

# Mass spectrometry-based phospholipid imaging: methods and findings

メタデータ	言語: English 出版者: 公開日: 2022-02-08 キーワード (Ja): キーワード (En): 作成者: Mamun, Md. Al, Islam, Ariful, Eto, Fumihiro, Sato, Tomohito, Kahyo, Tomoaki, Setou, Mitsutoshi メールアドレス: 所属:
URL	<a href="http://hdl.handle.net/10271/00003947">http://hdl.handle.net/10271/00003947</a>

1 Review Article

2 **Mass spectrometry-based phospholipid imaging: methods and findings**

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14 **Abstract**

15 **Introduction:** Imaging is a technique used for direct visualization of the internal structure  
16 or distribution of biomolecules of a living system in a two-dimensional or three-  
17 dimensional fashion. Phospholipids are important structural components of biological  
18 membranes and have been reported to be associated with various human diseases.  
19 Therefore, the visualization of phospholipids is crucial to understand the underlying  
20 mechanism of cellular and molecular processes in normal and diseased conditions.

21 **Areas covered:** Mass spectrometry imaging (MSI) has enabled the label-free imaging of  
22 individual phospholipids in biological tissues and cells. The commonly used MSI  
23 techniques include matrix-assisted laser desorption ionization-MSI (MALDI-MSI),  
24 desorption electrospray ionization-MSI (DESI-MSI), and secondary ion mass  
25 spectrometry (SIMS) imaging. This special report described those methods, summarized  
26 the findings, and discussed the future development for the imaging of phospholipids.

27 **Expert opinion:** Phospholipids imaging in complex biological samples has been  
28 significantly benefited from the development of MSI methods. In MALDI-MSI, novel  
29 matrix that produces homogenous crystals exclusively with polar lipids is important for  
30 phospholipids imaging with greater efficiency and higher spatial resolution. DESI-MSI  
31 has the potential of live imaging of the biological surface while SIMS is expected to image  
32 at the subcellular level in the near future.

33 **Keywords:** Phospholipid; Mass spectrometry imaging; MALDI-MSI; DESI-MSI; SIMS  
34 imaging.

35

36 **Highlights:**

- 37 1. Mass spectrometry imaging (MSI) is a powerful method for imaging specific  
38 phospholipids (PLs) in tissues and cells.
- 39 2. The potential of MSI of either single or multiple PLs for the development of  
40 therapeutic agents, biomarkers, and predictive factors for diseases is reviewed.
- 41 3. The current states of MALDI-MSI, DESI-MSI, and SIMS-MSI on tissue PL  
42 imaging are assessed and discussed.
- 43 4. By image reconstruction, conventional 2D imaging can be applied in 3D PL  
44 imaging.

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## 46 **1. Introduction**

47 In biology, imaging refers to the technique used to visualize the internal structure or  
48 biomolecules in tissues and cells of a living system in two- dimensional (2D) or three-  
49 dimensional (3D) style without perturbing the structure. The history of imaging dates  
50 back to 1895 when Wilhelm Roentgen discovered X-ray. X-ray was originally used in  
51 medical imaging to create 2D image of internal organs in an X-ray film [1]. With the  
52 advancement of computer vision and algorithms, several methods such as computed  
53 tomography [2], magnetic resonance imaging [3], positron emission tomography [4] and  
54 ultrasound [5] have been evolved to produce 2D as well as 3D image [6]. Besides  
55 anatomical imaging, those techniques play important role in molecular imaging where  
56 contrast agents are used for the noninvasive visualization, characterization, and  
57 measurement of the biological processes in the living system [6,7,8]. Discovery of  
58 electron microscope enabled the comprehensive visualization of cellular and subcellular  
59 ultrastructures [9]. Some other microscopy-based labelled molecular imaging techniques  
60 such as green fluorescent protein labelling [10], and immunohistochemistry [11] are used  
61 to visualize the distribution map of protein molecules in tissue as well as cell structure.

62 Phospholipids (PLs) are one of the major structural components of biological membranes  
63 [12,13] and play important roles in protecting the cells and organelles [14]. They also act  
64 as signaling molecules [15] and precursors of many signaling mediators for various  
65 biological processes [14,16]. An increasing amount of evidence indicates that the altered  
66 level of PLs and their metabolites in tissues are associated with various human diseases  
67 such as cancers [17], cardiovascular diseases [18], diabetes [19], Alzheimer's disease  
68 (AD) [20,21], and autoimmune inflammatory diseases [22]. Therefore, it is important to  
69 visualize individual PL species in tissue as well as in cellular level in order to explore the  
70 underlying mechanism of cellular and molecular processes in normal and diseased  
71 conditions. However, PLs in tissue sections cannot be imaged with conventional  
72 molecular imaging techniques. Some staining methods such as Nile red [23], Oil Red O  
73 [24,25] or osmium tetroxide [26] is commonly used to localize the PL fraction on frozen  
74 tissue sections. However, these methods localize either the complete lipid fraction or only  
75 one PL class on tissue sections, not individual PL species. Unlike immunohistochemistry  
76 of proteins, there is no such protocol for individual PL imaging due to the lack of  
77 antibodies or fluorescent probes.

78 Conventional mass spectrometry (MS) including liquid chromatography-MS (LC-MS)  
79 has been extensively employed in lipidomics, particularly in the identification and  
80 quantification of PLs in biological tissue [27,28]. Typical sample preparation protocol in  
81 LC-MS includes the homogenization of tissue sample followed by lipid extraction  
82 resulting in the loss of spatial information. LC-MS analysis of lipids extracted from  
83 various anatomical regions of a certain tissue such as the brain is often investigated in  
84 order to retain the spatial information [29,30]. However, it is a time-consuming process  
85 and cannot resolve the microscopic anatomical regions because of the challenges in the  
86 dissection and lipid extraction processes. In addition, the method requires a huge amount  
87 of sample that limits the analysis of important clinical samples such as biopsy tissues.  
88 Fortunately, MS has undergone tremendous development particularly in sample  
89 preparation protocol and instrumentation enabling the analysis of biomolecules directly  
90 from the thin tissue section to visualize individual molecule over the entire tissue [31,32].

91 So far, this method commonly known as imaging mass spectrometry (IMS) or mass  
92 spectrometry imaging (MSI) has revolutionized the comprehensive imaging (2D and 3D)  
93 of PLs in complex biological tissues. In this report, we focused on this technique in PLs  
94 imaging including the methodologies and findings.

