Early Detection of Ovarian Cancer Using Cells Sourced from a Local Microenvironment

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Abstract

Background. Sampling a local microenvironment represents a potentially informative method for monitoring disease progression due to proximity between the sample and diseased tissue. Mass spectrometry (MS) offers high levels of specificity and sensitivity in clinical applications, and we have previously been able to demonstrate that matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS is capable of distinguishing cell mixtures (two-component) at low limits of detection.

Methods. IVIS imaging of a murine xenograft model of high grade serous ovarian cancer (HGSOC) was performed on a weekly basis to track tumor burden. Vaginal lavages were also obtained weekly through disease progression and samples were subjected to our MALDI-TOF MS workflow followed by statistical analyses of the resulting protein fingerprints over the course of the study.

Results. Over the course of disease progression, the presence and absence of proteins were compiled to generate a unique protein fingerprint. These fingerprints were found to be largely stable across all mice and converge towards the end point of the study. Our MS limit of detection was found to be more sensitive than microscopy-based methods for the RFP-tagged cells.

Conclusion. MALDI-TOF MS serves as a unique analytical technique for sampling the vaginal microenvironment in a specific and sensitive manner for the detection of HGSOC in a murine model.

Highlights

1. **Vaginal lavages represent a unique microenvironment to monitor high grade serous ovarian cancer disease progression in a murine model.**
2. **MALDI-TOF mass spectrometry is capable of distinguishing disease progression *in vivo*.**
3. **Small proteins in the 4-20 kDa region represent a unique diagnostic mass spectrometry-based fingerprint.**

1. Introduction

Ovarian cancer is a severe gynecological disease that is currently the fifth leading cause of cancer deaths among women in the United States, with an estimated 22,530 newly diagnosed cases and 13,980 deaths expected in 2019.[1] This disease has a five-year survival rate of 93% if diagnosed when tumor growth is limited to reproductive organs such as the ovaries or fallopian tubes.[2,3] However, the majority of diagnoses occur during later stages of the disease when tumors have already metastasized, resulting in a five-year survival rate of less than 30%. Around 90% of these diagnoses are of epithelial origin, of which high-grade serous ovarian cancer (HGSOC) is the most common subtype.[3–5] These late-stage diagnoses can be attributed to the limited diagnostic methods available for implementation in routine women's health exams. Current diagnostic methods, such as transvaginal ultrasounds and CA-125 biomarker tests, are generally used only after patients present with symptoms as they are more invasive procedures than what is performed in a typical health

screening. These methods also suffer from inaccuracy and a lack of specificity, which limits their use in diagnosing ovarian cancer in its early stages.[6,7]However, these procedures are valuable in a clinical setting as they are commonly used to monitor patients throughout their treatment progress once cancer is detected as well as recurrence.[8]

In a routine well woman health screening, women receive a Pap smear to determine cervical health but recent data suggests that these samples may be repurposed for ovarian cancer detection. In a recent study, DNA from liquid Pap smears obtained from women diagnosed with either endometrial or ovarian cancer were sequenced, in which 100% and 41% of samples had identifiable mutations matching the tumor of origin, respectively.[9] This, along with other studies, shows that DNA from ovarian cancer cells can be detected in clinical samples, such as Pap smears, which represents a concentrated sample from the local microenvironment in the female reproductive system.[9–11] This clinical evidence would suggest that cells and/or cellular fragments from ovarian tumors reside outside of the proximal environment in areas such as the cervix or vagina. Further confirmation of the Pap smear linked to HGSOC detection was recently detailed in a case report, in which adenocarcinoma cells derived from HGSOC precursor lesions were observed in a cervical smear.[12] While conization of the patient's cervix did not result in any evidence of adenocarcinoma cells in the tissue, a bilateral salpingectomy, which occurred 16 months later, and an oophorectomy, four months after that, revealed serous tubal intraepithelial carcinoma lesions and HGSOC, respectively.[12] The fact that these abnormal cells found in Pap smears, which were unrelated to overall cervical health, were present prior to the detection of HGSOC in the patient demonstrates the value in developing technology for the early detection of ovarian cancer from the local vaginal microenvironment.

