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## **Biomarker identification in liver cancers using desorption electrospray ionization mass spectrometry (DESI-MS) imaging - an approach for spatially resolved metabolomics**

Hui Chen<sup>1,2,3</sup>, Sylvère Durand<sup>1,2</sup>, Olivia Bawa<sup>4</sup>, Mélanie Bourgin<sup>1,2</sup>, Léa Montégut<sup>1,2,3</sup>, Flavia Lambertucci<sup>1,2</sup>, Omar Motiño<sup>1,2</sup>, Sijing Li<sup>1,2,3</sup>, Uxía Nogueira-Recalde<sup>1,2,5</sup>, Gerasimos Anagnostopoulos<sup>1,2,3</sup>, Maria Chiara Maiuri<sup>1,2,6</sup>, Guido Kroemer<sup>1,2,7,#</sup> and Isabelle Martins<sup>1,2,#</sup>.

<sup>1</sup> Centre de Recherche des Cordeliers, Equipe labellisée par la Ligue contre le cancer, Inserm U1138, Université Paris Cité, Sorbonne Université, Institut Universitaire de France, Paris, France ;

<sup>2</sup> Metabolomics and Cell Biology Platforms, UMS AMICCa, Gustave Roussy, Villejuif, France;

<sup>3</sup> Faculté de Médecine, Université de Paris Saclay, Kremlin Bicêtre, France ;

<sup>4</sup> PETRA, UMS AMICCa, Gustave Roussy, Villejuif, France;

<sup>5</sup> Rheumatology Research Group (GIR), Biomedical Research Institute of A Coruña (INIBIC), Professor Novoa Santos Foundation, A Coruña, Spain;

<sup>6</sup> Department of Molecular Medicine and Medical Biotechnologies, University of Napoli Federico II, Napoli, Italy;

<sup>7</sup> Institut du Cancer Paris CARPEM, Department of Biology, Hôpital Européen Georges Pompidou, AP-HP, Paris, France.

#Correspondence: Guido Kroemer ([Kroemer@orange.fr](mailto:Kroemer@orange.fr)) or Isabelle Martins ([isabelle.martins@inserm.fr](mailto:isabelle.martins@inserm.fr)).

**Contact Information:**

Centre de Recherche des Cordeliers U1138

Metabolism, Cancer and Immunity laboratory

15 rue de l'Ecole de Médecine

75006 PARIS

FRANCE

**Running head:** Biomarker identification in liver cancers using DESI-MS imaging

## **Abstract**

Liver cancers are characterized by interindividual and intratumoral heterogeneity, which makes early diagnosis and the development of therapies challenging. Desorption electrospray ionization mass spectrometry (DESI-MS) imaging is a potent and sensitive MS ionization technique for direct, unaltered, 2D and 3D imaging of metabolites in complex biological samples. Indeed, DESI gently desorbs and ionizes analyte molecules from the sample surface using an electrospray source of highly charged aqueous spray droplets in ambient conditions. DESI-MS imaging of biological samples allows untargeted analysis and characterization of metabolites in liver cancers to identify new biomarkers of malignancy. In this chapter, we described a detailed protocol using liver cancer samples collected and stored for histopathology examination, either as frozen or as formalin-fixed, paraffin-embedded specimens. Such hepatocellular carcinoma samples can be subjected to DESI-MS analyses, illustrating the capacity of spatially resolved metabolomics to distinguish malignant lesions from adjacent normal liver tissue.

**Keywords:** DESI-MS; spatial metabolomics, liver, cancer

## **1. Introduction**

Metabolic syndrome is associated to cardiovascular disorders, insulin resistance (IR), diabetes mellitus, obesity, and non-alcoholic fatty liver disease (NAFLD) *(1)*. NAFLD is the clinical symptom of metabolic syndrome in the liver, and is characterized by an abnormal and excessive accumulation of intrahepatic fat in the form of triglycerides (steatosis) in the absence of excessive alcohol consumption (>5% of hepatocytes are affected on histology) *(2)*. NAFLD can progress to non-alcoholic steatohepatitis (NASH), which increases the risk of liver damage by developing liver fibrosis, cirrhosis, and leads to hepatocellular carcinoma (HCC) *(3)*.