95

## 96 **2. Structure and classification of PL species**

97 PLs are diverse in chemical structures consisting of a hydrophilic head group and one or  
98 more hydrophobic acyl chains attached to an alcohol moiety [14], and are commonly  
99 referred to glycerophospholipids (GPLs) [33]. GPLs are esters of glycerol, fatty acids,  
100 and phosphoric acid(s), where glycerol acts as the backbone. Two fatty acid chains are  
101 generally present at the sn-1 and sn-2 positions, whereas the phosphate group is linked to  
102 the sn-3 position of the glycerol backbone. The head group is attached to the phosphate  
103 group(s), and its chemical nature can be diverse, leading to different GPLs. The most  
104 common GPLs containing a single phosphate group are phosphatidylcholine (PC),  
105 phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS),  
106 phosphatidylglycerol (PG) and phosphatidic acid (PA). A subclass of GPLs is the  
107 lysophospholipids (LPLs), in which a single fatty acid is present at either sn-1 or sn-2  
108 position of the glycerol backbone. Examples of LPLs include lysophosphatidic acid  
109 (LPA), lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE),  
110 lysophosphatidylinositol (LPI), lysophosphatidylglycerol (LPG), and  
111 lysophosphatidylserine (LPS). Diphosphatidylglycerol, historically known as cardiolipin  
112 (CL), is a unique mitochondrial PL that contains two phosphate groups and four acyl  
113 chains linked to the glycerol backbone (Table 1).

114 Various combinations of fatty acids and head group to the alcohol backbone, and their  
115 oxidation products resulted in the remarkable structural diversity (Table 1) and biological  
116 functions of the PLs molecules.

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133 **Table 1: Names and structures of major phospholipids occurring in the living system.**

Major phospholipids	General structure
Phosphatidylcholine	$  \begin{array}{c}  \text{O} \\  \parallel \\  \text{R}^2\text{-C-O-CH} \\    \\  \text{H}_2\text{C-O-C-R}^1 \\    \\  \text{H}_2\text{C-O-P-O-CH}_2\text{-CH}_2\text{-N}^+\text{(CH}_3\text{)}_3 \\    \\  \text{O}^-  \end{array}  $
Phosphatidylethanolamine	$  \begin{array}{c}  \text{O} \\  \parallel \\  \text{R}^2\text{-C-O-CH} \\    \\  \text{H}_2\text{C-O-C-R}^1 \\    \\  \text{H}_2\text{C-O-P-O-CH}_2\text{-CH}_2\text{-NH}_2 \\    \\  \text{OH}  \end{array}  $
Phosphatidylserine	$  \begin{array}{c}  \text{O} \\  \parallel \\  \text{R}^2\text{-C-O-CH} \\    \\  \text{H}_2\text{C-O-C-R}^1 \\    \\  \text{H}_2\text{C-O-P-O-CH}_2\text{-CH-NH}_3^+ \\    \qquad \qquad \qquad   \\  \text{OH} \qquad \qquad \qquad \text{COO}^-  \end{array}  $
Phosphatidylinositol	$  \begin{array}{c}  \text{O} \\  \parallel \\  \text{R}^2\text{-C-O-CH} \\    \\  \text{H}_2\text{C-O-C-R}^1 \\    \\  \text{H}_2\text{C-O-P-O-} \\    \qquad \qquad \qquad   \\  \text{OH} \qquad \qquad \qquad \text{C}_6\text{H}_8\text{O}_5  \end{array}  $
Phosphatidylglycerol	$  \begin{array}{c}  \text{O} \\  \parallel \\  \text{R}^2\text{-C-O-CH} \\    \\  \text{H}_2\text{C-O-C-R}^1 \\    \\  \text{H}_2\text{C-O-P-O-CH}_2\text{-CH(OH)-CH}_2\text{(OH)} \\    \\  \text{OH}  \end{array}  $
Phosphatidic acid	$  \begin{array}{c}  \text{O} \\  \parallel \\  \text{R}^2\text{-C-O-CH} \\    \\  \text{H}_2\text{C-O-C-R}^1 \\    \\  \text{H}_2\text{C-O-P-OH} \\    \\  \text{OH}  \end{array}  $
Cardiolipin	$  \begin{array}{c}  \text{O} \qquad \qquad \qquad \text{OH} \qquad \qquad \qquad \text{O} \\  \parallel \qquad \qquad \qquad   \qquad \qquad \qquad \parallel \\  \text{HO-P-O-CH}_2\text{-CH-CH}_2\text{-O-P-OH} \\    \qquad \qquad \qquad   \qquad \qquad \qquad   \\  \text{O} \qquad \qquad \qquad \text{O} \qquad \qquad \qquad \text{O} \\    \qquad \qquad \qquad   \qquad \qquad \qquad   \\  \text{CH}_2 \qquad \qquad \qquad \text{CH}_2 \qquad \qquad \qquad \text{CH}_2 \\    \qquad \qquad \qquad   \qquad \qquad \qquad   \\  \text{R}^1\text{-C-O-CH} \qquad \qquad \qquad \text{HC-O-C-R}^3 \\    \qquad \qquad \qquad   \\  \text{R}^2\text{-C-O-CH}_2 \qquad \qquad \qquad \text{H}_2\text{C-O-C-R}^4 \\  \parallel \qquad \qquad \qquad \parallel \\  \text{O} \qquad \qquad \qquad \text{O}  \end{array}  $

134 N.B.: R<sup>1</sup>, R<sup>2</sup>, R<sup>3</sup> and R<sup>4</sup> indicate acyl chains of fatty acids.

### 135 **3. Methods of PLs imaging by MSI**

136 MSI is a powerful technique that allows the simultaneous imaging of hundreds of  
137 biomolecules in thin tissue sections without extraction, purification or separation [34]. It  
138 is a label-free molecular imaging method which was initially used to map proteins and  
139 peptides in biological tissues [35]. In the last several years, this technique has been  
140 utilized extensively in the visualization of individual PLs in a variety of human and  
141 animal samples [36].

#### 142 **3.1 Instrumentation for MSI**

143 Like conventional MS, the basic instrumentation of MSI includes (i) an ionization source  
144 to generate ions from neutral molecules, (ii) a mass analyzer to analyze ions based on the  
145 mass-to-charge ratio ( $m/z$ ), and (iii) a detector to subsequently detect the ions and convert  
146 into digital signals. The fundamental difference with the conventional MS is that it  
147 collects ions directly from the surface area of a sample under controlled motion resulting  
148 in the preservation of the spatial information.

