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Non-Destructive, Histologically Compatible Tissue Imaging by Desorption Electrospray Ionization Mass Spectrometry

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Imaging mass spectrometry (MS) is a powerful technique which has been gaining increased attention and broad use in many fields of science. Many biological applications are being pursued using the comprehensive information it provides on the distribution of multiple endogenous and exogenous molecules within animal and plant tissues^[1]. Amongst the imaging MS techniques^[2], those based on ambient ionization^[3] such as desorption electrospray ionization mass spectrometry (DESI-MS)^[4], laser ablation electrospray ionization (LAESI)^[5] and atmospheric pressure femtosecond laser imaging mass spectrometry (AP fs-LDI IMS)^[6] amongst others^[7], allow analysis at atmospheric pressure without significant sample preparation. In the past few years, much effort has gone into advancing ambient imaging mass spectrometry, especially in cancer diagnostics^[8]. The prospect of improving the accuracy of histopathological cancer evaluation by adding chemical information to morphological microscopic analysis now represents an attainable advance.

The capabilities of DESI-MS imaging as a diagnostic tool are demonstrated in many studies, in which the lipid profiles directly detected from different types of tissues, such as kidney^[9], bladder^[10], prostate^[11], and brain^[12] cancers enabled diagnosis of multiple types of human cancers. However, technical challenges remain and validation studies are still needed to successfully merge microscopic and mass spectrometric information into routine histopathology workflow. Correlation between histology and DESI-MS has thus far been performed by comparing the ion images obtained to the diagnosis from pathological evaluation of a serial section, typically a hematoxylin and eosin (H&E) stained section^[9]. Even though this strategy is sufficient for optical image evaluation under routine microscopic pathology, unambiguous correlation demands the use of the same tissue section for morphological and MS imaging evaluation^[13]. The primary reason for incompatibility of DESI-MS imaging with histochemical examination is that the most commonly used solvent systems, such as methanol/water and acetonitrile/water 1:1 (v/v), yield extensive chemical information but completely destroy the native tissue morphology in the process,

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precluding any subsequent analysis on the same tissue section. In an effort to overcome this limitation, we have developed a methodology which allows DESI-MS imaging to be performed while tissue integrity is preserved. New solvent systems, which we refer to as morphologically friendly, allow a spot-by-spot extraction of lipid species from tissue during DESI-MS analysis while the morphology of the tissue remains undisturbed. This is demonstrated here by imaging of lipids from multiple types of human cancerous and normal tissue to achieve a molecular diagnosis and then examining the same tissue section by H&E staining or immunohistochemistry to allow an independent histochemical diagnosis.

DESI-MS imaging is based on microscopic scale solvent extraction and many studies have shown that the chemical and physical properties of the solvent system chosen affect the molecular information obtained^[14]. However, the effect of the solvent system on the morphology of the tissue being analyzed by DESI-MS is an important property that has not yet been carefully explored. In this study, many solvents such as ACN, H₂O, MeOH, ethanol (EtOH), tetrahydrofuran (THF), N,N-dimethylformamide (DMF), chloroform (CHCl₃), and acetone as well as their mixtures, were explored in the analysis of 15 μ m thick serial coronary mouse brain tissue sections (Figure S1, Supporting Information). The majority of the ions observed in the mass spectra obtained from the solvent systems tested correspond to commonly observed lipid species in brain tissue when using standard MeOH:H₂O (1:1)^[15], such as deprotonated free fatty acids, phosphatidylserines (PS), phosphatidyl-inositols (PI) and sulfatides (ST), with variations in the relative abundances of the lipid species and in the total ion signal. Remarkably, the use of DMF as the spray solvent did not cause visible damage to the tissue section^[16]. The effect of DMF on the tissue was further explored by combining this solvent with other solvents in binary (1:1 v/v) and tertiary mixtures (Figure S2, supporting information). None of the DMF solvent combinations tested caused damage to the tissue sections analyzed, while they did provide extensive chemical information. For example, the combination of DMF with ACN, EtOH, THF or CHCl₃ yielded high ion signals and chemical information similar to that seen using standard MeOH:H₂O, while combinations with either H₂O or MeOH greatly enhanced the signal of low *m/z* compounds, such as small metabolites, fatty acids (FA) and FA dimers^[16].

To confirm preservation of tissue integrity, H&E staining was performed directly on the tissue sections after DESI-MS imaging, without any intermediate sample preparation steps. Microscopic examination of the H&E tissue sections revealed no damage or change in the cellular morphology of the sample after DESI-MS analysis using DMF:EtOH and DMF:H₂O solvent systems, while the tissue analyzed using MeOH:H₂O was found to be significantly damaged, as observed visually (Figure S3, Supporting Information).

