Supporting Information

Cholesterol Sulfate Imaging in Human Prostate Cancer Tissue by Desorption Electrospray Ionization Mass Spectrometry

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Material and Methods

Tissue Preparation:

All tissue samples were handled in accordance with approved institutional review board (IRB) protocols at Indiana University School of Medicine. Thirty four pairs of human prostatic tissue, obtained from 34 different patients were obtained from the Indiana University Medical School. All tissue samples were flash frozen in liquid nitrogen at the time of collection and subsequently stored at -80° C until sliced into 15 micrometers thick sections using a Shandon SME Cryotome cryostat (GMI, Inc., Ramsey, MN, USA). The thin tissue sections were thaw mounted to glass slides; each slide containing one section of tumor tissue and one section of adjacent normal tissue from the same patient. The glass slides were stored in closed containers at -80° C. Prior to analysis they were allowed to come to room temperature and then dried under nitrogen in a dessicator for approximately 20 minutes.

A serial section of each sample was formalin fixed and subsequently stained using hematoxylin and eosin (H&E) for pathological examination. These H&E stained slides were pathologically examined for tissue diagnosis. It is important to note that the serial sections subjected to pathological analysis are not ideal since they were obtained from flash frozen tissue and stained only using H&E, making pathological diagnosis of precancerous lesions more difficult. Paraffin embedded tissue pieces subjected to additional staining methods, including immunostaining are better for pathological examination and will be used in future studies.

In addition to these tissue samples, tissue from one patient (MH0107-01) was sectioned as described below (*Serial tissue sections from one patient)* and 20 sections were subjected to DESI-MS analysis and 10 interleafed sections where examined after H&E

staining. This sample was examined in such a way that a 3D image of the individual molecular ions could be obtained.

DESI Imaging:

The DESI ion source used in our experiments was a lab-built prototype, configured as described previously[1]. The spray solvent used for MS acquisition was acetonitrile:water (50:50) with a 5 kV spray voltage applied. Acetonitrile was purchased from Sigma-Alrich (St. Louis, MO, USA) and water (18.2 MΩ-cm) was from a PureLab ultra system by Elga LabWater (High Wycombe, UK). The nitrogen gas pressure was 150 psi and the solvent flow rate was 1.5 μL/min. The tissues were scanned using a 2D moving stage in horizontal rows separated by a 200 μm vertical step until the entire sample surface had been assayed. The surface moving stage included an XYZ integrated linear stage (Newport, Richmond, CA) and a rotary stage (Parker Automation, Irwin, PA). All experiments were carried out using a LTQ linear ion trap mass spectrometer controlled by XCalibur 2.0 software (Thermo Fisher Scientific, San Jose, CA, USA). An in-house program allowed the conversion of the XCalibur 2.0 mass spectra files (.raw) into a format compatible with the Biomap software (freeware, htto://www.maldo-msi.org). Spatially accurate images were assembled using the BioMap software. The color scale is normalized to the most intense (100% relative intensity) peak in the mass spectra.

PCA Analysis:

The DESI-MS imaging raw data were processed in Matlab 2009a, The MathWorks (Natick, MA, USA). Each pixel includes a full mass spectrum covering the full mass range recorded in the original data. All pixels were resampled to unit resolution and normalized by calculating the median area under the curve for the full image and scaling each pixel to this value. No background adjustment or smoothing filters were necessary. Resampling

dramatically decreases the computational complexity of the calculation. PCA was performed for each image individually. Eigenvalues and eigenvectors were ordered in terms of decreasing component variance. Only the first three principal components are used in the data in the present work. Each eigenvalue set corresponding to all pixels for a given principal component (e.g., all eigenvalues calculated for the first principal component eigenvector) were scaled between 0 and 1. For images developed using only one principal component, the eigenvalue set was plotted over the range blue to red (corresponding to 0 and 1, respectively) using the web color (RGB) scheme.

