INCREASE IMAGE CLARITY AND SPECIFICITY USING DUAL MSI SOURCES AND MULTI-REFLECTING TIME-OF-FLIGHT ANALYZING METABOLITES IN MOUSE TESTIS

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INTRODUCTION

Tight regulation in lipid homeostasis is critical for male fertility and in the normal process of germ cell development, extensive lipid remodelling occurs in different cell types which is poorly understood. Mass spectrometry imaging provides spatial localization of different molecular species in the adult mouse testis but there are challenges with conventional imaging given the highly complex tissue structure and marked heterogeneity in cell types[1,2].

With the improvements developed for desorption electrospray ionisation (DESI) and matrix-assisted laser desorption ionization (MALDI) sources such as higher spatial resolutions as well as advances in time-of-flight technology, we have investigated lipid metabolites of the different compartments within a mouse testis using high resolution mass spectrometry imaging (MSI) for increased specificity and high mass accuracy.

METHODS

Tissue sample preparation

Experiments were conducted on mouse testis tissue sections, which were sectioned using a cryotome, deposited onto standard microscope slides and preserved at -80°C until analysis by mass spectrometry was performed.

For MALDI experiments, a HTX M5 (HTX Technologies) nebulizing spray device was used to evenly apply several coatings of α-Cyano-4-hydroxycinnamic acid (CHCA), 5 mg/mL solution in acetonitrile/water (70/30 *v/v*).

DESI imaging experiments require no sample preparation as desorption and ionization are initiated by charged droplets (95% MeOH, 5% water with 100pg/µL Leu-enkephalin) impacting directly on the surface.

Mass spectrometry

MSI experiments were carried out on a SELECT SERIES™ MRT (a quadrupole Multi-Reflecting Time-of Flight) mass spectrometer and SYNAPT™ G2-*Si* mass spectrometers equipped with a MALDI source and a DESI XS source.

The intermediate vacuum MALDI source employed a solid-state diode-pumped Nd:YAG (355 nm) laser with a repetition rate of up to 2.5 KHz.

	MRT MS	SYNAPT G2-*Si* MS
	MALDI	
Laser pulse rate	**1000 Hz**	**1000 Hz**
Ionisation mode	**(+)**	**(+)**
Pixel size (lateral)	**15 µm**	**30 µm**
	DESI	
Ionisation mode	**(+) and (-)**	**(+) and (-)**
Flow rate	**2 µl/min**	**2 µl/min**
DESI sprayer	**High Performance**	**1st generation sprayer**
Capillary voltage	**0.85 kV**	**4.5 kV**
Nebulising gas	**10 psi**	**70 psi**
Pixel size (lateral)	**25 µm**	**50 µm**

Data management

DESI and MALDI imaging datasets were mined using MassLynx™ as well as processed and visualized using High Definition Imaging Software (HDI™) v1.6 (Waters).

Regions of Interest (ROIs) defined in HDI and associated intensities were averaged and TIC normalized in the form of a .csv file which was loaded directly into Metaboanalyst (https://www.metaboanalyst.ca/MetaboAnalyst/faces/home.xhtml) for extensive statistical analyses.

Putative lipid identification were performed using suitable databases (LIPID MAPS® Lipidomics Gateway website).

RESULTS

Initial experiments were carried out by DESI imaging in positive ion mode using a SYNAPT G2-*Si* (Q-oaTOF) and MRT mass spectrometers. Mainly glycerophospholipids[3] and triglycerides were detected with a mix of potential cation types such as H+, Na+ and K+, increasing the complexity in lipid identification.

MS spectra from DESI ionisation on the SYNAT G2-*Si* and SELECT SERIES MRT are displayed in figure 1, when samples were analysed in position and negative ionisation mode. Ion images of the most abundant lipids can be viewed in figure 2 for the MRT MS experiment in positive ion mode.

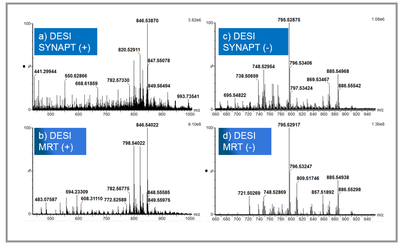

*Figure 1. **Positive ion mode** MS spectra comparing (a) SYNAPT G2-Si and (b) MRT mass spectrometers from the DESI imaging analyses of mouse testis.*

***Negative ion mode** MS spectra comparing (c) SYNAPT G2-Si and (d) MRT mass spectrometers from the DESI imaging analyses of mouse testis.*

From the positive ionisation mode, principal component analysis (PCA) was performed using eight ROIs drawn on the three main compartments of the testis using MetaboAnalyst. From the score plot (PC1 vs PC2), the groups were clearly separated.

