techniques include vibrational spectroscopies in addition to a range of powerful mass spectrometry imaging methods. These techniques offer complementary information, with different strengths and limitations. By combining datasets from several of these techniques using image registration, powerful visualization of cells and tissues can be achieved, enabling high chemical specificity and sensitivity with sub-micron spatial resolution. Here we present several correlative imaging examples that combine vibrational spectroscopic with mass spectrometric imaging, in addition to single and multiphoton fluorescence. This multimodal approach offers optimal visualization of biological structure, in addition to the measured drug distribution.

Keywords: Correlative imaging, SRS microscopy, fluorescence microscopy, mass spectrometry imaging, SIMS.

1. INTRODUCTION

Fluorescence microscopy is ubiquitous in life sciences research, providing powerful subcellular visualization. However, the requirement for a fluorophore can be restrictive when the molecules of interest are not inherently fluorescent. Conjugation of a probe introduces uncertainty due to the impact of the dye molecule on the system under study, potential for deconjugation and photobleaching. Therefore, for certain applications, the use of label-free approaches is preferred.

Raman spectroscopy (RS) provides chemical imaging based on molecular vibrations and has been widely used to interrogate drug delivery[1, 2]. However, to generate high resolution images, the data acquisition time is very long, potentially compromising data integrity. Stimulated Raman scattering (SRS) microscopy overcomes this problem by using two lasers, with their frequency difference tuned to match a vibrational mode of interest. This facilitates much more rapid image acquisition at speeds approaching video rate[3]. In addition, SRS can be performed simultaneously to other optical methods, such as second harmonic generation (SHG) and two-photon fluorescence microscopies (TPEF) to image connective tissues[4]. Nevertheless, SRS can lack sensitivity for many physiologically relevant drug concentrations.

Mass spectrometry imaging (MSI) provides label-free chemical analysis and includes a range of both ambient and high vacuum techniques[5]. Secondary ion mass spectrometry (SIMS), for example, utilizes a focused ion beam to sputter the molecules that compose the sample outermost surface. Sputtered molecules are then extracted and identified through a mass analyzer with a time-of-flight (ToF) or Orbitrap analyzer[6]. SIMS offers high-sensitivity chemical analysis with a lateral resolution comparable to optical microscopies.

The aforementioned techniques are powerful when applied individually, but they perform differently across important metrics such as chemical sensitivity, specificity, and spatial resolution. It is advantageous, therefore, to combine the complementary information they offer[7].

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Visualizing and Quantifying Drug Distribution in Tissue IX, edited by Kin Foong Chan, Conor L. Evans, Proc. of SPIE Vol. 13315, 1331505 · © 2025 SPIE
1605-7422 · doi: 10.1117/12.3043629

2. METHODOLOGY

2.1 Sample preparation

When preparing samples for correlative imaging by multiple techniques sequentially, sample preparation can be particularly challenging owing to the pooled compatibility requirements. In particular, the choice of substrate is key. For optical methods operated in transmission, glass is usually required, and for multiphoton techniques such as SRS microscopy, thin glass (#1.5 coverslips) is preferred due to the short working distances of the lenses. Whereas for confocal Raman spectroscopy, calcium fluoride or reflective substrates such as silicon wafer may be preferred, depending on the setup. MSI methods also have different requirements depending on the nature of the sampling, with some requiring conductive substrates.

Consideration must also be given to the impact of probes and media used on other techniques, for example fluorescent dye molecules are highly absorbing and can lead to spurious signals in SRS microscopy. Typical mounting media and embedding resins can negatively impact molecular imaging methods including Raman and MSI.

Prior to beginning any measurements, the order of analysis must be carefully planned: it is usually best to perform fluorescent methods first to minimize photobleaching. If the analysis is truly correlative, the destructive technique evidently must be performed last. However sometimes flexibility exists if different methods are analyzing different faces of the sample, e.g. mass spectrometry analysis might be performed on the top face of a tissue section, with optical microscopy acquired on the bottom face, e.g. if using an inverted microscope. When combining ambient with high vacuum methods, the impact of exposure to ultra low pressures also needs to be considered and where possible, minimized, to aid eventual image registration.