HCC is the most common primary liver cancer and accounts for 90% of cases in the world *(4)*. The risk factors of HCC include hepatitis B and C virus infection, alcohol consumption and non-alcoholic steatohepatitis developing in the context of diabetes and obesity *(5)*. Metabolic reprogramming has been characterized as hallmarks of liver cancer and presents opportunities for cancer diagnosis and devise novel targeted cancer treatment *(6, 7)*. The alpha- fetoprotein (AFP) level is the only validated marker for HCC and is no longer recommended by several learned societies for HCC screening due to a lack of sensitivity *(8)*. However, it represents a prognostic factor and is widely used in postoperative recurrence monitoring *(9)*. In the case of small HCC accessible to treatment with curative intent, its prognostic value is controversial *(10)*. The best treatment option for HCC is orthotopic liver transplantation (OLT) as this simultaneously treats the cancerous disease and its cause, cirrhosis *(11)*. Unfortunately, the majority of HCC patients are diagnosed at an advanced stage and hence are not candidates for OLT. The other therapeutic treatments used against HCC are chemoembolization, local oncolysis, radiotherapy, surgical resection and chemotherapy *(12)*. Currently, therapeutic options for the treatment of advanced HCC are insufficient due to limited responses to the treatments. Overcoming the limitations of these therapies represents an urgent medical need.

It is therefore important to identify new prognostic markers and to develop new therapeutic strategies against HCC.

The high-throughput detection and identification of small molecules (metabolites) in tissues or biofluids is referred to as metabolomics (*13*). Metabolomics has found diverse applications in liver studies in both healthy and diseased tissue and allows biomarker discovery for future evaluation (*14*). Metabolomic analyses of plasma or serum have identified shifts in systemic metabolism that might contribute to HCC pathogenesis (*15*). The development of spatial metabolomics offers the chance to identify the localization of specific metabolites in tissues. Thus, changes in the abundance of small molecules can be directly linked to anatomical characteristics of metabolically heterogeneous tissues including the cancerous liver (*16, 17*). With the aid of a "soft" ionization technique known as DESI-MS (desorption electrospray ionization mass spectrometry imaging), metabolite changes can be studied including in complex lipid species (*18, 19*). The type of organic solvent and its specific composition used in DESI-MS have a significant impact on the metabolites that can be desorbed from the surface. Current publications have made it possible to optimize sample preparations to achieve ideal conditions for the use of DESI-MS (*20*). In this chapter, we propose a protocol allowing to subject tissue samples that have been collected and stored for histopathological purposes (rather than specifically for metabolomics) to DESI-MS, thereby retrieving spatially resolved information on HCC.

## **2. Materials**

### **2.1. Disposables**

1. Polysine slide 75x25 mm
2. Histological cassette
3. Microtome
4. Drying plate
5. Coverslip
6. -20°C fridge
7. -80°C fridge
8. Cupules
9. SGE syringe
10. Liquid nitrogen portable tank

### **2.2. Equipment**

1. MilliQ water generator Reference A+
2. Cryostat-microtome
3. Microtome
4. Oven at 37°C
5. -20°C fridge
6. -80°C fridge
7. Mass spectrometer QTOF SYNAPT XS (Waters Inc.)
8. Source DESI XS (Waters Inc.)
9. Source ESI (Waters Inc.)

### **2.3. Software**

1. MassLynx (v. 4.2) (Waters Inc.)
2. HDI (v. 1.6) (Waters Inc.)

## **2.4. Reagents**

1. Methanol (MeOH)
2. Formic acid
3. Leucine-Enkephaline (Waters Ink.)
4. Paraformaldehyde (PFA)
5. Optimal cutting temperature Quality (OCT)
6. Paraffin
7. Water
8. Xylene
9. Ethanol
10. Mayer's Hemalun
11. Eosine
12. Safranin
13. Mounting medium
14. Propan-2-ol
15. MilliQ Water
16. Liquid Nitrogen
17. PBS



### 3. Methods

#### 3.1. Sample preparations (Figure 1)

The presented protocols of sample preparations are not optimized for Mass Spectrometry Imaging (MSI). The protocol of this chapter follows the strict requirements of anatomical pathology studies. Further sample preparation development should be processed following innovative protocols to obtain a better compromise between MSI detection quality and staining analyses.

##### 3.1.1. Tissue in PFA (Paraformaldehyde) (*see Note 1*)

1. Collect fresh liver from mice.
2. Fix the liver in PFA 4% for 24 hours maximum (depending on the sample's size) at 4°C (*See Note 2*).
3. Put the sample in a histological cassette.
4. Dehydrate and embed in paraffin the sample with a LOGOS automation: ethanol 70%, ethanol 100% (2 baths), propan-2-ol (2 baths), paraffin.
5. Include the sample in paraffin blocks.
6. Cut paraffin blocks to obtain from 3 to 10  $\mu\text{m}$  thickness section with a microtome at room temperature (*see Note 3 and 4*): One slice for Hematoxylin and eosin staining (*see Note 5*) and two white slices (*see Note 6 and 7*) for the DESI-MS analysis (*see Note 8*).
7. Put a drop of sterilized distilled water on an adherent slide and spread the cut.
8. Put slides on a drying plate for 10 seconds.
9. Place slides in an oven at 37°C overnight (*see Note 9*).
10. Store the paraffined slices at -20°C.
11. Deparaffinize *ex-temporanea* the white slide, with a protocol including several baths of xylene, ethanol and water.
12. Dry the White slide at room temperature before analysis by DESI-MS.