The detection of DNA originating from ovarian cancer cells in cervical samples is promising but also leads to complications as these mutations may arise in several anatomical sites therefore making the surgical removal of the responsible lesion difficult. Since the common treatment for women with ovarian cancer is resection of the reproductive tract and ovaires, an ideal routine diagnostic test would need to be extremely sensitive and specific to avoid unnecessary medical procedures. Recent advances have been made in this area such as with the Safe-SeqS assay or PapSEEK test, in which somatic mutations are detected from liquid Pap smear samples.[9,13] While PapSEEK was found to have a false positive rate of 1.4%, it was only able to detect mutations in 33% of patients with ovarian cancer although the percentage of women with endometrial cancer for which mutations were detected was much higher at 81%.[13] Perhaps in combination with genomic sequencing, the ability to directly detect proteins, particularly those that change in abundance over time from a local source, may improve strategies that can be leveraged for detection.

Without using amplification strategies, work has recently been done using MS to investigate Pap smears under the assumption that cells from the female genital tract would be present.[14] Bottom-up proteomics using a LTQ Orbitrap mass spectrometer was performed on liquid Pap smear samples from women considered to healthy to create a 'Normal Pap test Core Proteome.'[14] This analytical method is highly sensitive but it does require significant sample preparation such as sample cleanup to remove salts and insoluble particulates, protein digestion, and lengthy liquid chromatography times leading to a time investment, as well as a high degree of expertise needed to operate a high mass resolution tandem mass spectrometer (LC-MS/MS). We chose to investigate the use of matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS since this is considered a versatile analytical technique used in a variety of applications ranging from the characterization of microbial species to biomarker detection using protein signatures.[15–17] For example, the MALDI-TOF Biotyper system (Bruker Daltonics) is currently FDA approved for use in clinical diagnostic laboratories for microbial typing.[18] MALDI-TOF MS has also been used to characterize mammalian cells based on spectral fingerprints, which in turn, allows for their identification.[19–21]

To test the feasibility of MALDI-TOF MS to differentiate cell mixtures, we used whole-cell fingerprinting to characterize two-component cell mixtures based on protein signatures.[21] We identified that the small protein mass range (4 - 20 kDa) is valuable for characterizing cell types; this mass range was found to have a higher number of characteristic peaks overall as well as the most distinctive peaks when comparing cell types, particularly ovarian carcinoma (OVCAR) and murine oviductal (MOE) cell lines.[21] This study also laid the foundation for our standard sample preparation of cell

mixtures. We have taken these studies as the basis to adapt this MALDI-TOF-based technique to detect the presence of ovarian cancer cells *in vivo*. To study this concept *in vivo*, we used a murine model of HGSOC. OVCAR-8 has the ability to cause aggressive tumor formation and ascites following intraperitoneal (IP) xenografts, making it an acceptable model for HGSOC in a murine model system. [22,23] [24] In this study, we use vaginal lavages in a murine xenograft model to detect the increasing burden of ovarian cancer based on protein fingerprints obtained using MALDI-TOF MS, as shown in **Figure 1**. Statistical analysis of the protein signatures found candidate proteins that both increase and decrease over time as disease progresses using vaginal lavages highlighting the importance of sampling the vaginal microenvironment.

2. Materials and Methods

2.1. Cell Culture

OVCAR-8 cells expressing red fluorescent protein (OVCAR-8-RFP) were a gift from M. Sharon Stack at the University of Notre Dame. OVCAR-8-RFP were grown in DMEM with 10% FBS and 1% penicillin/streptomycin. Cultured cells were maintained in a humidified incubator at 37°C in 5% CO_2. Cells were passaged a maximum of 20 times. Cell lines were validated by short tandem repeat analysis and tested mycoplasma-free in 2017.