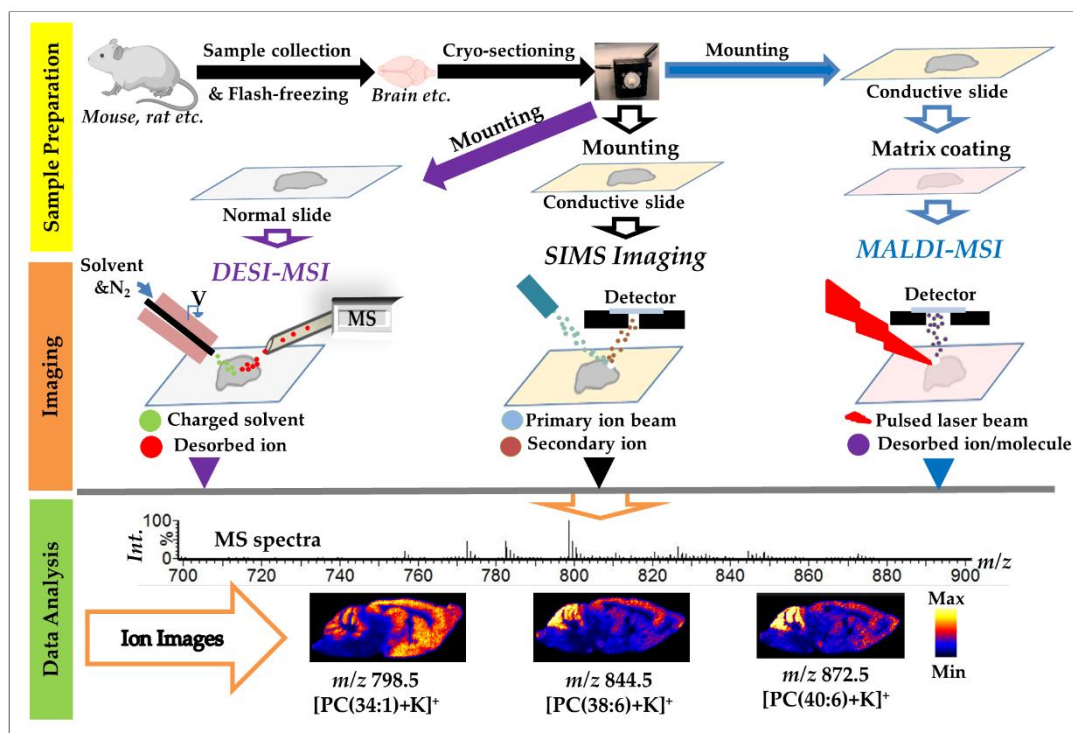
149 Numerous ionization sources originally developed for MS have been utilized further for  
150 MSI analysis. Among the various ionization sources used for MSI, matrix-assisted laser  
151 desorption ionization-MSI (MALDI-MSI) [37], desorption electrospray ionization-MSI  
152 (DESI-MSI) [38], and secondary ion mass spectrometry (SIMS) imaging [39] are most  
153 prominent. Several variants of MALDI-MSI include infrared-matrix-assisted laser  
154 desorption electrospray ionization (IR-MALDESI) [40,41], atmospheric pressure  
155 scanning microprobe MALDI (AP-SMALDI) [42], atmospheric pressure-MALDI-MSI  
156 (AP-MALDI-MSI) [43], and matrix-free laser desorption ionization (LDI) techniques  
157 such as nano-assisted laser desorption ionization-MSI (NALDI-MSI) [44] and desorption  
158 ionization using through-hole alumina membrane-MSI (DIUTHAME-MSI) [45]. Some  
159 other techniques such as laser ablation electrospray ionization (LAESI) [46], air flow-  
160 assisted ionization-MSI (AFAI-MSI) [47], and easy ambient sonic spray ionization  
161 (EASI) [48], have also been developed for imaging. The current report mainly focused  
162 on the three major ionization techniques namely MALDI, DESI, and SIMS for PLs  
163 imaging.

164 A variety of mass analyzers compatible with ionization sources are available for MSI  
165 analysis. Time-of-flight (TOF) is the most common mass analyzer used for MSI due to  
166 their high mass range, sensitivity and fragmentation capabilities in tandem MS. Ion trap  
167 and quadrupole time-of-flight (QTOF) are also widely used in MALDI-MSI and DESI-  
168 MSI. The use of Fourier-transform ion cyclotron resonance (FT-ICR) mass analyzer in  
169 MALDI-MSI has significantly increased the molecular imaging with high mass resolution.  
170 Each analyzer separates ions in a physically distinct mechanism, and has specific  
171 advantages and limitations.

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**Figure 1:** Overview of phospholipid imaging by mass spectrometry imaging. Tissue samples collected from human or animal sources are flash-frozen, cryosectioned, and thaw-mounted on a glass slide. For MALDI-MSI and SIMS imaging, the tissue section is generally mounted on indium tin oxide (ITO)-coated glass slide whereas DESI-MSI uses normal glass slide. Unlike MALDI-MSI, SIMS imaging and DESI-MSI do not require matrix-coating. The sample is placed onto a 2D moving stage and subjected to ionization usually in a raster fashion as the sample stage moves in x, y coordinates at a controlled speed. The ions are then analyzed based on the mass-to-charge ratio ( $m/z$ ) in a mass analyzer. A detector subsequently detects those ions and converts them into digital signals. Results are displayed as mass spectra as well as two-dimensional ion-images. The mass spectra and the ion images shown here were acquired by DESI-MSI of mice brain sections.

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### 184 3.2 Sample preparation for MSI

185 In MSI of PLs, careful sample preparation is crucial for successful imaging as the  
186 polyunsaturated fatty acid containing-PLs (PUFA-PLs) are prone to oxidation.

#### 187 3.2.1 Tissue sample preparation

188 *Collection of samples* – The use of thin sections of human and animal (usually mice and  
189 rats) tissues is very common in PLs imaging. Human samples used for imaging include  
190 the biopsy and the archived post-mortem tissues. Animals are usually euthanized by  
191 cervical dislocation or using anesthetics prior to tissue collection. In order to preserve the  
192 tissue anatomy and to halt the enzymatic degradation, tissues should be dissected rapidly



193 followed by immediate flash-freezing using powdered-dry ice or liquid nitrogen (Figure  
194 1). The frozen tissues are then stored at -80 °C until sectioning.