### **3.1.2. HES staining:**

1. Put the slides in four successive baths of xylene for 2 minutes and three successive baths of ethanol and water for dewaxing and rehydration.
2. Stain the nucleus with Mayer's Hemalun for 4'30 then rinse in water.
3. Stain the cytoplasm with eosin for 2'30 and rinse in water and ethanol 100%.
4. Stain the fibers with safranin for 3'30 and rinse in ethanol 100%.
5. Dehydrate the slides with three successive baths of ethanol 100% and xylene.
6. Mount the slides into the coverslip with a permanent mounting medium.

### **3.1.3. Tissue in OCT (Optimal Cutting Temperature) compound**

1. Pick up fresh liver from mice.
2. Enclose the liver in a cupule filled with OCT compound.
3. Throw the cupule in a bath of liquid nitrogen (*see Note 10*).
4. Cut OCT blocks to obtain a 4  $\mu\text{m}$  thickness section with a microtome-cryostat at  $-20^{\circ}\text{C}$  (*see Note 11 and 12*): one slice for Hematoxylin and eosin staining (*see Note 5*) and two white slices (*see Note 6 and 7*) for the DESI-MS analysis (*see Note 8*).
5. Remove OCT from tissue (*see Note 13*).
6. Store the slices at  $-80^{\circ}\text{C}$ .
7. Unfrozen *ex-temporanea* the white slide.
8. Dry the white slide at room temperature before analysis by DESI-MS.

### **3.2. MSI acquisition (Figure 2 and Figure 3)**

Mass spectrometry imaging (MSI) analysis is performed with a Synapt XS Waters equipped with a DESI XS source (Desorption Electrospray source ionization). Calibrated Mass

spectrometer (MS) instrumentation weekly with NaF clusters in negative and positive modes. Instrumentation is controlled by MassLynx (v 4.2).

1. Operate successive acquisitions in a positive and negative mode in full scan *Sensitivity* MS mode (*see Note 14*).
2. Do not activate the Ion mobility separation (IMS) for this experiment (*see Note 15*).
3. Set DESI source parameters: capillary 0.7 kV in positive mode (*see Note 16*), capillary 0.5 kV in negative mode (*see Note 17*), source temperature at 150 °C, and heat transfer line at 250 °C.
4. Use the sprayed buffer (methanol/water (9/1) with 0.1 % of formic acid and 500 pg/ml of Leu-Enkephaline as a lock spray to desorb the biological tissue.
5. Use a 10ml SGE syringe (*see Note 18*).
6. Set the Flow of the buffer to 2 µl/min (*see Note 19*).
7. Set the Gaz of nebulization to 1.1 bar.
8. Set the head sprayer angle to 75° (*see Note 20*).
9. Set Imaging parameters in HDI Waters' software.
10. Set the spatial resolution to 50 µm<sup>2</sup>, and the scan speed to 150 µm/s, implying the scan time to 0.319 s (*see Note 21*).
11. Set the mass range from m/z 100 to m/z 1200, with a lock spray (Leu-Enkephaline) of m/z 556.28 in positive mode, and of m/z 554.26 in negative mode (*see Note 22*).
12. Set Trap CE to 4.
13. Set transfer CE to 2 V (*see Note 23*).

### **3.3 Post-acquisition data treatment**

1. Use the HDI waters' software to perform data treatment.

2. Choose parameters to scan the totality of the mass range (from  $m/z$  100 to  $m/z$  1200) at a resolution of 10000.
3. Search 1000 highest ion signals in the unbiased approach of the software.
4. Normalize the ion response of the 1000 ion signal with the ion signal of the lock spray.
5. Search for a biological relevant map of ion signal (*see Note 24*) (**Figure 4**).
6. Once an interesting metabolite is selected, process a Spearman correlation via HDI to find out other relevant metabolites correlated with the first one.
7. Once a list of ion signals is settled, perform a manual attempt of annotation matching  $m/z$  obtained from HDI and online databases (Human Metabolome Database, lipid maps, KEGG, Pubchem, chebi).