2.2. *In Vivo* Study

All animals were humanely treated in accordance with the Animal Care and Use Committee guidelines at the University of Illinois at Chicago (UIC) using Protocol #17-174. NCr *nu/nu* athymic female mice aged 10 to 12 weeks (Taconic) were housed in facilities managed by the Biological Resources Laboratory at UIC and were provided food and water *ad libitum*. Vaginal lavage samples using 200 μL of sterile PBS as described by McLean *et. al*. at various time points prior to xenografts to collect healthy samples.[25]

2.3. IP Xenograft and Collection of Murine Vaginal Lavages

OVCAR-8-RFP ($5x10^6$) cells were xenografted IP for each mouse (N=5). Tumor burden was monitored with a Xenogen IVIS® Spectrum *In Vivo* Imaging System (PerkinElmer) as described by Lewellen *et al.* using an exposure time of one second, an Fstop of two, and an excitation and emission wavelength of 535 and 620 nm, respectively.[26] Mice were imaged and lavage samples were collected on a weekly basis. After week 8, mice were sacrificed followed by collection of tumors and reproductive organs.

2.4. Cell Counting and Preparation of Cells for Analysis

Cells from the murine vaginal lavages were counted using a K2 Cellometer (Nexcelom). 20 μL of lavage sample was pipetted into disposable counting chambers and placed into the instrument for imaging. Bright-field images were taken of each sample for accurate counts of leukocytes and epithelial cornified cells present which were then used to calculate the concentration of the lavage samples using FCS Express software (De Novo). To determine if OVCAR-8-RFP cells were present in the lavages, fluorescent images were also taken using a 660 nm filter. All lavages were spun down at 150 rcf for five minutes, at which point, excess PBS was removed. Cells were resuspended in deionized water to reach a final concentration of 10,000 cells/μL.

2.5 Sample Preparation for MALDI-TOF MS Analysis

A sinapic acid matrix solution was prepared by dissolving 20 mg of sinapic acid (Sigma Aldrich) into one milliliter of 30/70 ACN/H_2O with 0.1% trifluoroacetic acid (TFA). Frozen lavage cell suspensions were thawed on ice and diluted to 5,000 cells/μL using deionized water. Equal volumes of lavage cells and a 20 mg/mL sinapic acid matrix solution were mixed together for a final concentration of 2,500 cells/μL and placed on ice for ten minutes before spotting the samples. 1.5 μL of the sample was then spotted onto a 384-well ground steel MALDI target plate with 24 replicates per spot with Protein Standard I (Bruker Daltonics) used as a calibrant. This procedure was previously optimized and described in depth in Petukhova *et. al*.[21]

2.6. MALDI-TOF MS and Data Analysis

Mass spectra of murine lavage samples were acquired on an Autoflex Speed LRF MALDI-TOF mass spectrometer (Bruker Daltonics) in positive linear mode with a mass range of 4 to 20 kDa. Protein spectra was acquired using AutoXecute, an automated run function of FlexControl v. 3.4 software. Four thousand laser shots were accumulated in fifty shot increments for each sample using random walk. All mass spectra were externally calibrated using Protein Standard 1, which had been spotted directly adjacent to each sample spot in a square pattern. Spectra were processed using FlexAnalysis v. 3.4 (Bruker Daltonics) with baseline subtraction (TopHat algorithm) and smoothing (SavitzkyGolay algorithm, width of five mass-to-charge ratios (*m/z*) over five cycles). Peak peaking was performed using a signal-to-noise (SNR) threshold of four following processing. Data is publically available at ftp://MSV000083628@massive.ucsd.edu.

2.7. Statistical Analysis

For statistical evaluation, profile mass spectra were peak picked and converted to mzML format. Mass spectra in the mzML format were pre-processed using MALDIquant software following the workflow suggested in the software documentation (https://cran.r-project.org/web/packages/MALDIquant/MALDIquant.pdf) and shown in **S1**.[27] Mass spectrometry features across all samples were assembled into a matrix.

