

- **Understanding Primary Metastasis of Ovarian Cancer via Imaging Mass Spectrometry of a Novel 3D Tissue Explant and Cellular Coculture**

Cellular and molecular signaling have important roles in the development and progression of cancer. However, as this communication is often dynamic and environment-dependent, it is difficult to characterize using traditional analytical techniques.

A specific example of this challenge is to understand the primary metastasis of high grade serous ovarian cancer (HGSOC).

It has been suggested that HGSOC first develops in the fallopian tube, and then transformed carcinogenic cells migrate to

the ovary to form fallopian-tube-epithelium-derived (FTE-derived) HGSOC.

Keywords:
MALDI-TOF, autoflex,
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ovarian cancer

In this study, an innovative 3D cell culture was generated using agarose to allow for the diffusion of small molecules within the culture. Analysis of these cultures by matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry imaging enabled the visualization of different chemical signals between the FTE cells and healthy ovarian explant tissue.

Experimental

Animal Handling and Sample Collection

CD-1 mice were obtained from in-house breeding. Animals were housed in a temperature and light (12L:12D) controlled environment. Water and food were provided *ad libitum*. All animals were treated in accordance with the National Institutes of Health Guide for the Care and use of Laboratory Animals. Day 16–18 after birth, ovaries were removed, and the explant ovaries were maintained in warm media until plating. Immediately prior to plating, the explants were separated from the bursa and halved.

Cell Culture

A total of 4 different murine cell lines were cultured, as previously described (Table 1). Cells were kept in T-75 flasks of 10 mL of α MEM media at 5% CO₂ and 37°C. Cells were passaged every 3–4 days. In preparation for slide plating, cells were washed with 10 mL of PBS and collected with 1 mL of 1x trypsin in 10 mL of α MEM. Cells were counted with a hemocytometer and then centrifuged for 5 minutes at 800 rpm (Eppendorf 5810R). The α MEM media was aspirated and cells were resuspended in 1x DMEM with 10% FBS and 2x penicillin-streptomycin (Sigma D5030-10L) to a concentration

Table 1: Names and descriptions of the 4 different cell lines used in this study.

Cell Line	Description
MOE	Spontaneously immortalized murine oviductal epithelial cells (equivalent of human FTE)
MOSE	Murine ovarian surface epithelial cells
SCR ^{shRNA}	MOE cells stably expressing scrambled control shRNA
PTEN ^{shRNA}	MOE cells stably expressing a shRNA targeting PTEN

of 333 cells/ μ L. These cells were then mixed with a volume equivalent of 2% melted agarose for a final sample concentration of 166 cells/ μ L in 1% agarose.

3D Cell Culture Optimization

Many factors had to be considered in the design of the 3D cell and tissue explant culture. First, it was necessary to create a culture where cellular signaling molecules were able to move, but cells were also homogeneously distributed and immobile in culture. While agar is typically used for MALDI Imaging of bacterial colonies, agarose was used for this culture as it kept cells in place, while allowing for the diffusion of signaling molecules. It was found that a uniform mixture of cells, media and agarose (150 μ L of 1X DMEM containing 50K cells mixed with 150 μ L of 2% agarose) yielded a more homogenous distribution of cells than various cell, agarose, and media layering techniques. Homogeneity in the cellular distribution ensured that observed signals were not artifacts due to cellular clustering. In addition, a uniform sample height is necessary for accurate MALDI-TOF Imaging analysis. The optimal plating condition was found to be an 8-well Permanox chamber (Lab-Tek 177445)

adhered on an ITO-coated glass slide. These chambers required only 300 μ L of material and the resulting agarose plugs dried flat in 4 hours.

Matrix Application Optimization

For MALDI Imaging of bacterial cultures, dry matrix is typically sieved onto wet agar. However, in these experiments, it was found that sieving matrix onto the agarose plugs prior to desiccation resulted in either uneven matrix coverage or ion suppression due to the quantity of matrix applied to obtain even coverage. Spraying the matrix using the TM-Sprayer onto the agarose plugs post-desiccation not only provided the opportunity to increase sample adherence by pre-coating the slide with matrix, but also resulted in a 75% reduction in crystal size, used significantly less matrix than sieving, and provided homogeneous matrix coverage while maintaining a consistent sample height for MALDI-TOF analysis (Figures 2 and 3). Prior to spraying, all matrix was recrystallized by dissolving the matrix in warm ethanol, filtering it, and adding two volumes of deionized water. The solution was left to stand overnight in a refrigerator and then the precipitate was filtered and dissolved in hot deionized water.