The physical and chemical effects of the DMF:EtOH solvent system were further investigated by performing ten consecutive DESI-MS image acquisition experiments on the same region of a 15 μ m thick mouse brain tissue section (Supporting Information). After these DESI-MS imaging analyses the section was H&E stained and it still showed no morphologic damage. A plot of the ion count of a representative ion observed in the gray matter region, *m/z* 834.4 (PS 18:0/22:6), as a function of the DESI-MS analysis number revealed an approximately exponential decay in ion count, consistent with the spot-by-spot micro-extraction mechanism proposed for DESI-MS (Figure S4, Supporting Information)^[17]. The extraction process that occurs in DESI-MS can be compared to the fixative procedures commonly used in histology in which lipids are removed while the nucleoproteins and the intracellular and extracellular proteins stained in the histochemical treatment are preserved.

All combinations of DMF with other solvents tested in this particular study on mouse brain tissue sections (2, 3, 5, 10 and 15 μ m thicknesses) by DESI-MS imaging were found to be

morphologically friendly (Supporting Information). Some other pure solvents, such as ACN, DMF, THF, EtOH and a few other solvent combinations that did not contain DMF, such as ACN:EtOH (1:1), MeOH:CHCl₃ (1:1) and ACN:CHCl₃ (1:1), also appeared to be morphologically friendly while many more, such as pure H₂O, THF:H₂O (1:1), EtOH:H₂O (1:1), MeOH:H₂O (1:1) and ACN:H₂O (1:1), were not and the tissue after DESI-MS imaging was damaged physically. The morphological effect that the DESI-MS spray has on tissue is related to the physical and chemical properties of the solvent system^[17]. The relative solubility of lipids and insolubility of the cellular and extracellular protein components of the tissue section in the DESI spray solvent helps to conserve tissue morphology while also providing high quality lipid mass spectra. The physical properties of the solvent such as surface tension and its effects on the dynamics of the DESI spray are the proximate cause of the damage to the tissue. Solubilization of cellular and extracellular constituents of the cells allows the tissue to become more susceptible to the mechanical action of the DESI spray droplets, which appears to be the case for conventional solvents such as MeOH:H₂O^[18]. In the experiments shown, our choice of DMF combinations such as DMF:EtOH and DMF:ACN, among others, arises from the fact that high ion signal intensity with low background was achieved for the compounds of interest, and that a very stable and very well defined spray spot was obtained, which is ideal for imaging applications. Our focus in this communication is to demonstrate the ability of DESI-MS imaging to be histologically compatible for biomedical applications. The underlying reasons for this compatibility must be related to the physical and chemical effects of these solvents on tissue but the nature of these effects remains to be elucidated.

Chemical information and image quality are the most important factors in DESI-MS imaging applications. Figure 1a shows an average mass spectra of a mouse brain tissue section obtained using DMF:EtOH as the solvent system. The spray geometry, gas pressure and solvent flow rate used in the imaging experiments were optimized so that a spot size of 180 μ m was obtained (see Supporting Information)^[15]. An optical image of the histologically compatible DESI-MS imaging experiment is shown in Figure 1b. The two distinctive negative ion mode MS patterns associated with the lipid compositions of the gray and the white matter of the mouse are seen in the ion images shown in Figure 1 c-f^[15]. Lipids are tentatively assigned based on tandem MS experiments, although isomers are known to occur. Figure 1g shows the optical image of the same tissue section which was H&E stained after DESI-MS imaging. The high-quality 2D DESI-MS ion images can be directly compared and overlaid with the H&E stained tissue section for better correlation between the spatial distribution of the lipid species and brain substructures.

The capability to perform DESI-MS imaging and histochemical analysis of the same tissue section is important for accurate correlation between molecular signatures and disease state. Because the information from the two methods is orthogonal, it is expected to greatly improve discrimination and disease state diagnosis. This is especially true in the analysis of cancerous tissue sections which are very often highly heterogeneous, with regions containing various tumor cell concentrations^[19], infiltrative normal tissue^[10] and precancerous lesions^[11], within other features. Integration of DESI-MS imaging into a traditional histopathology workflow required that the MS analysis before histology would not interfere with the morphology of the tissue section. To investigate this capability, human bladder, kidney and prostate cancer and adjacent normal tissues were analyzed by DESI-MS imaging in the negative ion mode using one of the histology compatible solvents and sequentially H&E stained or subjected to immunohistochemical (IHC) analysis with p63 antibody^[20]. Figure 2 shows a series of negative ion mode DESI-MS ion images of human bladder transitional cell carcinoma and adjacent normal tissue. As previously reported by DESI-MS imaging^[10], the ions that most significantly contribute to the discrimination between cancerous and normal bladder tissue are the free fatty acids (FA) and their dimers