Differential Abundance Between Time Points

Features were searched that were differentially expressed throughout the longitudinal sampling points in every mouse. Utilizing the abundances for each feature, a Wilcoxon rank-sum test was applied to assess whether the abundance differences between all pairs of days within one mouse were statistically significant. The Wilcoxon rank-sum test was chosen because it makes very few assumptions about the distribution of the data, e.g. normality in the case of t-tests. Bonferroni-correction was applied to the p-value of each test to account for multiple hypothesis testing and a threshold of 0.01 was applied on the corrected p-value for each test. Source code is available here[https://github.com/mwang87/ometa_mousemaldi].

3. Results

In order to determine if vaginal samples could be analyzed by MALDI-TOF MS as disease progressed in a mouse model, vaginal samples and fluorescent imaging were performed. All five of the OVCAR-8-RFP xenografted mice showed abundant tumor burden that increased weekly based on IVIS imaging. As seen in a representative image in **Figure 2A**, tumors increased in size and colonized the peritoneum over time. Quantitatively the average radiant efficiency increased throughout the time course of the study, as shown in **Figure 2B**. In addition to the fluorescence data, it was also observed that five mice presented with ascites at the conclusion of the *in vivo* study.

Along with the IVIS imaging, vaginal lavages were also imaged to determine if any OVCAR-8-RFP cells could be detected. While no cells were noted to escape from the murine reproductive organs, it is also possible that too few RFP-tagged cells escaped for detection purposes. To determine the limit of detection, vaginal lavages were collected from healthy mice and cells were counted, pelleted and resuspended to reach a final concentration of 10,000 cells/μL. OVCAR-8-RFP cells were then spiked into healthy cell mixtures at 10% and 1% for detection. Based on brightfield and fluorescence imaging using a K2 Cellometer and a 20 μL sample size, it was determined that an otherwise healthy cell mixture needed to be composed of at least 10% OVCAR-8-RFP cells in order to reproducibly detect fluorescence. Representative images of lavages used for fluorescence studies along with accompanying statistics can be found in **S2**. Previous work by *Petukhova et al.* demonstrated that MALDI-TOF MS was able to detect as low as 1% of cancer cells in an otherwise healthy heterogeneous cell mixture. [21]

Protein fingerprints, obtained from lavages in weeks one through seven of the xenograft study, were used for analysis of disease progression over time. The first week of the study was chosen as the first time point in our statistical analysis for comparative purposes due to irregularities in the samples taken at earlier time points including insufficient sample ionization. Additionally, the seventh week of the study was used as the final time point as one mouse expired prior to the

end of the study. All spectra were pre-processed using MALDIquant, resulting in a feature intensity matrix which provided intensity information on all spectral features over a SNR of four for all five mice. These values were the basis of our analysis to determine statistical significance in spectral features throughout ovarian cancer progression.

Following analysis using a Wilcoxon rank-sum test, specific features, shared between all mice, were found to be significantly up- and downregulated throughout the time course of the study. The number of features that change between time points are shown in **Figure 3** along with the top ten *m/z* values showing significant differences in either direction between the first and seventh week of the study. Across the study, there are 19 *m/z* values and 10 *m/z* values that are significantly upregulated and downregulated, respectively, in all mice ($p < 0.001$). These zoomed-in regions of the mass spectra as well as average spectra of the two time points of interest can be found in **S3**. The dot matrix plot, shown in **Figure 4**, shows low similarity when comparing early time points in the study. However, it also shows that the spectra appear to coalesce in the later stages of the study such as between weeks six and seven, which is confirmed in the pivot tables from **Figure 3**. All detected peaks that were found to be differentially expressed across time points can be found in **Table S1**.

While this data analysis was promising, we also wanted to verify that the protein fingerprints were unique to HGSOC and not a result of any peritoneal disease. Our previous work with cell lines suggested that this would not be the case as specific cell lines give rise to distinct spectra. Nevertheless, murine lavages were also collected from mice (N=9) with non-alcoholic steatohepatitis (NASH), which results in severe liver damage. These mice were sex- and age-matched to the mice from the OVCAR-8-RFP xenograft study in its seventh week and comparative mass spectra can be found in **S4**. This comparison between two separate disease states resulted in an observation that the protein signatures were distinct between the different peritoneal diseases. Therefore, our MALDI-TOF MS methodology yields distinct, disease specific protein fingerprints from the vaginal microenvironment.