2.2 Locating the same region of interest on different instruments

Several approaches are available to locate the same region of interest of a sample when moved between instruments for sequential analysis. Combining different measurement modalities within the same device offers a clear advantage; however, this is not always practical or even possible. Tissue sections with distinctive shapes or morphological features are easier to locate, but differences in instrument contrast can obfuscate them, and it is therefore helpful to acquire a low-resolution survey image of the whole tissue, to verify positioning. Homogenous tissues and cell cultures present a greater challenge, and thus the use of gridded coverslips or fiducial markers are recommended.

2.3 Image registration

There are many potential methods to perform image registration, including feature-based methods e.g. algorithms such as scale-invariant feature transform (SIFT)[8] and speeded-up robust features (SURF)[9]; intensity-based methods and transform models. Resolution discrepancies can introduce uncertainty due to the need for interpolation or down sampling. High data dimensionality such as in MSI presents another challenge, i.e., deciding which channel to use for registration. A helpful approach here is to reduce the datasets to 3 dimensions using non-negative matrix factorization, to visualize as red, green and blue color channels to find matching features for registration [10, 11].

3. RESULTS

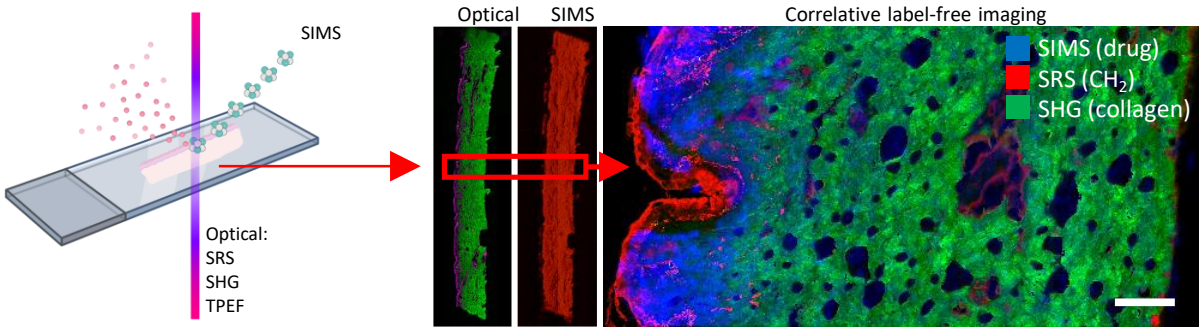

Figure 1. Schematic illustrating how multiphoton microscopies and secondary ion mass spectrometry imaging can be performed on the same tissue sample. Figure created with BioRender.com. Scale bar represents 100 μm.

The ability to locate and analyze the same tissue sections with different chemical imaging techniques facilitates the possibility to obtain detailed structural information and to compare measured chemical distributions. Figure 1 depicts how non-linear optical microscopy can be combined with SIMS to visualize the tissue structure and analyze the drug distribution. In this example, the region of interest was identified using the inherently distinctive structural features of the skin section, visible in both modalities. Further experimental details can be found in Belsey et al [7].

When comparing chemico-spatial information acquired by different imaging modalities it should be noted that discrepancies are expected for several reasons. For example, ToF-SIMS and multiphoton methods offer similar lateral resolution, but the sampling depth is markedly different: the multiphoton depth resolution is on the order of a micron, whereas the ToF-SIMS probes only the top few nm of the sample. In addition, combining MSI methods with the use of an inverted optical microscope, would result in opposite faces of the tissue section being analysed, and depending on the section thickness, and heterogeneity of the sample, significant differences might be expected. There are also likely further deviations due to non-linearities with chemical concentration. For optical methods, these could be caused by heterogeneity in sample thickness (due to detection in transmission), optical density and refractive index. MSI signal intensities are commonly impacted by matrix effects, i.e., ion enhancement or suppression in different chemical microenvironments, and topological distortions from sample roughness. A systematic study of all causes of signal non-linearity with concentration would be required for both techniques to obtain the most accurate representation and the 'ground truth'.