195 *Cryo-sectioning*– Before slicing, frozen tissues are transferred into a cryostat chamber  
196 and left for 15 – 20 minutes at – 10 °C to – 20 °C to equilibrate to the sectioning  
197 temperature. Tissue block is affixed to the stage of a cryostat machine using an embedding  
198 media and then sliced at a thickness of around 5 – 20 µm. For 3D MSI, a series of serial  
199 sections are collected with minimal error and registered carefully. During sectioning,  
200 optimized temperature, usually at – 20 °C (can vary depending on the type of tissue), must  
201 be maintained in the cryostat. Particular care should be taken when using optimal cutting  
202 temperature embedding media (OCT) or other embedding media containing polymer as  
203 they cause serious contamination reducing the quality of the spectra. The thickness of the  
204 section should be as thin as possible to achieve higher quality spectral data in MALDI-  
205 MSI [49], although DESI-MSI has the flexibility regarding the sample thickness.

206 *Sample pre-treatment* – For MALDI, the tissue section is generally thaw-mounted on an  
207 indium tin oxide (ITO)-coated glass slide followed by a homogenous coating with a  
208 suitable matrix solution allowing the formation of co-crystals between the matrix  
209 molecules and the analytes. The matrix molecules must have the property of absorbing  
210 laser energy to aid in the ionization process. Various matrix compounds have been  
211 developed due to the fact that a single matrix does not work for all PLs. For instance, 2,5-  
212 Dihydroxybenzoic acid is commonly used to image PC molecules [50,51] in positive ion  
213 mode while 9-aminoacridine is used for imaging various PLs including PI, PG, PS, PA  
214 and CL in negative ion mode [52]. Other matrices that have been used for the imaging of  
215 PLs include 2,5-dihydroxyacetophenone [53],  $\alpha$ -cyano-4-hydroxycinnamic acid [50],  
216 graphene oxide [54], and 1,6-Diphenyl-1,3,5-hexatriene [55]. The matrix can be sprayed  
217 either manually (e.g., using artistic air brush) or automatically (e.g., using TM-Sprayer<sup>TM</sup>  
218 manufactured by HTX Technologies, and ImagePrep manufactured by Bruker Daltonics  
219 Inc.) [56].

220 Unlike MALDI, DESI uses normal glass slide for tissue mounting and does not require  
221 sample pre-treatment reducing the time significantly for sample preparation.

222 For SIMS imaging, the tissue section is generally transferred onto the ITO-coated glass  
223 slide and does not require matrix coating. Unfortunately, the high energy primary ion  
224 beam used in typical SIMS causes increased fragmentation of the PLs resulting in the  
225 poor molecular ion yield. In order to reduce the fragmentations, several approaches for  
226 sample preparation such as matrix enhancement (similar to MALDI protocol) and surface  
227 metallization (coating with a thin layer of gold or silver) have been developed [57].

### 228 **3.2.2 Sample preparation for single-cell imaging**

229 PLs imaging at the cellular level has been made possible by SIMS imaging as it offers the  
230 capability of resolving very small features which can be as low as a hundred nanometers.  
231 Sample for single-cell analysis includes the isolated cells from the specimen or cultured  
232 cell. Unfortunately, live cells cannot be analyzed directly in SIMS as it works under a  
233 high vacuum condition. Generally, cells are extracted or grown on an appropriate  
234 substrate such as silicon wafer or gold-coated silicon wafer [58] followed by fixation to  
235 minimize sample degradation. Two fixation methods are commonly applied: (i) chemical  
236 fixation using glutaraldehyde, and (ii) cryofixation. A cryofixation method, namely

237 plunge fixation, has been shown to be advantageous for single-cell lipid imaging [59]. In  
238 plunge freezing, samples are stored in liquid nitrogen at -196 °C after washing by a  
239 mixture of propane and isopentane (3:1). Interestingly, enhanced signal intensity for PLs  
240 has been reported when matrix solution is added on the cell surface prior to the fixation  
241 [58]. After fixation, frozen samples are freeze-dried and stored until analysis.

### 242 **3.3 Analysis by MSI**

243 To minimize the degradation or oxidation of the PLs, imaging should be performed as  
244 soon as possible once the sample is ready for analysis. The sample is placed onto a 2D  
245 moving stage and subjected to ionization either in positive or negative ion mode. Ions are  
246 collected from the surface usually in a raster fashion as the sample stage moves in x, y  
247 coordinates at a controlled speed. The ions are then sent into the mass analyzer for  
248 analysis and subsequently detected by the detector system.

249 PLs are a large group of molecules and show diversity in ion production. PC and PE are  
250 typically ionized in positive ion mode as protonated/sodiated/potassiated adducts while  
251 PS, PA, PI, and PG are ionized in negative ion mode as deprotonated ions in the mass  
252 range of  $m/z$  700 – 900. LPLs are detected either in positive or negative ion mode in the  
253 mass range of  $m/z$  400 – 600, on the other hand, singly charged CLs are usually detected  
254 in the negative ion mode in the mass range of  $m/z$  1000 – 1600.

255 MALDI is a widely used soft ionization process that primarily produces singly-charged  
256 ions in both positive and negative ion modes. In this method, the matrix-coated sample  
257 surface is irradiated by pulsed laser causing the rapid excitation and heating of the matrix  
258 molecules [60,61] (Figure 1). The heated molecules undergo evaporation resulting in the  
259 desorption of neutral and charged analytes, matrix molecules, analyte-matrix, and matrix-  
260 matrix clusters, all under the vacuum conditions. Ionization of the analytes are thought to  
261 occur during the desorption process, or in the expanding plume through the charge  
262 transfer between the excited matrix molecules and the neutral analytes. Ultraviolet lasers  
263 such as frequency-tripled Nd:YAG lasers (355 nm) are commonly used for MALDI-MSI  
264 as they are strongly absorbed by the matrix molecules [62].