4. Discussion

While reliable early diagnostic markers for ovarian cancer have remained elusive, research has moved closer towards characterizing the proteome of a healthy female reproductive system in humans using MS.[14] The use of MS in a clinical setting is not novel concept; the iKnife and MassSpec Pen are both innovative devices that couple mass spectrometry signatures in lipids to the detection of cancerous tissue in a surgical setting.[28,29] Ovarian cancer is typically diagnosed after it has metastasized, and surgical removal and chemotherapy while effective typically do not eradicate the tumors completely resulting in recurrence. Therefore, being able to diagnose this disease state during its early stages, when tumors are localized to the ovaries and fallopian tubes, would increase survival.

Data based on sequencing and the patient case report highlights the fact that ovarian cancer cells have the ability to travel through the female reproductive system to the cervix. Therefore, we felt it would be possible to detect these cells using MALDI-TOF MS and use their resulting protein signatures for diagnostics.[9,11] By sourcing cells from a local microenvironment, we hoped to achieve greater sensitivity and specificity while also exploring the ability of small proteins to lend themselves as possible biomarkers of disease progression.

While we were able to track the tumor burden using IVIS imaging, this acted solely as a check as to whether or not the xenograft had been successful and to determine how far the tumors had progressed *in vivo*. We had originally anticipated using the fluorescence emission from the OVCAR-8-RFP cells during the counting process throughout the study. However, when we tested this approach using lavages spiked with OVCAR-8-RFP at known concentrations, as described above, we found that at least 10% of RFP-tagged cells had to be present in a heterogenous mixture to in order for cells to be reliably detected. Our lab has previously shown that MALDI-TOF MS can distinguish as low as 1% of cancer cells/cellular proteins (cells lyse in workup) from a heterogenous mixtures based on the resulting protein fingerprint, indicating that MS will be a more sensitive technique for detecting the presence of aberrant cells in *in vivo* samples. [21] While we did not observe fluorescence in murine lavages at any time point, changes were detected in the protein fingerprint over time using MALDI-TOF MS, which is of interest as changes in the local microenvironment are occurring

as the disease progresses. As cells are lysed prior to analysis, our MS method relies on the detection of proteins from lavages instead of whole cells, making the source of these proteins ambiguous. For instance, we cannot differentiate between cellular origination from tumor cells versus those found in the murine reproductive system. Moreover, it could also be possible that we are observing the cellular response to cancer, but this provided a unique fingerprint over mice with another peritoneal disease such as NASH. The observance of statistically significant changes in feature intensity, regardless of whether the proteins are from ovarian cancer tumor cells or not, is of importance for this study as we are not focused on the appearance of tumor cells in the murine lavage but rather changes in the overall system.

Following the evident appearance of tumor burden, 1056 mass spectra (N=5, n=24) were collected across eight weeks using MALDI-TOF MS and analyzed. The MALDI-TOF mass analyzer had several advantages for this study such as solvent-free, rapid data acquisition (milliseconds), minimal sample requirement, and the availability of this technology in clinical laboratories. [15–18] The use of MALDI-TOF MS also allows rapid collection of a protein fingerprint over a large mass range (4,000 - 20,000 Da) and provides an intact mass as it is a soft ionization technique. This allows us to observe proteins with high abundance from the murine vaginal lavages, which form a unique fingerprint and get more information regarding the overall mass of the detected proteins. Similar to how the Biotyper system (Bruker Daltonics) works for microbial identifications, the collection of a protein fingerprint allows for the comparison of different spectral patterns, in terms of the appearance and absence of peaks, to look for matches based on characteristic peaks.[18] This is advantageous to our study as we have the ability to statistically analyze and compare protein fingerprints collected at various time points throughout the study to look for changes in protein peaks during tumor progression.