The MRT instrument yields ppb mass accuracy, allowing confidence in the putative identification of PC (36:1) and PC (38:4) (both detected with a sodium and potassium cations) which was strongly localised in the interstitial regions where Leydig cells and blood vessels reside. PC (34:1) and PC (36:4) (Na^+ and K^+) ions were mainly localised in the Sertoli cells/early germ cell type and PC (38:5) and PC 38:6) were localised in the more mature germ cells.

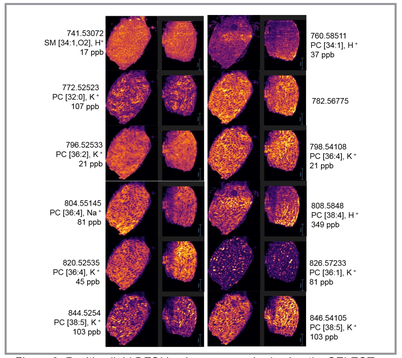

Figure 2. Positive lipid DESI ion images acquired using the SELECT SERIES MRT mass spectrometer .

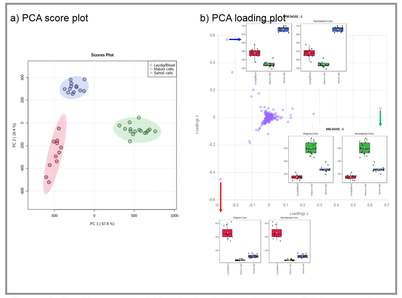

Figure 3. Positive mode of MetaboAnalyst statistical a) PCA score plot and b) Loadings plot.

Detection of isotopic fine structure for unambiguous elemental composition.

Comparing the datasets from Q-oaTOF (FWHM ~ 25,000) and SELECT SERIES MRT MS(FWHM ~ 200,000), there are numerous examples where the increased mass resolution obtained with the MRT MS allows several peaks to be resolved whereas only one was detected from the SYNAPT G2-*Si* MS datasets.

For example, at m/z 848.55676, potassiated PC 38:4 is mainly localised in the interstitial regions where Leydig cells and blood vessels reside. With the ultra high MS resolution, it is possible to differentiate isotope fine structure. The two other peaks with similar localisations correspond to isotope ^{41}K and 2 ^{13}C of potassiated PC 38:5 (figure 4).

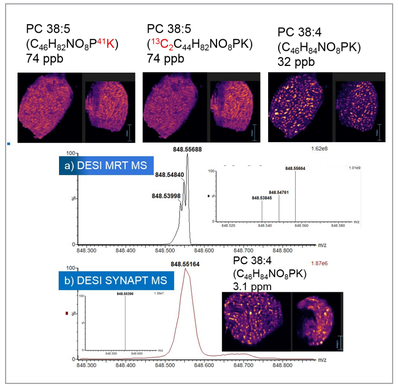

Figure 4. Increased MS resolution can distinguish isotope fine structure to clarify the MS ion images a) DESI MRT MS and b) DESI SYNAPT G2 -Si MS.

The example displayed in figure 5 highlights the images of three lipids with less than 21 mDa and having different localisation within the testes. There were identified with a mass accuracy better than 500 ppb as PS [36:0]+K+, PC [O-38:6]+K^+ and PC [38:5]+Na^+.

However from the SYNAPT XS MS data, one peak was detected which was an average of the two most intense peaks. The m/z was compromised and therefore the mass tolerance for the database search needs to be increased for a matching putative identification.

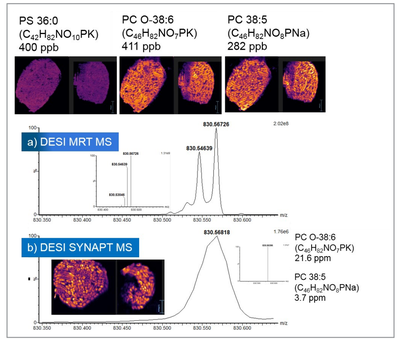

Figure 5. Increased MS resolution highlighting three different lipids with specific distribution within the testes. Sub 500 ppb mass accuracy increase identification confidence with a) DESI MRT MS data vs. b) DESI SYNAPT G2-Si.

Putative identification of lipids in DESI negative mode.

Consecutive testes tissue sections were analysed using the DESI XS MRT mass spectrometer. Figure 6 shows the overlay RGB ion images of m/z 795.52991, 782.49768 and 885.54987, respectively localized in Sertoli cells/early germ cells (red), mature germ cells (green) and Leydig/blood vessel cells (blue).

Putative identification based on mass accuracy sub 500 ppb was performed. The main lipid classes were Fatty acid (FA), Phosphatidylglycerol (PG), Phosphatidylserine (PS), Phosphatidylethanolamine (PS) and Phosphatidylinositol (PI).

More interestingly, the most abundant peak *m/z* 795.53, localized through the tubules, was identified as (C16:0-alkyl-C16:0-acyl seminolipid which is in accordance with the study carried out by Goto-Inoue et al[5]. Two reported seminolipids were also detected *m/z* 767.5 (C14:0-alkyl-C16:0-acyl seminolipid), *m/z* 821.5 (C18:1-alkyl-C16:0-acyl seminolipid).