Imaging cell populations using multiple instruments presents additional challenges, in particular live cell analysis, owing to additional environmental requirements, coupled with cell movement. However recent work by Dondi et al has demonstrated excellent spatial correlation can be achieved when imaging fixed cells.

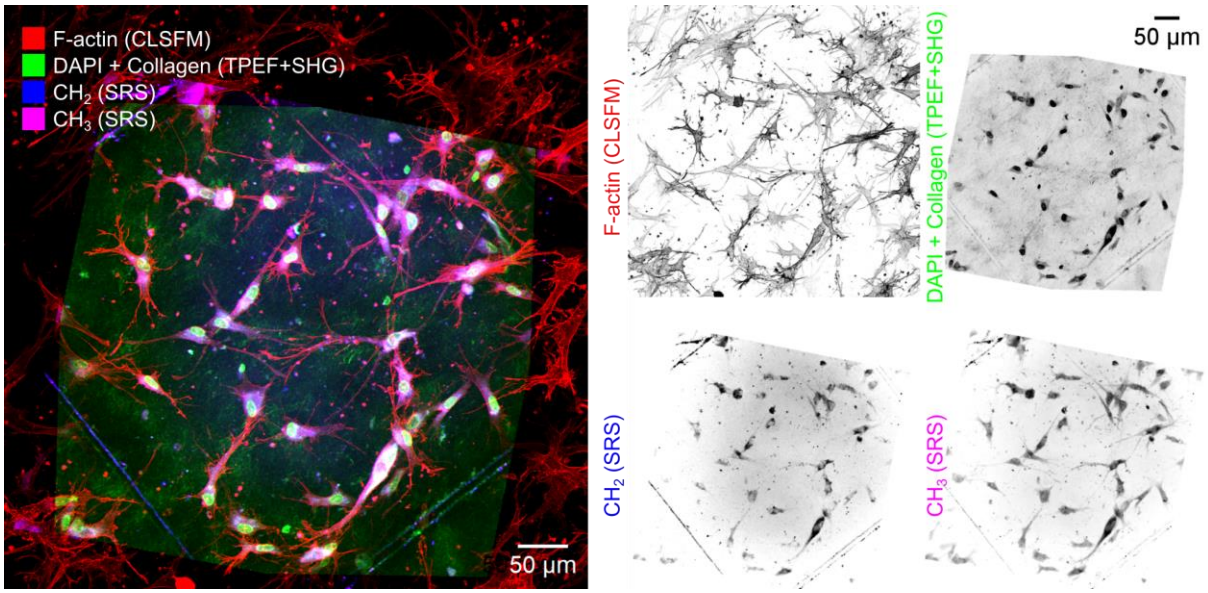

Figure 2. Example multimodal images of human dermal fibroblasts cultured in a type 1 collagen scaffold acquired by confocal laser scanning fluorescence (CLSFM) and multiphoton microscopy (SRS and SHG). Left panel shows composite image in which four image channels are displayed using different colors. Right panel shows the same image data with individual channels displayed in inverted grayscale.

Human dermal fibroblasts were cultured in a type 1 collagen scaffold, stained for F-actin and DNA and fixed after 24 hours. Multimodal optical image data captured using two different microscope platforms (a confocal laser scanning microscope and a multiphoton microscope) were registered using SIFT with landmarks based on common features identified in 1PEF and 2PEF images of cell nuclei. Figure 2 shows cell structures including F-actin visible in confocal laser scanning image data, allowing the assessment of cellular morphology, with bundling of F-actin in the cytoskeleton indicating the formation of filopodia. The chemical information accessible through SRS enabled measurement of CH_2

and CH_3 content in the cell membrane and nuclei, whilst SHG signals allowed direct label-free visualization of collagen fibrils of the matrix. The combination of different imaging modalities enables the compilation of comprehensive structural and chemical information.