265 Unlike MALDI, DESI works in an open environment at atmospheric pressure. In DESI,  
266 a beam of charged microdroplets of solvent coupled with a nebulizer nitrogen gas is  
267 directed continuously onto the tissue surface where molecules are extracted, ionized and  
268 desorbed into the mass spectrometer for analysis [63] (Figure 1). Methanol (usually 98%)  
269 is commonly sprayed at a flow rate of 2 — 5  $\mu\text{L}/\text{min}$  using a solvent pump, and a capillary  
270 voltage of 2 — 4.0 kV is generally applied to charge the solvent. A 2-D stage moves the  
271 tissue at a controlled speed to record the mass spectra from different spatial coordinates.  
272 The spatial resolution of DESI is lower (can range between 50—200  $\mu\text{m}$ ) than that of  
273 MALDI (typically <50  $\mu\text{m}$ ). However, nano-DESI can offer much higher spatial  
274 resolution (better than 10  $\mu\text{m}$ ) [64,65,66]. Prior to the analysis, optimization of several  
275 parameters such as solvent composition, solvent flow rate, gas flow rate, capillary voltage,  
276 cone voltage, inlet temperature, and the geometry of the sprayer is required in order to  
277 obtain the highest desorption and ionization as well as the best spatial resolution of the  
278 ion image [67,68].

279 Conventionally, SIMS uses a hard ionization technique where the tissue surface is  
280 bombarded with a focused high energy primary ion beam, causing desorption of

281 secondary ions into the mass analyzer. It operates either in dynamic or static modes, while  
282 the latter is used for PLs imaging as the intact molecular ions are typically detected under  
283 that condition. The emergence of cluster ion sources (e.g., C<sub>60</sub><sup>+</sup>, Bi<sub>3</sub><sup>+</sup>, and Au<sub>3</sub><sup>+</sup>) as a  
284 primary ion beam has significantly improved the PLs imaging with high sensitivity [57].  
285 Like MALDI, ionization events in SIMS occur under the vacuum conditions.

286 For single-cell analysis, freeze-dried cells are typically subjected to SIMS imaging.  
287 Interestingly, the increased signal intensity of PLs was observed when the sample is  
288 analyzed in frozen-hydrated conditions where the cryo-fixed cells are kept frozen with a  
289 liquid nitrogen-cooled stage during the analysis [69,57].

### 290 **3.4 Data analysis**

291 MSI data are multidimensional, and usually processed by in-built software visualizing as  
292 mass spectra (a plot of intensity vs.  $m/z$ ), and 2D-ion images corresponding to each ion  
293 signal (Figure 1). In 3D MSI, suitable 2D ion images acquired from a series of serial  
294 sections are combined to construct a 3D image. Each pixel of the ion image contains a  
295 mass spectrum of all molecules detected at that irradiated/sprayed area. Data  
296 normalization to the total ion current is often used to eliminate artifacts. MSI has the  
297 strength of combining (e.g., overlay, merge etc.) an ion image corresponding to a certain  
298 signal with the microscopic data (e.g. H&E stained image) of the same or serial tissue  
299 section allowing the interpretation of the spatial distribution within the histological  
300 context. Mass spectra from different regions of interest (ROIs) of an ion image can be  
301 extracted and compared. Multivariate analysis methods (MVA), particularly, principal  
302 component analysis (unsupervised method) and partial least-squares regression  
303 (supervised method) are widely used to classify or differentiate between ROIs or samples  
304 by reducing the dimensionality of the MSI data. MVA is often used in combination with  
305 discriminant analysis to examine how well the ROIs or samples are differentiated.  
306 Statistical analyses such as t-tests and ANOVA are performed to evaluate whether the  
307 observed distributional changes are significant. For biomarker discovery, a receiver  
308 operator characteristic curve analysis is often performed that calculates the sensitivity and  
309 specificity of individual ions.

310 Unambiguous molecular assignment to an  $m/z$  of interest is an important task in MSI. A  
311 careful assignment is highly recommended since an  $m/z$  value often presents a number of  
312 different molecular ions. In addition, two or more PLs can be detected at a single  $m/z$   
313 value in the spectra as PLs show diversity in their head group, acyl chain moieties, and  
314 adduct forms. The tentative assignments of PLs are performed by matching the observed  
315  $m/z$  value in the mass spectra to the reported literature or database of known compounds  
316 within an acceptable mass error range (typically 1-20 ppm, varies with the mass resolution  
317 of the analyzer). Several online databases such as human metabolome database  
318 (<https://hmdb.ca/>) [70], and lipid map (<https://www.lipidmaps.org/>) [71] are dedicated for  
319 this purpose. For unambiguous assignments, tandem mass spectrometry analysis (e.g.,  
320 on-tissue MS/MS, LC-MS/MS of lipid extracts) is routinely performed where individual  
321 lipid species yield characteristic fragment ions (e.g., fragments specific to the head groups,  
322 sn-1 and sn-2 fatty acyl groups etc.) [72]. The fragmentation patterns of the ion of interest  
323 are compared with those of a pure compound analyzed under the identical conditions.  
324 Immunohistochemical analyses of protein(s) related to the PL of interest are often  
325 performed for further confirmations of the specific PL assignment [73,74].

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#### 327 **4. Reports on PLs imaging by MSI**

328 MSI has been employed extensively to explore the spatial distribution of PL species in a  
329 variety of biological samples [75]. MALDI-MSI is the most widely used techniques for  
330 PLs imaging followed by the DESI-MSI and SIMS imaging. By far the most detected PL  
331 species are the PCs while the most imaged tissues are brain and tumors.

#### 332 **4.1 MSI revealed the region-specific distributions of individual PLs**

333 Distribution of specific PLs in different normal anatomical locations of biological tissues  
334 has been extensively studied. MALDI-MSI allowed visualizing the cell-selective  
335 distribution of polyunsaturated fatty acid-containing PCs (PUFA-PCs) in mouse brain:  
336 arachidonic acid-containing PCs and DHA-PCs were distributed in the hippocampal  
337 neurons and cerebellar Purkinje cells, respectively [76]. Two PLs, PI(38:4) and PC(36:1)  
338 were preferentially localized to the gray matter while PC(32:0) were preferentially  
339 localized to the white matter of rat brain [51]. PLs imaging in the human term placenta  
340 showed the differential distribution of PC(16:0\_20:4) between the stem and terminal villi  
341 [77]. Two PC molecules, PC(16:0\_18:2) and PC(16:0\_18:1) were detected as the  
342 dominant molecules in the human gastric mucosa near the fundic glands [78]. DESI-MSI  
343 was capable of imaging 1-O-alkyl phosphatidylethanolamines and phosphatidylserines,  
344 PE (18:1e/18:1), and PS (18:1e/18:1) in a thin ring in the outermost region of the human  
345 lens [79]. Some other MALDI-MSI studies showed the distributions of PLs in various  
346 biological tissues including rat brain [80,81,82], heart [83], liver [84], mouse kidney [85],  
347 lung [86] and human lens [87]. ToF-SIMS imaging revealed the distribution of some  
348 specific PLs in the sections of the mouse brain [88], rat brain [89] and human skeletal  
349 muscle [90]. While the most imaged samples in the literature are tissues, several studies  
350 applied MALDI-MSI to profile PLs distribution in the whole body of some species  
351 including *Caenorhabditis elegans* [91], and *Drosophila melanogaster* [92].