Tentative ID	Formula	Expected mass	Observed mass	ppb error
FA (18:1)	C18H34O2	281.248604	281.24863	-92.4
FA (18:1;O)	C18H34O3	297.243518	297.24353	-40.4
FA (20:4) or ST (20:1;O2)	C20H32O2	303.232954	303.23294	46.2
FA (18:1;O2)	C18H34O4	313.238433	313.23837	201.1
FA (22:5)	C22H34O2	329.248604	329.24857	103.3
FA (22:4)	C22H36O2	331.264254	331.26422	102.6
PG (32:0)	C38H75O10P	721.502509	721.50269	-250.9
PE (36:4) or PE (O-36:5;O)	C41H74NO8P	738.507928	738.50787	78.5
PE (O-38:7)	C43H74NO7P	746.513014	746.513	18.8
PS 934:1)	C41H78NO10P	760.513408	760.51337	50.0
PS (O-36:5)	C42H74NO9P	766.502843	766.50299	-191.8
PE (38:4) or PE (O-38:5;O)	C43H78NO8P	766.539229	766.53906	220.5
C16:0-alkyl-C14:0-acyl	**C39H76O12S**	**767.498472**	**767.49811**	**471.7**
PE (39:6)	C43H78NO7P	776.523578	776.52374	-208.6
PS (36:4)	C42H74NO10P	782.497758	782.49768	99.7
C16:0-alkyl-C16:0-acyl	**C41H80O12S**	**795.529772**	**795.52991**	**-173.5**
PS (38:5) or PS (O-38:6;O)	C44H76NO10P	808.513408	808.51349	-101.4
C18:1-alkyl-C16:0-acyl	**C43H82O12S**	**821.545422**	**821.54547**	**-58.4**
PS (40:5)	C46H80NO10P	836.544708	836.54492	-253.4
PS (41:4) or PS (O-41:5;O)	C47H84NO10P	852.576008	852.57568	384.7
PI (36:4)	C45H79O13P	857.518553	857.51862	-78.1
PG (44:12)	C50H75O10P	865.502509	865.50262	-128.2
PG (44:11) or PG (O-44:12;O)	C50H77O10P	867.518159	867.51862	-531.4
PI (38:4)	C47H83O13P	885.549853	885.54987	-19.2
PI (40:5) or PI (O-40:6;O)	C49H85O13P	911.565503	911.56549	14.3
			RMS	203.4

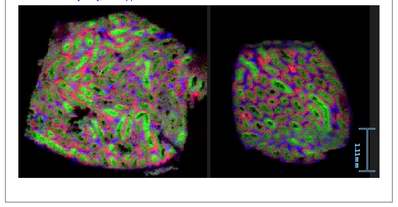

*Figure 6. Table reporting the putative lipid identification with a RMS of 203 ppb. RGB overlay of **m/z 795.52991 in Sertoli cells/early germ** cells , **782.49768 in mature** germ cells and **885.54987 in Leydig/blood vessel** cells.*

MALDI *vs.* DESI for MSI on the MRT mass spectrometer.

Finally analyses of the testis sections were performed in positive mode using the MALDI MRT mass spectrometer.

MS spectra between MALDI and DESI experiments show similar lipids in positive mode (figure 7), although after closer inspection some subtle differences were observed. Comparable RGB overlay ion images were generated showing certain localisation of lipids in specific tissue types of the testes.

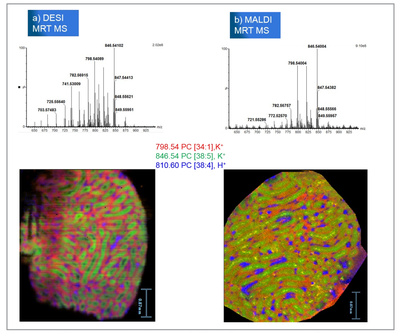

*Figure 7. Comparison MALDI vs. DESI MS spectra and RGB overlay ion images.(**m/z 798.54 in Sertoli cells/early germ** cells , **846.54 in mature** germ cells and **810.60 in Leydig/blood vessel** cells.*

CONCLUSION

- **Lipid profiles in the different tissue types within a mouse testis are radically different, in both positive and negative mode.**
- **With the 200,000 FWHM MS resolution on the SELECT SERIES MRT MS, baseline resolution of lipids with a less than 20 mDa mass difference were successfully imaged, allowing the detection of fine isotopic structure species.**
- **Mass accuracy on a wide range of lipids demonstrated sub-500 ppb and a RMS error of 203 ppb, increasing identification confidence.**
- **MALDI and DESI MSI allows the differentiation of specific tissue types in positive and negative ionisation mode. The use of the two ionisation techniques on the same mass spectrometer increase ionisation efficiency for complementary molecular detection.**

References

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