4. CONCLUSIONS

Optical and MSI methods offer rich and diverse information, which can offer powerful insight to drug delivery in cells and tissues. This study demonstrates the analytical possibilities of combining complementary optical methods with one another, and with mass spectrometry imaging. The main advantages of the combined approach are the considerably higher sensitivity for drug detection by MSI compared to vibrational spectroscopies, together with sub-micron spatial resolution, and a greater degree of structural information offered by optical methods; for example, connective tissue visualization using SHG. Correlative analysis between instruments may be important to better understand each respective technique; for example, to reveal signal non-linearity with concentration, or to study artefacts. However, care is required when interpreting such datasets, since these techniques probe different information depths and have different sensitivities towards different chemistries. While methods such as SIMS can only be performed *in vitro*, the rich information it provides can aid the interpretation of complex Raman optical signals and better inform, therefore, the future *in vivo* application of multiphoton microscopies.

ACKNOWLEDGEMENTS

This work was funded by the UK Department for Science, Innovation and Technology through the National Measurement System Programme. Development of the methodologies used in this work was funded in part by Haleon CH SARL, and the authors are grateful to Dr Mila Boncheva Bettex for helpful discussions. Support by the Community for Analytical Measurement Science is also gratefully acknowledged for a 2020 CAMS Fellowship Award to Natalie Belsey, funded by the Analytical Chemistry Trust Fund.

REFERENCES

1. Franzen, L. and M. Windbergs, *Applications of Raman spectroscopy in skin research — From skin physiology and diagnosis up to risk assessment and dermal drug delivery.* Advanced Drug Delivery Reviews, 2015. **89**: p. 91-104.
2. Zarmpi, P., et al., *Confocal Raman Spectroscopic Characterization of Dermatopharmacokinetics Ex Vivo.* Molecular Pharmaceutics, 2023. **20**(11): p. 5910-5920.
3. Freudiger Christian, W., et al., *Label-Free Biomedical Imaging with High Sensitivity by Stimulated Raman Scattering Microscopy.* Science, 2008. **322**(5909): p. 1857-1861.
4. Mansfield, J., et al., *Chemically specific imaging and in-situ chemical analysis of articular cartilage with stimulated Raman scattering.* Journal of Biophotonics, 2013. **6**(10): p. 803-814.
5. Buchberger, A.R., et al., *Mass Spectrometry Imaging: A Review of Emerging Advancements and Future Insights.* Analytical Chemistry, 2018. **90**(1): p. 240-265.
6. Passarelli, M.K., et al., *The 3D OrbiSIMS—label-free metabolic imaging with subcellular lateral resolution and high mass-resolving power.* Nature Methods, 2017. **14**(12): p. 1175-1183.
7. Belsey, N.A., et al., *Visualisation of drug distribution in skin using correlative optical spectroscopy and mass spectrometry imaging.* Journal of Controlled Release, 2023. **364**: p. 79-89.
8. Lowe, D.G. *Object recognition from local scale-invariant features*. in *Proceedings of the Seventh IEEE International Conference on Computer Vision*. 1999.
9. Bay, H., et al., *Speeded-Up Robust Features (SURF).* Computer Vision and Image Understanding, 2008. **110**(3): p. 346-359.
10. Xiong, X.-C., et al., *Feature Extraction Approach for Mass Spectrometry Imaging Data Using Non-negative Matrix Factorization.* Chinese Journal of Analytical Chemistry, 2012. **40**(5): p. 663-669.
11. Fonville, J.M., et al., *Hyperspectral Visualization of Mass Spectrometry Imaging Data.* Analytical Chemistry, 2013. **85**(3): p. 1415-1423.