For plots consisting of data from multiple principal components, each set of eigenvalues, corresponding to one principal component, was scaled over the range 0 to 255 and convolved with a hexadecimal value characteristic of a primary color in the RGB scheme. That is, PC1 eigenvalues are convolved with red contribution, PC2 with green contribution, and PC3 with blue contribution. A full hexadecimal number is therefore generated for each pixel in the image and is characteristic of the first three principal components. This "web color" was then plotted to create the image.

Contributions to the developed PCA images may be understood by analyzing the loading plots associated with each principal component. An example of this is given for sample MH0301-17 in Figure S2. Cholesterol sulfate is (m/z 465.4) is a primary contribution to the variation in all three principal components, but most notably for PC2. In the mixed color PCA plot, PC2 is convolved with green, which is seen to correspond well to the existence of cholesterol sulfate in the single ion images.

Pathological Analysis:

An experienced pathologist, Liang Cheng, examined H&E stained tissue sections, prepared as described above. The pathology examination was made without knowledge of the DESI data. Table S1 summarizes the conclusions for the 68 sections examined.

Table S1. Summary of Results of Pathological Examination

	Pathological Diagnosis				
	NORMAL	**CANCER**	**PIN**	**CANCER/PIN**	**Probable PIN**
Patient Case Codes	MH0107-01	MH0107-01			
		MH0202-37	MH0202-37		
			MH0301-11	MH0301-11	
		MH0211-06			MH0211-06
	MH0301-17	MH0301-17			
		MH0212-04	MH0212-04		
	MH0108-32	MH0108-32			
			MH0206-29	MH0206-29	
		MH0204-30	MH0204-30		
	MH0205-17		MH0205-17		
			MH0112-01 (2)		
				MH0112-17	MH0112-17
		MH0203-51	MH0203-51		
		UH0001-38	UH0001-38		
				MH0305-06	MH0305-06
		MH0001-05	MH0001-05		
		UH0002-20	UH0002-20		
			MH0205-22 (2)		
			MH0204-24		MH0204-24
					MH0201-51 (2)
		MH0304-06	MH0304-06		
		MH0302-46 (2)			
		UH0002-08	UH0002-08		
	MH0301-40		MH0301-40		
		UH0005-17	UH0005-17		
			UH0002-28 (2)		
		UH0002-33	UH0002-33		
	UH0002-25	UH0002-25			
		UH0003-38	UH0003-38		
			UH0003-33 (2)		
					UH0004-18 (2)
			UH0002-29 (2)		
			UH0003-32 (2)		
	UH0003-12		UH0003-12		
Total	7	19	30	4	8

Of the 68 total tissue samples, 7 were diagnosed as normal tissue, 19 as cancerous tissue, 30 as containing PIN within normal tissue, 4 as containing cancer and PIN and 8 as probably PIN.

Comparison of DESI-MS with H&E pathological analysis:

Full mass spectra were recorded for each pixel, typically about 10^3 per tissue section. Comparisons between DESI images and H&E stains were made down to the single-pixel level (200 micron spot diameter). A signal at m/z 465 with a S/N greater than 5 was considered as positive for cholesterol sulfate.

Serial tissue sections from one patient:

After seeing the smallest amount of PIN in normal tissue in one patient (MH0107-01) tissue section, we performed a 3D experiment in which we sectioned the entire remaining block of tissue. The PIN was followed throughout the tissue piece by DESI-MS analysis on 20 sections, essentially recording a 3D distribution of cholesterol sulfate. The results were checked with and validated by pathological examination of H&E stains on 10 interleaved sections. To produce the necessary samples, the ca 2.7 mm thick tissue block was cut in 20 μm sections (total of 135 sections) and one section at approximately every 100 μm was used for DESI-MS analysis (of the 27 sections mounted for DESI after this procedure, results are reported for 20 and the remainder have been keep as archive slides). A section at every 220 μm was used for H&E staining and pathological examination. After the entire tissue pieces were sectioned, a total of 37 slides was obtained, 10 for H&E analysis and slide and 27 for DESI-MS analysis. This experiment greatly increases our confidence in the ability to detect normal and PIN tissue by DESI-MS.