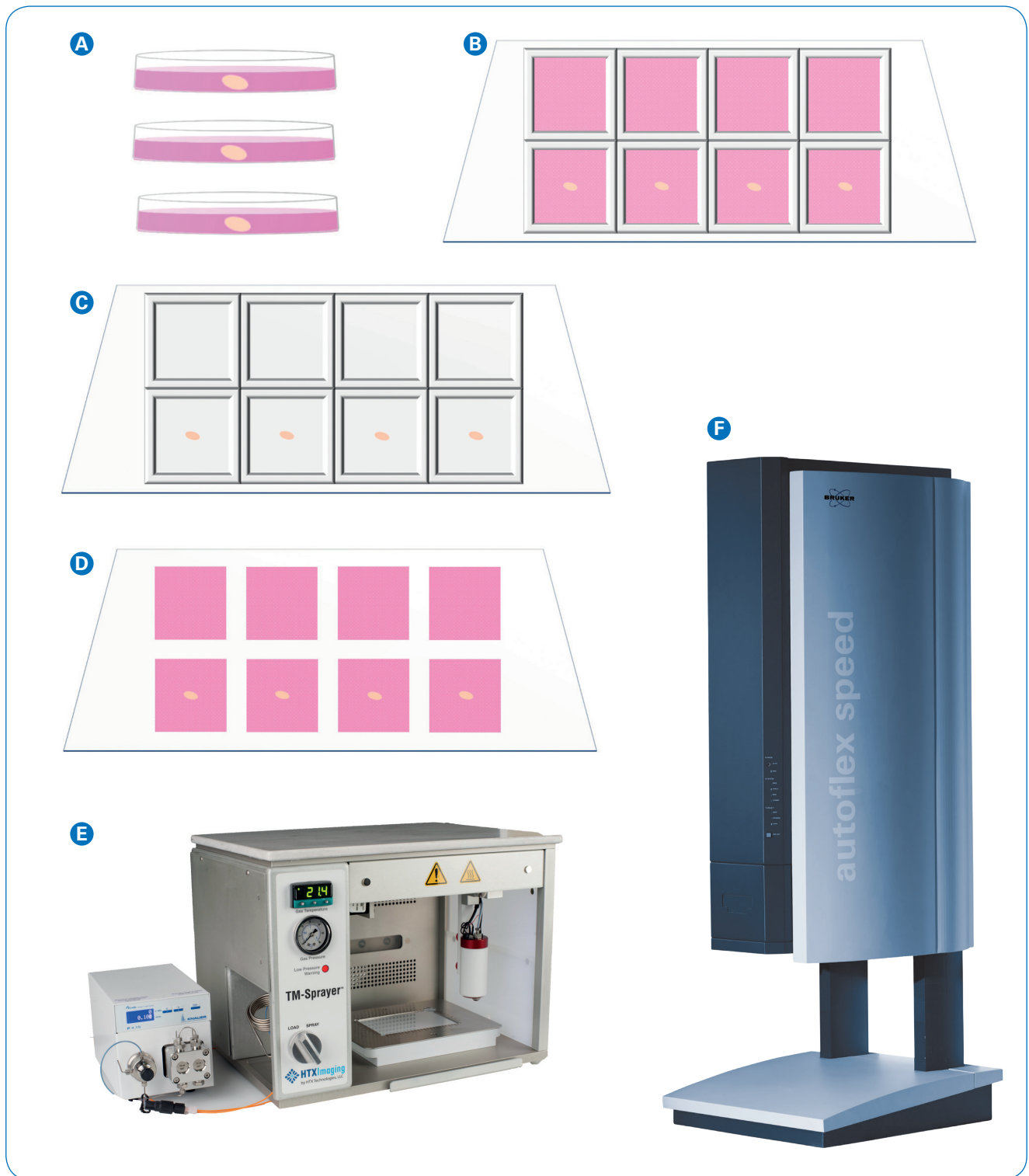


Figure 1: Overview of workflow for MALDI Imaging. **A** Ovaries from 16-18 day old female mice were extracted and maintained in warm media. **B** Ovaries are separated from bursa and halved immediately prior to plating. Explants are placed in the center of half of the wells of the 8-well chamber mounted to an ITO-coated glass slide. **C** 300 μ L of various cell cultures prepared in 1% agarose are plated in each well. Each cell type was plated with an ovarian explant and as a pure cell culture. The entire slide was covered with a lid and incubated for 4 days at 5% CO_2 and 37°C. **D** The 8-well chamber is removed. The 1% agarose 3D cell cultures maintain their shape, ensuring that the cultures do not come into contact with one another. The slide is dried in a 37°C oven for 4 hours and is rotated 90° every hour, resulting in flat and secure agarose plugs for sampling. **E** Slides were sprayed with matrix using the HTX TM-Sprayer. **F** Slides were imaged using a Bruker autoflex speed LRF MALDI-TOF.