In order to begin to visualize the data, the cosine similarity of the resulting vaginal lavages in a dot matrix plot was determined, shown in in **Figure 4**. This allows for an easily accessible view of how the profiles may differ over time when compared to specific weeks in the study. At the beginning of the study, each biological replicate (N=5) had a largely unique profile when compared to others at or around the same time point (red dots). However, one of the striking results was that at the end of the study during the seventh week, the protein fingerprints from the vaginal lavages are largely conserved (green dots), which would indicate that all five mice have similar protein profiles once the tumor burden is representative of late stage ovarian cancer. This would imply that there are unique processes and/or cellular responses associated with the progression of HGSOC that can be captured in a spectral protein fingerprint.

This late stage similarity prompted us to search for the presence and absence of protein features across all mice across paired time points (i.e. week one versus week seven). We found that multiple proteins began to dominate the mass spectra throughout disease progression, as expected from **Figure 4**, and that a larger number of proteins either appeared and become absent from the protein fingerprint over time. This led to the investigation of specific regions of the spectrum and the verification that specific peaks became either significantly up- or downregulated over time, which would suggest that tumor progression does, in fact, induce change in specific processes in our murine model system. Several research groups have studied the proteome of cervical-vaginal fluid obtained from women found to be in good health and while some of the proteins they were identified fell within the mass range detailed in this study, such as thioredoxin and profilin-1, there is not much known about the reproductive proteome between *m/z* 4,000 - 20,000, prompting additional research.[14,30,31] Some have speculated that this mass range contains an abundance of histones or ribosomal proteins based on previous whole-cell fingerprinting research and we hope to expand the knowledge of this area in the future through the identification of detected proteins using MS.[19] While the spectral fingerprint obtained from the murine lavages is a promising start to better understanding the cellular processes that occur throughout the progression of ovarian cancer, it is of note that while MALDI-TOF mass spectrometers allow for the observance of intact protein masses, they do not have the mass resolving power in linear mode to provide an exact mass for the detected spectral features. Bottom-up proteomics, performed on a high-resolution instruments such as a hybrid quadrupole-orbitrap, will be required to definitively identify these proteins via analysis of peptide fragments. Bottom-up proteomics has its own limitations such as potentially not having enough cleavage sites for the digestion enzyme due to the small size of the proteins. However, using a combination of both MS methods to obtain an intact mass along with tandem fragmentation will allow us to identify these proteins in the future.

To identify features that were up- or downregulated in a statistically significant manner, we used a Wilcoxon rank-sum test as the basis for our statistical analyses. This test makes minimal assumptions about the data, which allows it to be used in cases of non-normal distributions. The Wilcoxon rank-sum test uses the normalized intensity values from each observed peak and ranks them in terms of value for comparison. The determination of the test statistic in each comparison (week to week) allowed for the calculation of a p-value for significance. The use of this test allowed for the identification of 118 upregulated and 97 downregulated spectral features when comparing data from different time points, implying that there were multiple significant changes over time in our mouse model.

5. Conclusion

Based on statistical analysis with the Wilcoxon rank-sum test, we have been able to identify intact *m/z* values of small proteins obtained from murine lavages. We conclude that the protein fingerprint region obtained from MALDI-TOF MS analysis of vaginal lavages can be used to detect the presence of ovarian cancer. By sourcing samples from a local microenvironment, such as the vaginal cavity or cervix, we have shown that significant change in protein signatures can be observed over time in the progression of ovarian cancer in a murine model. Our next step will be to further investigate and identify these proteins of interest using tandem MS to gain a better understanding of their role in disease progression in our *in vivo* model. Additionally, we plan to perform imaging mass spectrometry on reproductive tissue and tumors collected during the study to gain information regarding spatial distribution of these proteins as well as their tissue of origin. A deeper understanding of these signature proteins and the processes they are involved in may further enhance our ability to design a diagnostic test.

Conflict of Interest

The authors have no conflicts of interest to disclose related to the findings of this study.