Statistical validity of the data:

We consider the results to be preliminary mainly due to the limited number of samples (34 individuals) and the possibility of some unknown selection bias in the sample population since all tissue samples originated from the same location.

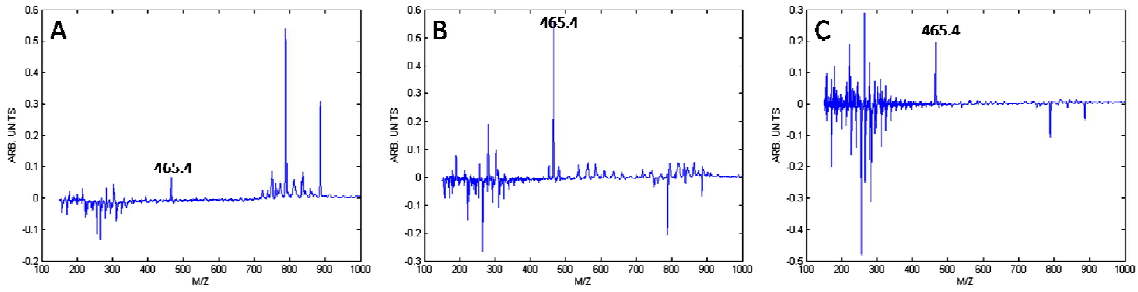

Figure S1. Loading plots for the first three principal components of PCA analysis of sample MH0301-17; (**A**) PC1, (**B**) PC2, and (**C**) PC3.