The solution was immediately filtered through a fritted glass filter, and the crystals were collected and dried under air and then under a high vacuum.

MALDI-TOF Imaging

Samples were first scanned at 1200 dpi to integrate an image for data acquisition. Then, samples were analyzed using a Bruker autoflex speed LRF mass spectrometer over m/z 100-2000 Da. The instrument

was calibrated manually using red phosphorus and operated in positive mode, with laser power set to 40%, laser width set to 2 (small) and reflector gain set to 2.0x. At each sample location, 500 laser shots were fired at 2000 Hz in a random walk method. Image pitch was set to 50 μm . Data were analyzed using flexImaging v 4.1 and SCiLS Lab v2015b was used to identify significant m/z signals at regions of interest. All spectra were normalized to the total ion count (TIC).

Results

An initial experiment was performed to determine unique signals associated with the explant/MOE PTEN^{shRNA} coculture. Four separate conditions were prepared on one slide: (1) agarose and media only (control), (2) MOE PTEN^{shRNA} cells alone, (3) an ovarian explant alone, and (4) an ovarian explant and MOE PTEN^{shRNA} cell coculture. A total of 44 m/z signals were found to be of statistical ($p < 0.05$) and visual significance.

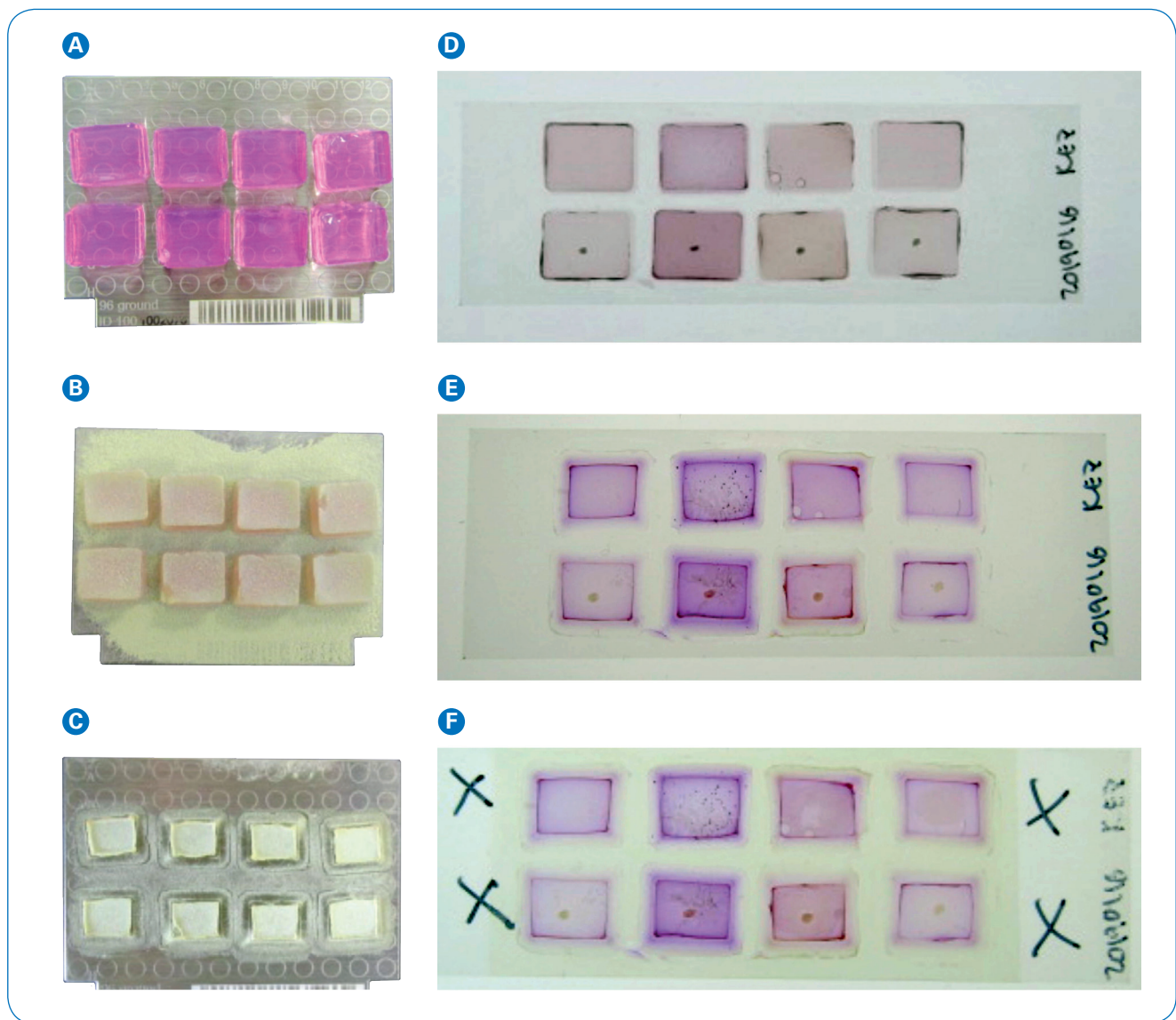


Figure 2. **A** Samples post-incubation; **B** Samples after sieved matrix application; **C** Samples post-desiccation; **D** Samples post-incubation; **E** Samples post-desiccation; **F** Samples after sprayed matrix application.