#### 352 **4.2 PLs imaging determined the margin of diseased tissue**

353 MSI has successfully revealed the altered distribution of some specific PLs in several  
354 diseased tissues thereby discriminating between diseased and healthy tissues [93,94]. This  
355 power of demarcation is of great help for the successful surgery of diseased tissue,  
356 particularly the tumors. In a MALDI-MSI study, our group reported that PC (36:1) was  
357 highly abundant in human breast cancer tissues than in the references [73] Margulis et al.  
358 used DESI-MSI and reported the distinct distribution of PG(18:1\_16:0) and the relatively  
359 higher distribution of PS(18:1\_18:0) and PI(18:0\_20:4) in the human basal cell carcinoma  
360 tissue than in the normal skin [95]. Distinct distributions of PLs including PI(18:0/20:4)  
361 were seen in human seminoma and adjacent normal tissues [94]. In a study, 37 resected  
362 hepatocellular carcinoma tissues were analyzed by MALDI-MSI and revealed an increase  
363 of PC(16:0\_16:1) and a decrease of LPC(16:0) in the cancerous region than in the normal  
364 region [96]. MALDI-MSI showed a higher accumulation of stearic acid and arachidonic  
365 acid-containing PI, PI(18:0\_20:4), in the thickened wall than in the thinned wall of  
366 intracranial aneurysms [97]. Imaging of the ischemic rat brain revealed the production of  
367 LPC(16:0) in the area of focal cerebral ischemia [98].

#### 368 **4.3 PLs imaging showed the potentiality to the therapeutics discovery**

369 A large number of MSI studies were performed to examine the changes of PLs level in  
370 tissues after a certain intervention in animal or insect, and thereby revealed the promising  
371 therapeutic agents for diseases. Recently, our group imaged PLs in the dorsal root  
372 ganglion (DRG) of mice after sciatic nerve transection (SNT) using MALDI-FTICR  
373 imaging at a spatial resolution of 25 $\mu$ M. The study showed that the arachidonic acid-  
374 containing PC (AA-PC), PC(16:0\_20:4) highly increased while other two PCs and one  
375 PA(36:2) molecule decreased in the DRG following nerve transection [99]. LPA, an  
376 initiator of neuropathic pain, is generated from PA by phospholipase A2 enzymes [100]  
377 speculating that PA(36:2) is consumed to produce LPA [99]. Thus, the study suggested  
378 the potential use of LPA blocker for the treatment of neuropathic pain [99]. Our group  
379 employed MALDI-MSI and showed that a stearate and docosahexaenoic acid (DHA)  
380 containing PC (DHA-PC), PC(18:0\_22:6), depleted in the grey matter of a post-mortem  
381 human AD brain [101] which was in excellent agreement with another study that revealed  
382 the improved memory function and distribution levels of brain DHA in senescence-  
383 accelerated mice P8 (SAMP8 mice), a model of dementia, supplemented with green nut  
384 oil (a rich source of  $\alpha$ -linolenic acid) or DHA [102]. Recently, we used DESI-MSI  
385 technique to image the DHA-PCs in the brain of SAMP8 mice fed with green nut oil or  
386 DHA. SAMP8 mice brain showed the lower distribution of PC(16:0\_22:6), and  
387 PC(18:0\_22:6) compared to that of wild type mice. Interestingly, green nut oil or DHA  
388 treatment restored the decreased distribution of PC(16:0\_22:6) and PC(18:0\_22:6) in the  
389 brain of SAMP8 mice [72] suggesting the potential use of DHA and green nut oil in the  
390 prevention and treatment of dementia. Philipsen et al. examined the distribution of some  
391 individual phospholipids in Drosophila brain after the administration of cocaine and  
392 methylphenidate by ToF-SIMS imaging with a spatial resolution around 3  $\mu$ M and  
393 revealed the possible involvement of brain lipids in learning and memory. Before  
394 treatment, two PCs, PC(34:1) and PC(34:2) were found to be abundant in the central brain  
395 while PE(34:1) and PI(36:4) were distributed throughout the brain. Cocaine  
396 administration largely changed the distribution of those PLs: PCs became more abundant  
397 in the central brain and optical lobe areas whereas the overall intensities of PE and PI  
398 decreased [103].

#### 399 **4.4 MSI identified PLs as a biomarker and predictive factor of diseases**

400 MSI of PLs showed the promising power to the discovery of cancer biomarker and  
401 predictive factor of tumor recurrence which can potentially contribute to the improved  
402 disease management and monitoring of the disease progression. In a MALDI-MSI study,  
403 PC(16:0\_16:1) was revealed as a novel biomarker in colorectal cancer [104]. PC (32:1)  
404 is highly abundant in recurrent triple-negative breast cancer (TNBC) tissues compared to  
405 the non-recurrent TNBC tissues making it as a potential predictive factor of TNBC  
406 recurrence [105]. High resolution-MALDI-MSI revealed the lower expression of  
407 LPC(16:0/OH) in prostate cancer than that in the benign prostate epithelium of human.  
408 These differences in expression of PLs will potentially help predict prostate cancer  
409 aggressiveness, and provide novel insights into the lipid metabolism in prostate cancer  
410 tissue [106]. In DESI-MSI study, lipids including several individual PCs, PGs and CLs  
411 were identified as biomarkers as well as predictive factors of serous ovarian cancer [93].  
412 This methodology was utilized in classifying disease status, different subtypes and grades  
413 of several cancers including human bladder carcinomas [107], gliomas [108], renal cell  
414 carcinomas [109], and breast cancer [110]. Furthermore, Mao et al. showed the

415 application of AFAI-MSI in the rapid classification of human breast tumors based on the  
416 abundance of lipids including PLs [111].

#### 417 **4.5 Potential mechanism of cancer invasion revealed by PLs imaging**

418 Membrane fluidity is thought to largely depend on the fatty acyl species linked to the PCs  
419 [112]. Compositional changes in the acyl chains may thus contribute to the altered fluidity  
420 influencing the behavior of the cancer cells, such as invasion and metastasis. Mapping of  
421 superficial-type pharyngeal carcinoma tissue sections from five human patients showed  
422 the higher distributions of three AA-PCs, PC(16:0\_20:4), PC(18:1\_20:4) and  
423 PC(18:0\_20:4) in the subepithelial invasive region indicating the potential role of AA-  
424 PCs in the invasion mechanism [113].