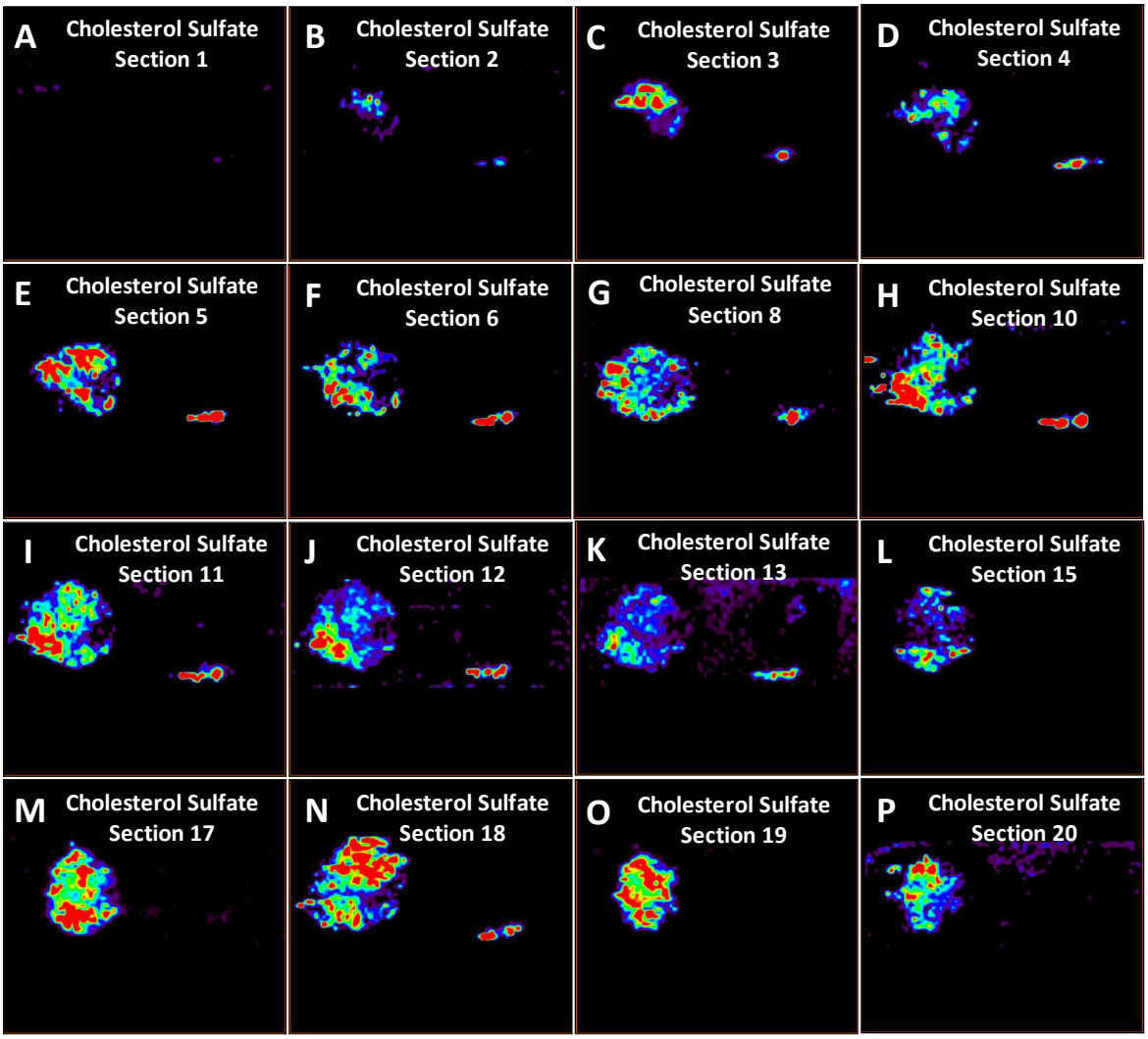

Figure S2. Negative ion mode tissue imaging of prostate tissue showing areas of cancer and adjacent normal tissue with PIN in sample MH0107-01. (A)-(P) Ion images of m/z 465.4, cholesterol sulfate for selected serial sections. Numbered serial sections from 1 to 20 are approximately 100 μm apart.

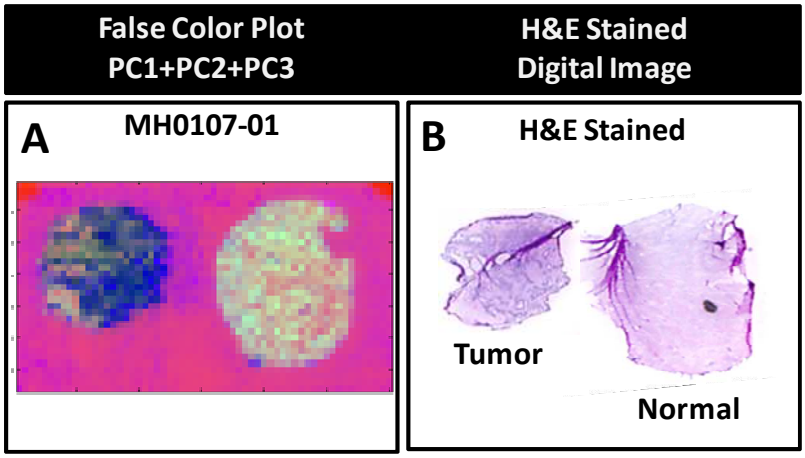

Figure S3. Sample MH0107-01 (A) PCA-developed images, false color plot of PC1, PC2, and PC3. (B) H&E stained tissue sections of the tumor tissue and normal with PIN.