A subsequent experiment was then performed to determine if these 44 m/z signals were unique to tumori-genic FTE-derived cells. Eight separate conditions were prepared on one slide: (1) agarose and media only (control), (2) an ovarian explant alone, (3) MOE SCR^{shRNA} cells, (4) an ovarian and MOE SCR^{shRNA} cell coculture, (5) MOE PTEN^{shRNA} cells, (6) an ovarian explant and MOE PTEN^{shRNA} cells coculture, (7) MOSE cells, and (8) an ovarian explant and MOSE cell coculture. The ovarian explant and MOE SCR^{shRNA} cell coculture served as a control to determine if the observed signals were also elicited by nontumorigenic FTE-derived cells, and the ovarian explant and MOSE cell coculture served as control to determine if the chemical signals were unique to FTE-derived cells or could also be induced by cells derived from the ovarian surface epithelium. It was found that 33 m/z signals were significantly increased ($p < 0.05$) in the ovarian explant and MOE PTEN^{shRNA} cell coculture compared to each of the other 7 conditions (Figure 4). Among these 33 signals, m/z 170 was identified as norepinephrine, which is known to have a role in development of ovarian cancer.

Table 2: TM-Sprayer method parameters for precoat and post-desiccation spraying

	Precoat	Matrix Application Post Desiccation
Matrix	50:50 CHCA:DHB	
Solvent	90:10 ACN:H ₂ O + 0.1% TFA	
Matrix Concentration (mg/mL)	5	
Flow Rate (mL/min, FR)	0.2	
Spray Nozzle Velocity (mm/min, V)	1100	
Spray Nozzle Temperature (°C)	30	
Track Spacing (mm, TS)	3	
Number of Passes (NP)	4	8
Nitrogen Pressure (psi)	10	
Spray Pattern	CC	
Drying Time (sec)	0	
Nozzle Height (mm)	40	

Precoat Parameters	
Matrix density (W)	$W = \frac{NP \times C \times FR}{V \times TS} = 0.0012 \text{ mg/mm}^2$
Linear Flow Rate (LFR)	$LFR = \frac{FR}{V} = 1.8e-4 \text{ mm/min}^*$
Post-Desiccation Matrix Application Parameters	
Matrix density (W)	$W = \frac{NP \times C \times FR}{V \times TS} = 0.0024 \text{ mg/mm}^2$
Linear Flow Rate (LFR)	$LFR = \frac{FR}{V} = 1.8e-4 \text{ mm/min}^*$

* Corresponds to wet spray, defined by $LFR > 8.3e-5$.