#### 425 **4.6 CLs imaging**

426 It is now clear that MSI has shown tremendous progress on PCs imaging in the last several  
427 years. PCs are the most abundant molecules in the membranes and also dominate the mass  
428 spectra due to their higher ionization efficiency than the other PL species. Unfortunately,  
429 these highly abundant molecules contribute to the ion suppression of some other PLs of  
430 low abundance. Due to this fact, the most reported PLs in the literature are the PCs,  
431 followed by LPLs and CLs.

432 CLs are unique mitochondrial PLs that play important structural as well as functional  
433 roles in bioenergetics and signaling [114]. They exist in a relatively small amount with  
434 remarkable diversification and are difficult to detect due to the ion suppressive effect of  
435 the highly abundant molecules present on the tissue surface. So far, a decrease level of  
436 CLs were reported in the kidney cortex of non-alcoholic steatohepatitis mice using the  
437 conventional MALDI-MSI protocol [115]. Several methods have been developed for CLs  
438 imaging by reducing or eliminating the ion suppressive effects. Amoscato et al. showed  
439 that the treatment of the tissue surface (prior to MALDI-MSI) with phospholipase C and  
440 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide hydrochloride (EDC) eliminates the  
441 ion suppressive effects by removing the highly abundant phosphatidylcholine head  
442 groups and cross-linking the accessible carboxyl/amino-containing molecules on the  
443 tissue, respectively [116]. Using this approach, authors successfully mapped multiple CLs  
444 and demonstrated a nonrandom distribution of PUFA and non-PUFA containing CLs in  
445 different anatomical locations of the male rat brain. Interestingly, the habenular  
446 nuclear/dorsal third ventricle and lateral ventricle areas of the brain showed a robust  
447 signal for CLs and were defined as the CL “hot spot” [116]. By employing similar  
448 methods, it was revealed that the traumatic brain injury in rats resulted in the early  
449 depletions in polyunsaturated CLs in the contusional cortex, ipsilateral hippocampus, and  
450 thalamus [117]. Recently, Yang et al. demonstrated an effective MALDI-MSI method for  
451 CLs mapping based on the sample preparation with norharmane matrix that does not  
452 require additional digestion procedure [118]. DESI-MSI showed the capability of direct  
453 CLs imaging in normal and cancer tissues. Several individual CLs with a smaller number  
454 of double bonds were shown to significantly increased in v-myc avian myelocytomatosis  
455 viral oncogene homolog (MYC)-induced lymphomas compared with normal tissue [119].  
456 Furthermore, DESI-MSI identified cardiolipins as a molecular signature of mitochondria-  
457 rich thyroid oncocytic tumors [120].

#### 458 **4.7 Imaging of oxidized PLs**

459 PUFA- containing PLs (PUFA-PLs) in the membrane undergoes oxidation by various  
460 enzymes or reactive oxygen species producing oxidized PLs. Oxidized PLs are highly  
461 bioactive molecules having both pro-inflammatory and anti-inflammatory effects and are  
462 now considered as markers of biological oxidative stress [121]. Oxidized PLs in human  
463 and animal tissues have been extensively studied by the LC-MS techniques [122].  
464 Recently, there is a growing interest in imaging these modified PLs to discover biomarker  
465 for disease states and to advance the understanding of pathology. MALDI-MSI revealed  
466 a decrease of oxidizable PUFA-PLs including PS(18:0\_22:6) in traumatic brain injury  
467 [75]. However, no reports on oxidized PLs imaging have been demonstrated using that  
468 method. So far, DESI-MSI identified several oxidized-CL species in mitochondria-rich  
469 thyroid oncocytic tumors [120].

#### 470 **4.8 Imaging of single-cell PLs**

471 MALDI-MSI is capable of imaging at a spatial resolution less than 50  $\mu\text{M}$  [99] and has  
472 been applied in PLs imaging at the cellular level. In a MALDI-MSI approach, flow  
473 cytometry-sorted single multiple myeloma cells and normal plasma cells were imaged  
474 with 5  $\mu\text{M}$  laser ablation diameter and revealed a decrease of PC(16:0\_20:4) in the  
475 malignant cells [123]. AP-MALDI imaging with 7  $\mu\text{M}$  pixel size detected PC(32:1) and  
476 PC(34:1) primarily in the center of the HeLa cells where a large volume of membranes  
477 are expected [124]. However, these methods revealed only the gross PLs distribution on  
478 the cell surface.

479 So far, SIMS offers spatial resolution at a nanometer scale which is suitable for single-  
480 cell imaging. Unfortunately, PLs undergo severe fragmentations by the high energy  
481 primary ion beam resulting in the decreased spectral and image quality. Fragments of the  
482 phosphocholine head group are commonly seen in the mass spectra of SIMS, and they  
483 are often used to record the total PCs distribution. Ostrowski et al. employed TOF-SIMS  
484 under frozen-hydrated conditions to study the changes in the membrane lipid distribution  
485 during mating of *Tetrahymena thermophila* and observed a significant decrease of  
486 phosphocholine head group fragment along the conjugation junction [125].

487 Introduction of cluster ion sources and modifications in the sample preparation protocol  
488 have been developed discussed previously to reduce the fragmentations. Addition of  
489 matrix increased the signal intensity of several PCs including PC(34:1) on single-cell  
490 surface cultured on gold-coated silicon wafers [58]. Unfortunately, the crystals formed  
491 by matrix-coating mask some structural details on the cell surface, resulting in the poor  
492 image quality.

#### 493 **4.9 3D imaging of PLs**

494 With the advancement of the image reconstruction method and the high-throughput  
495 analysis, MSI showed the ability to generate 3D images of PLs distribution in an entire  
496 organ or tissue. Patterson and coworkers developed a robust 3D MSI method using open-  
497 source software and revealed the distributions of LPC and PC molecules in human and  
498 mice carotid atherosclerosis tissues [126]. 3D MALDI-MSI technique has been applied  
499 to map PLs in newly fertilized zebrafish embryos: PCs were mostly distributed inside the  
500 yolk as well as in the blastodisc, while PEs and PIs were distributed minimally inside the  
501 yolk [127]. Paine et al. performed 3D MALDI-MSI on whole brains from a mouse model  
502 of human medulloblastoma and identified several PA, PE, PS, and PI molecules

503 associated with the metastasis [128]. DESI-MSI and SIMS imaging have also been  
504 utilized for 3D imaging of PLs in various tissues that include mice brain [129],  
505 glioblastoma xenograft [130], and *Aedes aegypti* ovarian follicles [131].