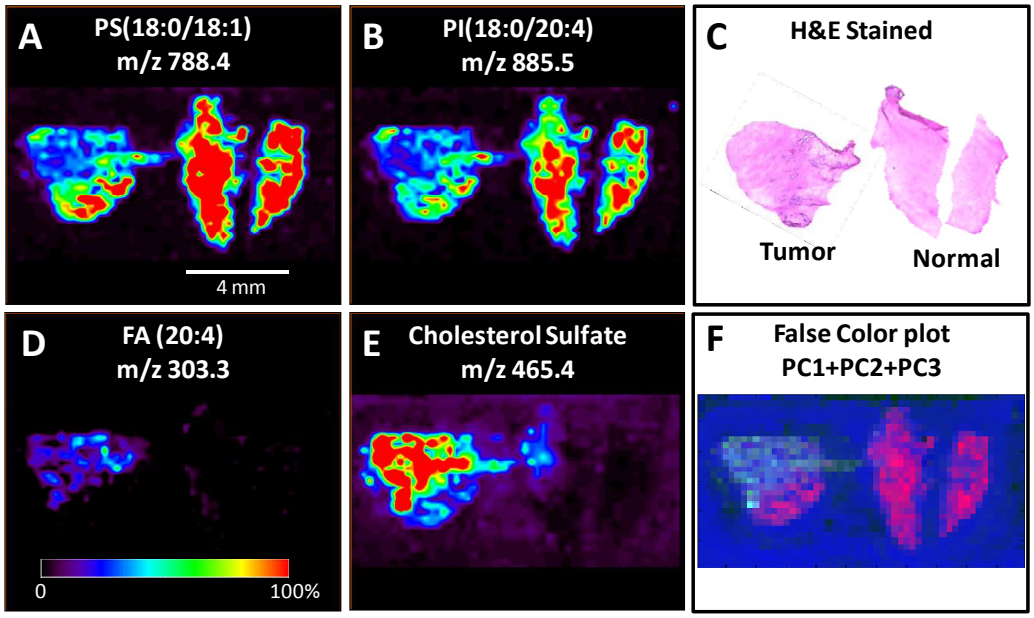

Figure S4. Negative ion mode tissue imaging of prostate tissues including areas of cancer and adjacent normal tissue of sample MH0301-17; (**A**) Ion image of m/z 788.4, PS(18:0:18:1), (**B**) Ion image of m/z 885.5, PI(18:0/20:4), (**C**) H&E stained tissue sections of the tumor tissue and normal. (**D**) Ion image of m/z 303.3, FA(20:4), (**E**) Ion image of m/z 465.4, cholesterol sulfate and (**F**) PCA-developed images, false color plot of PC1, PC2, and PC3.