However, due to the mixed coculture design of this experiment, it was unclear whether the norepinephrine was originating from the ovarian explant or MOE PTEN^{shRNA} cells. An experiment utilizing plastic tabs to divide the 8-well chamber was designed to embed the ovary separately from the cells in order to visualize the localization of the norepinephrine signal. Nine of the 33 signals identified as significant in the original experimental design were also detected here, including norepinephrine (Figure 5). It was clear from the spatial distribution of the signals that the ovary was the source of the chemical signals that then migrated through the agarose and media towards the MOE PTEN^{shRNA} cells.

Experimental Summary	
Sample Type	3D cell culture +/- murine ovarian explant
Incubation	4 days at 5% CO ₂ and 37°C
Drying Protocol	4 hours at 37°C, rotated 90° every hour
Instrumentation and Supplies	
MALDI Plate Matrix	ITO-coated glass slides Recrystallized 50:50 CHCA:DHB (Sigma Aldrich, 5 mg/mL in 90:10:0.1 ACN:H ₂ O:TFA)
MatrixSprayer	HTX TM-Sprayer™
MALDI MS	Bruker autoflex speed LRF
Acquisition Mode	Fullscan m/z 100-2000, Positive Mode FlexImaging v4.1 x 64
Imaging Software	SCiLS Lab 2015b

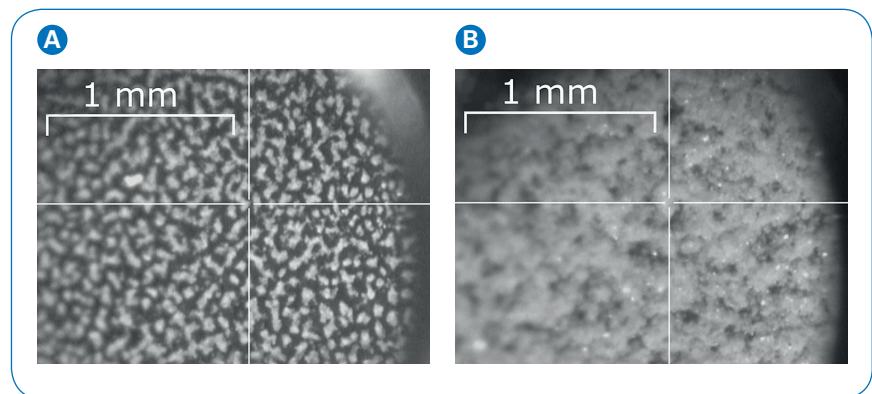


Figure 3: **A** Images of crystals sprayed onto agarose plugs post-desiccation using the TM-Sprayer. **B** Images of crystals sieved onto agarose plugs prior to desiccation.