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Author Contributions

JEB and LMS were responsible for the conception of the study. MMG and ANY were responsible for cell and animal work, including vaginal lavages, imaging and tumor collection following sacrifice. MMG and VZP were responsible for MS data collection and analysis. MW and JW were responsible for performing the statistical analysis required for this study. All authors were responsible for preparation of the manuscript with input and review received from all authors.

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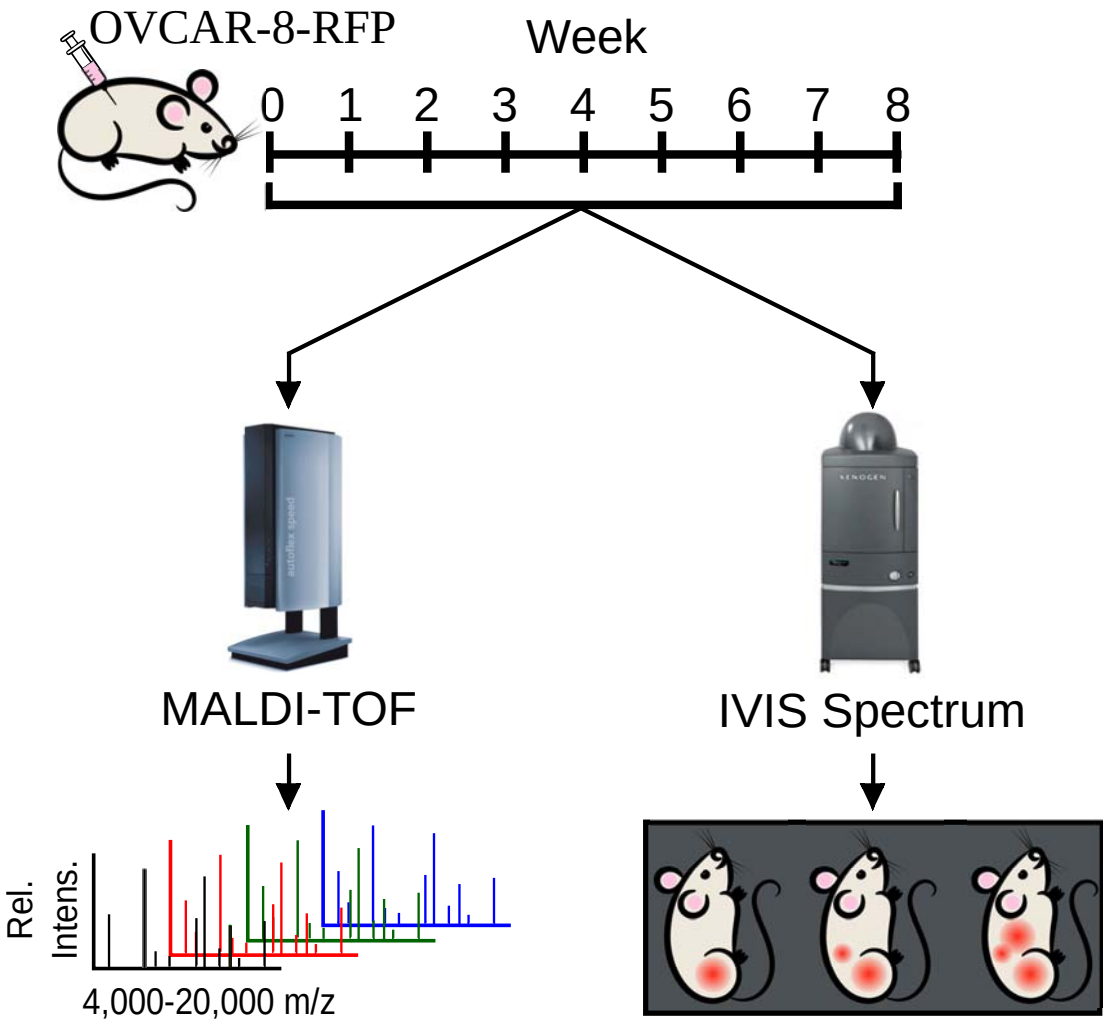

Figure 1: Workflow of murine xenograft study. Female athymic mice are IP injected with OVCAR-8-RFP and tumors were allowed to develop over an eight week period. Each week, mice were given vaginal lavages and were imaged using an IVIS imaging system to track tumor burden. Vaginal lavages were analyzed using MALDI-TOF MS to obtain mass spectra for statistical analyses.