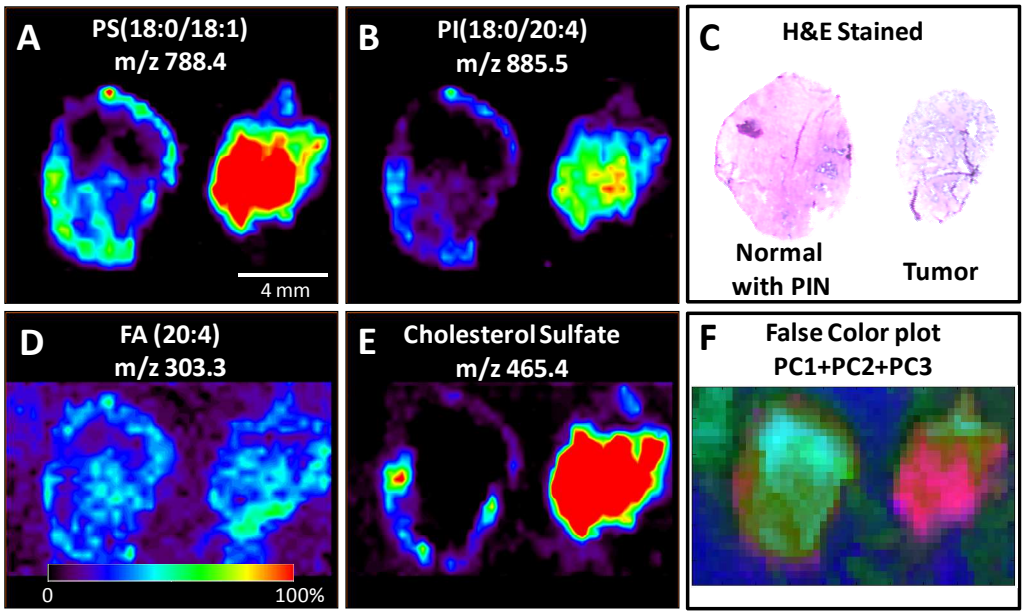

Figure S5. Negative ion mode tissue imaging of prostate tissues including areas of adjacent normal with PIN and cancer tissue of sample MH0301-11; (**A**) Ion image of m/z 788.4, PS(18:0:18:1), (**B**) Ion image of m/z 885.5, PI(18:0/20:4), (**C**) H&E stained tissue sections of the tumor tissue and normal. (**D**) Ion image of m/z 303.3, FA(20:4), (**E**) Ion image of m/z 465.4, cholesterol sulfate and (**F**) PCA-developed images, false color plot of PC1, PC2, and PC3.

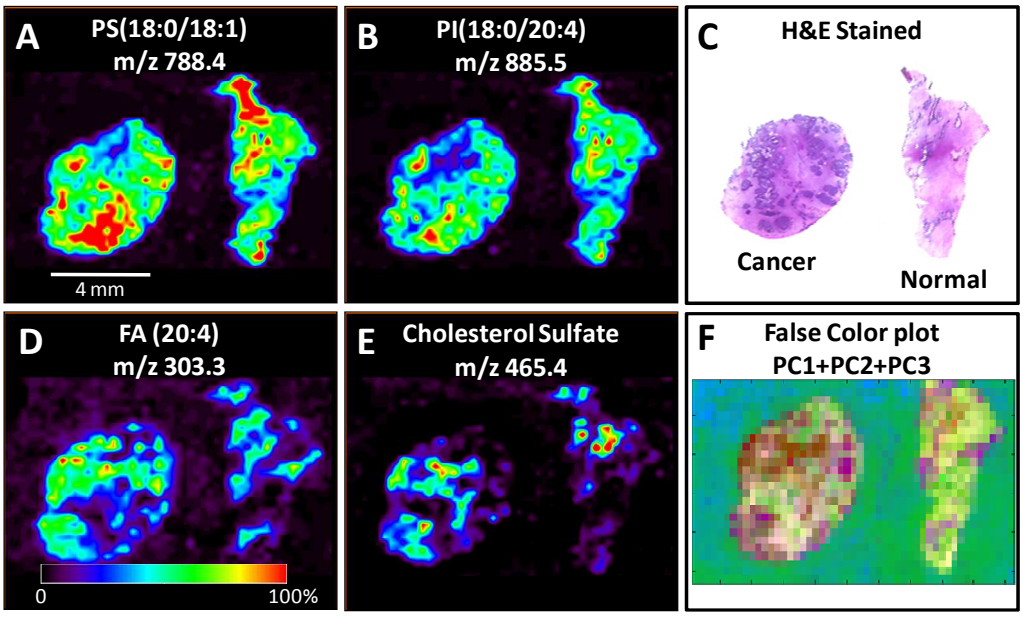

Figure S6. Negative ion mode tissue imaging of normal prostate tissues of sample MH0108-32; (**A**) Ion image of m/z 788.4, PS(18:0:18:1), (**B**) Ion image of m/z 885.5, PI(18:0/20:4), (**C**) H&E stained tissue sections of the tumor tissue and normal. (**D**) Ion image of m/z 303.3, FA(20:4), (**E**) Ion image of m/z 465.4, cholesterol sulfate and (**F**) PCA-developed images, false color plot of PC1, PC2, and PC3.

References for Supporting Online Material

(1) Ifa, D.R.; Wiseman, J.M; Song, Q. Y.; Cooks, R.G. *Int. J. Mass Spectrom.* **2007**, *259*, 8-15.