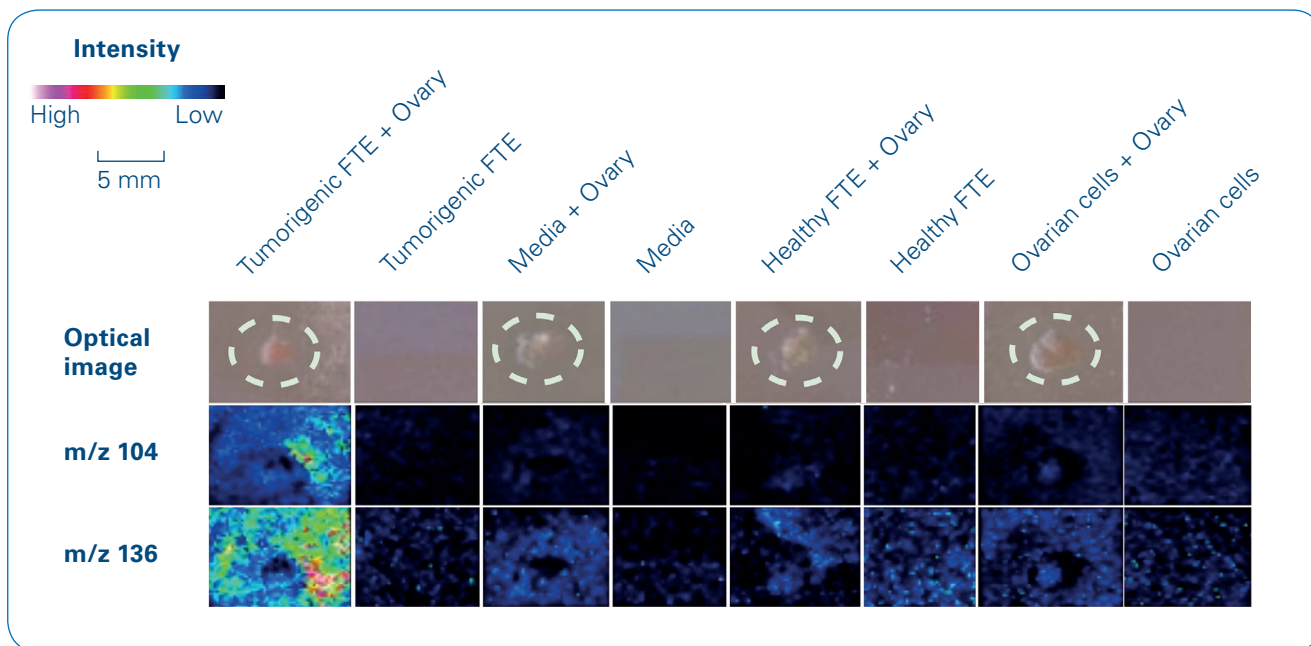


Figure 4: m/z 104 and m/z 136 were two of the 33 m/z signals that were identified to be significantly higher in the ovarian explant and MOE $PTEN^{shRNA}$ cells coculture than in any of the other 7 conditions.

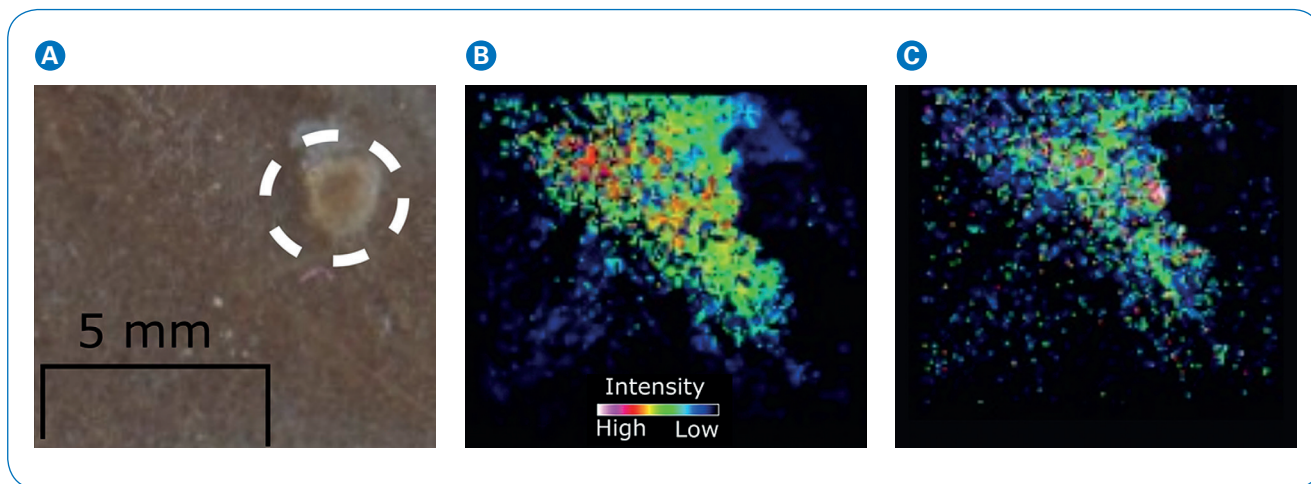


Figure 5: **A** Optical image of divided well of an 8-well chamber; **B** m/z 112 and **C** m/z 144 are two signals that were clearly originated from the ovary explant and migrated through the agarose towards the MOE $PTEN^{shRNA}$ cells.

Conclusion

- In this study, a MALDI Imaging protocol was optimized for a novel 3D cell and tissue explant coculture. Specifically, norepinephrine was identified as putative metastatic signaling molecule released by the ovary in response to tumorigenic FTE-derived cells. These data demonstrate the applicability of Imaging to study tissue and cell interactions to better understand *in vivo* biology.

The tissue images and MS data presented here were provided by Dr. Laura Sanchez and Katherine Zink from the University of Illinois at Chicago. More details about this work can be found in: *Imaging Mass Spectrometry Reveals Crosstalk between the Fallopian Tube and the Ovary that Drives Primary Metastasis of Ovarian Cancer*. Katherine E. Zink, Matthew Dean, Joanna E. Burdette, and Laura M. Sanchez. *ACS Central Science*: 2018 **4** (10), 1360-1370. DOI: 10.1021/acscentsci.8b00405.



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