#### 4. Notes

1. This fixation protocol is also well known as FFPE, formalin-fixed paraffin-embedded.
2. Use fresh PFA 4%; make a dilution (1:4) of PFA 16% with a clean PBS extemporaneously to obtain a better fixation of the tissue.
3. The width of the slice has minor importance for the DESI-SYNAPT XS analysis. However, it is crucial that the cut is with no imperfection.
4. The width of the slice is driven by the requirement of the HES (Hematoxylin eosin safranin stain) analysis which doubles the Mass Spectrometry Imaging (MSI) analysis.
5. HES is considered as a classical standard for histology characterization. We use HES here as a technical positive control for MSI interpretation.
6. The white slide is non-colored tissue.
7. The first slice is for analysis, and the second is for backup.
8. It is also possible to produce only one white slide, proceed to analyze the DESI-MS, then treat the slice for HES and proceed to histology. This overcomes the concern about the thick cells and very fragile tissue (e.g. “swiss roll” intestine histology). Nevertheless, this implies a sharp synchronization between the different platforms involved in the analyses.
9. For fragile tissues, dry the slides for 2 days in an oven at 37°C. This will improve the adhesion of the tissue to the slide and avoid detachment.
10. This freezing technique is also called snap freezing.
11. This sample preparation avoids temperature variation unlike PFA treatment and is recommended for MS analysis of labile molecules. We should consider the fact that PFA is widely used by users’ platforms, and we propose the two sample preparations.
12. To note, as we work in an unbiased MS method, the ideal procedure would be to perform analyses of both OCT and PFA samples. This can be reached by cutting in two pieces the fresh

liver and doing in parallel the two sample preparations. In this latter case, however, we would lose the capacity to superimpose layers of data to correlate multimodal imaging analyses.

13. When the glass slide with the frozen OCT-imbibed tissue reaches room temperature, OCT becomes liquid and can be easily removed from the tissue.

14. The Sensitivity mode allows a better ion response, and the lower mass resolved mode with a resolution of 12500.

15. IMS could improve accuracy in molecule detection but on another hand can reduce the dynamic range, and dramatically increase the acquisition file size.

16. This value is enough to correctly ionize metabolites without leading to electrical discharges.

17. Voltage is lower in negative mode due to a higher risk of discharge in this polarity.

18. This syringe allows a long acquisition time. Suitable infusion syringe pumps are required to stand counter pressure.

19. It is mandatory to always get flow in the emitter of the head sprayer, and the capillary silicate tube connected to it, in order to avoid sedimentation. When instrumentation is on standby, flow is usually set at 0.5  $\mu\text{l}/\text{min}$ .

20. It is highly recommended to not change this angle.

21. This allows a faster acquisition. However, it is recommended to be closed with a scan time of 1 s.

22. Lock spray is a standard molecule, diluted in the syringe containing the sprayer's buffer. The concentration of the lock spray is 500 pg/ml.

23. These parameters are typical for full-scan MS without any selection or fragmentation by CID.

24. This relevance was determined by the data interpretation of the HES slide from the same tissue section and processed by the anatomy pathologists.

## **Conflicts of interest**

GK has been holding research contracts with Daiichi Sankyo, Eleor, Kaleido, Lytix Pharma, PharmaMar, Osasuna Therapeutics, Samsara Therapeutics, Sanofi, Sotio, Tollys, Vascage and Vasculox/Tioma. GK has been consulting for Reithera. GK is on the Board of Directors of the Bristol Myers Squibb Foundation France. GK is a scientific co-founder of everImmune, Osasuna Therapeutics, Samsara Therapeutics and Therafast Bio. GK is the inventor of patents covering therapeutic targeting of aging, cancer, cystic fibrosis and metabolic disorders.

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## Figures Legends

**Figure 1. Sample preparation for DESI-MS analysis.** OCT and FFPE (paraformaldehyde-based) tissue preparations are the two main processes used by histopathological platforms.

**Figure 2. DESI-MS technology.** (A) In the blue circle, the pump pushes the buffer of methanol/water (9/1) comprised in a 10ml syringe. The buffer is driven towards the spray header within a silicate capillary (yellow). (B) The silicate capillary is plugged into the sprayer head (purple rectangle). (C) Spray header and heated transfer tube.

**Figure 3: DESI-MSI ionization process.** The position of the head sprayer is critical for the optimization of the ion signal and spatial resolution. Typically, the values presented here are the default parameters in the DESI XS Waters apparatus.

**Figure 4: Analysis on FFPE slide of mouse liver cancer.** Figure 4 shows (A) Hematoxylin and eosin stain (HES) and (B)(C)(D) three MSI acquisitions representing a three m/z ratio. (A) shows in light purple the tumor part of the tissue (on the left), and in pink (right part) the healthy liver. The HES is the standard control to compare with the MSI imaging. (B) and (C) images show images corresponding to two metabolites (namely, m/z 217.1129 and m/z 409.3051) that are similarly abundant in malignant and normal tissue. (D) shows an image of the spatial distribution of a metabolite (m/z 295.2032), which is only present in the healthy part of the liver.