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508

## 509 **5. Conclusions**

510 PLs imaging in complex biological tissues and cells has been revolutionized by the  
511 development of label-free MSI techniques. MALDI-MSI, DESI-MSI and SIMS imaging  
512 are the widely used MSI methods for the imaging of specific PLs, so far. MSI revealed  
513 the region-specific distributions of specific PLs in various tissues. Imaging of individual  
514 PLs demarcated the margins of cancers and contributed to the discovery of potential  
515 therapeutics, biomarkers, and predictive factors of diseases. Interestingly, the potential  
516 role of PLs in cancer invasion has been revealed by this technique. The abundance and  
517 the higher ionization efficiency of PCs contribute to the suppression of other PLS  
518 including CLs and oxidized PLs. Development in the sample preparation protocol enabled  
519 the successful imaging of CLs by reducing the ion suppression.

520

## 521 **6. Expert opinion**

522 MSI is a powerful method that has the capability of untargeted analysis of hundreds of  
523 molecules simultaneously in complex biological tissues and cells without labelling. These  
524 unique features made it a valuable tool for the visualization of proteins [132], lipids [36],  
525 nucleotides [133], neurotransmitters [134], small metabolites [135], and exogenous  
526 compounds [136] in a wide range of biological samples. It has become an indispensable  
527 tool for the imaging of individual PLs so far and greatly contributed to explore biomarkers  
528 as well as altered lipid distribution in various diseased states. However, there are many  
529 challenges in MSI of PLs exist.

530 Because of the variations of the acyl chains and the head groups, thousands of PL species  
531 could be present in a living cell. The head group attached to the phosphoric acid as well  
532 as the acyl chains of the glycerol backbone are detected and identified in tandem mass  
533 spectrometry. Unfortunately, the positional analysis of these chains (acyl chains specific  
534 to sn-1 and sn-2) is not possible in the current situation. In addition, on-tissue tandem  
535 MSI is often problematic due to the less sensitivity and the difficulty to isolate a target  
536 molecular ion from a myriad of molecules of the complex tissue sections. Therefore,  
537 imaging of PLs warrants further advancement of the mass analyzer for positional analysis  
538 of acyl chains and on-tissue MS/MS.

539 MSI is a surface analysis method and provides only the semi-quantitative information of  
540 the analytes. Although a lot of progress have been made in the development of methods,  
541 quantification is still one of the greatest challenges in MSI. A strategy for quantitative  
542 analysis could be the development of calibration curves using internal standards.



543 MALDI-MSI offers a better sensitivity for PLs detection than the other currently used  
544 MSI techniques. However, the choice of a matrix and the spray conditions directly affect  
545 the ionization efficiency and spatial resolution. A lot of efforts have been made to explore  
546 novel matrix and optimized spray conditions for successful imaging. Our group  
547 developed a two-step matrix application technique namely “spray-droplet method” that  
548 produce homogenous layer with minute crystals resulting in the improved ionization  
549 efficiency for MALDI-MSI [137]. Further development of matrix or alternative to the  
550 matrix that form homogenous crystals exclusively with polar lipids will potentially  
551 contribute to the PLs imaging with higher ionization efficiency and higher spatial  
552 resolution. The use of nanoparticle instead of matrix enabled the visualization of several  
553 PLs at an improved resolution (15  $\mu\text{m}$ ) in mammalian tissues [138].

554 DESI-MSI, a relatively new and nondestructive imaging method, has attracted much  
555 attention due to its capability of matrix-free simple analysis. In our experience, DESI-  
556 MSI is a suitable technique for analyzing small molecules [139,140,141] although it’s  
557 spatial resolution and the ionization efficiency for PLs are lower than that of MALDI-  
558 MSI. DESI-MSI has a great potential of live imaging of biological surface molecules in  
559 clinical settings since it does not require any special sample preparation. Higher spatial  
560 resolution can be achieved through the advancement of the sprayer conditions (such as  
561 solvent flow rate, gas flow rate etc.) and the geometry of the ionization chamber while  
562 ionization efficiency for PLs can be improved by exploring the right solvent composition.

563 Nowadays, the need for PLs imaging at the sub-cellular level is increasing. Unlike tissue  
564 section imaging, single-cell imaging presents a great challenge as it requires techniques  
565 with high spatial resolution as well as high sensitivity because of the microscopic size  
566 and extremely small volume of analytes in a single cell. In DESI-MSI, pixel size is  
567 typically in the range of 50–200  $\mu\text{m}$ , and thus single-cell structures cannot be resolved  
568 with this spatial resolution. Single-cell imaging by MALDI-MSI has revealed only the  
569 gross distributions of PLs. So far, SIMS has shown the capability of resolving features at  
570 the nanometer scale which allows for the imaging at the sub-cellular level. Unfortunately,  
571 it is difficult to detect PLs by this technique since fragmentation occurs during analysis.  
572 Some advancement including the use of cluster ion sources and the sample analysis under  
573 frozen-hydrated state significantly improved the PLs imaging.

574 Although MSI was primarily developed for 2D imaging, it has been applied for 3D  
575 imaging that significantly enhanced our understanding of the molecular processes.  
576 However, the current methodology for 3D MSI is laborious and time consuming.  
577 Interestingly, an automated 3D DESI-MSI method has been demonstrated for rapid  
578 analysis. In this technique, a robotic slide loader is installed to autonomously mount and  
579 change slides on the sample stage while the 2D ion images are automatically constructed  
580 and aligned to generate a 3D image [130].

581 Imaging of low abundance PLs including the CLs and oxidized PLs is still in its infancy.  
582 Methodological developments that reduce the ion suppression effect, and increase the  
583 sensitivity are warranted for the imaging of these molecules.

584

585

## 586 **7. Acknowledgements**

587 This work was supported by MEXT/JSPS KAKENHI (Grant Number JP15H05898B1),  
588 AMED (Grant Number JP20gm0910004), JSPS KAKENHI (Grant Number  
589 JP18H05268), and MEXT Project for promoting public utilization of advanced research  
590 infrastructure (Imaging Platform) under grant number JPMXS0410300220, Japan.

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