(Figure S5, Supporting Information), which consistently appear at increased intensities in the cancerous tissue (Figure 2d and 2e). Detailed pathological examination of the same tissue sections stained with H&E after DESI-MS imaging (Figure 2f) compared to a control section confirmed that no damage to the cells morphology or to the overall tissue integrity was caused as a result of DESI-MS imaging analysis (Figure S6, Supporting Information). The non-destructive nature of the DMF based solvent system enables ion images to be overlaid with the H&E stain of the same tissue section for unambiguous diagnosis and correlation. For example, a small region of tissue within the cancerous section detected by DESI-MS as negative for bladder cancer based on the distribution of FA dimer (m/z 537.2) was confirmed as normal tissue by pathological evaluation of the overlaid DESI-MS ion image and H&E stain of the same tissue section (Figure 2g). Compatibility between tissue DESI-MS imaging and IHC was also investigated. Figure 3 shows the magnified brightfield optical images of prostate cancer tissue sections which were first imaged using DMF:EtOH or conventional ACN:H₂O and sequentially H&E stained or subjected to p63 IHC, and the control tissues. No spatial delocalization of the cellular proteic content stained during the IHC or H&E protocols was observed as a consequence of DESI-MS imaging analysis using DMF:EtOH, when compared to the respective control tissues, as confirmed by detailed pathological evaluation. The destructive nature of conventional solvents system is clearly observed and prevents any further morphological analysis. The same histological observations were true for analysis of other samples of human bladder, prostate and kidney cancers (Figure S7-S9, Supporting Information, respectively) and their adjacent normal tissue. Previous molecular information that allowed a diagnosis to be obtained for these types of cancer was consistent with information obtained using the new solvent systems^[9, 11].

The results reported here introduce a novel capability of DESI-MS tissue imaging - histological compatibility. The feasibility of performing DESI-MS imaging while maintaining tissue integrity and cell morphology allow ambient MS analysis of tissue sections to be combined with traditional histopathology to provide more accurate disease diagnosis. This means that DESI-MS imaging can now be inserted at any point into a work flow profile dealing with tissue analysis. We anticipate that these advances will allow DESI-MS to be more broadly applied as a molecular diagnostic tool. DESI-MS imaging could be included as the initial step in the clinical tissue analysis workflow. Furthermore, the morphologically compatible solvent system allows DESI-MS imaging to be combined with other analytical techniques for chemical analysis of the same tissue section. These include immunofluorescence and other spectroscopic and imaging experiments. We are currently investigating higher spatial resolution applications of DESI-MS imaging using the developed morphologically friendly solvent systems. The methodology being introduced here now allows DESI-MS to be considered as an essentially non-destructive technique in terms of tissue morphology preservation, introducing new possibilities to further expand the applications of DESI-MS imaging in the biomedical field, to incorporate this technique into routine histopathological analysis for better accuracy of cancer diagnosis and grading, and ultimately to the strategy of intraoperative DESI-MS analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

MS	Mass spectrometry
DESI-MS	desorption electrospray ionization mass spectrometry
LAESI	laser ablation electrospray ionization
AP fs-LDI IMS	atmospheric pressure femtosecond laser imaging mass spectrometry
H&E	hematoxylin and eosin
ACN	acetonitrile
H₂O	water
MeOH	methanol
EtOH	ethanol
THF	tetrahydrofuran
DMF	N,N-dimethylformamide
CHCl₃	chloroform
PS	phosphatidylserines
PI	phosphatidylinositols
ST	sulfatides
FA	fatty acids
m/z	mass-to-charge ratio

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Figure 1.

Average DESI-MS mass spectra of a mouse brain coronal section including regions of white and grey matter obtained using DMF:EtOH (1:1) as the solvent system (A). Optical image of the DESI-MS imaging experiment (B). DESI-MS ion images show the distribution of (C) m/z 834.3, PS(18:0/22:6); (D) m/z 888.6, ST(24:1); (E) m/z 885.6, PI(18:0/20:4) and (F) m/z 303.3, FA(20:4). Optical image of the same tissue section after first being imaged by DESI-MS and then H&E stained is shown in (G).

Figure 2.

DESI-MS imaging of human bladder cancerous and adjacent normal tissue sections using morphologically friendly DMF:EtOH (1:1) as the solvent system. Ion images show the distribution of (A) m/z 788.4, PS(18:0/18:1); (B) m/z 885.6, PI (18:0/20:4); (C) m/z 835.6, PI(16:0/18:1); (D) m/z 281.6, FA (18:1) and (E) m/z 537.2 (FA dimer). After the DESI-MS imaging experiment, the tissue sections were subjected to H&E staining (F), evaluated by an expert pathologist, and diagnosed. Overlay of the ion image of m/z 537.2 and H&E stain of the same tissue section (G).

Figure 3.

Magnified brightfield optical images of prostate cancer tissue sections subjected to H&E stain or p63 IHC. Optical images of the entire tissue sections are shown to the left of each magnification. When compared to controls (A) no effect on tissue morphology was observed in tissue sections first imaged by DESI-MS using (B) DMF:EtOH (1:1). Tissue destruction occurs using conventional solvent systems, such as (C) ACN:H₂O (1:1).