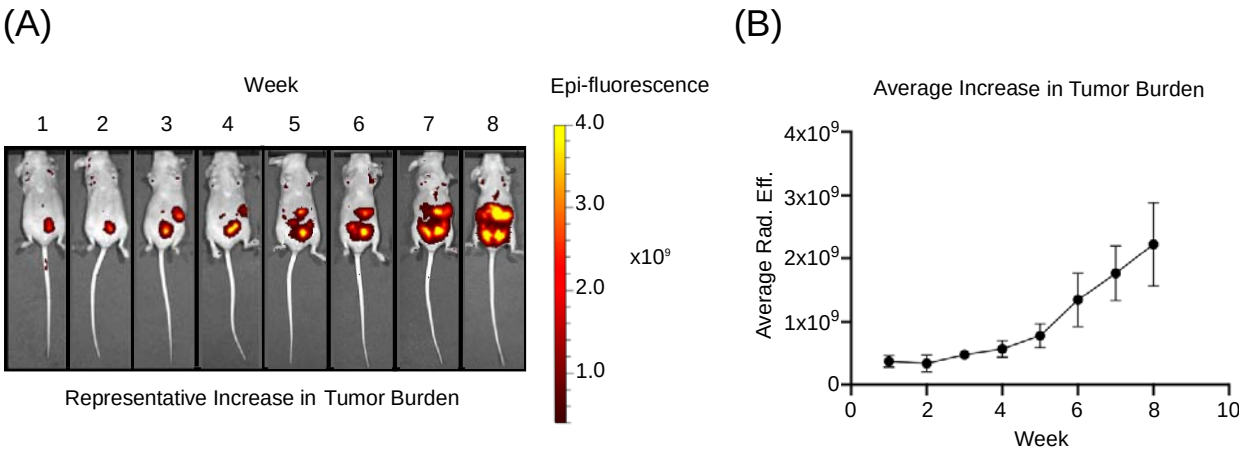

Figure 2: (A) *In vivo* fluorescence imaging of OVCAR-8-RFP tumors in athymic nude mice over an eight week period (B) Average radiant efficiency over time for all mice indicating an increase in tumor burden

(A)

Week	2	3	4	5	6	7
1	-	-	3	12	42	19
2	-	1	-	5	1	2
3	-	-	3	2	2	4
4	-	-	-	15	-	5
5	-	-	-	-	-	1
6	-	-	-	-	-	1

(B)

Week	2	3	4	5	6	7
1	-	1	-	9	4	10
2	-	-	-	-	-	6
3	-	-	-	2	-	25
4	-	-	-	12	2	25
5	-	-	-	-	-	1
6	-	-	-	-	-	-

(C)

	Peak	*m/z*	Avg. p-value
Upregulated	1	13770.4	6.20×10^{-14}
	2	13777.6	6.20×10^{-14}
	3	13786.8	6.20×10^{-14}
	4	13805.6	6.20×10^{-14}
	5	13997.2	6.20×10^{-14}
	6	14008.2	6.20×10^{-14}
	7	14016.7	6.20×10^{-14}
	8	14025.3	6.20×10^{-14}
	9	14048.4	6.20×10^{-14}
	10	14069.3	9.92×10^{-14}
Downregulated	1	5640.7	1.98×10^{-13}
	2	6753.3	9.45×10^{-11}
	3	6759.6	1.75×10^{-10}
	4	6756.0	1.83×10^{-10}
	5	4795.7	4.93×10^{-10}
	6	6744.0	5.43×10^{-10}
	7	4862.5	6.43×10^{-9}
	8	4792.9	9.09×10^{-9}
	9	4714.2	4.62×10^{-8}
	10	7196.1	3.14×10^{-7}

Figure 3: (A) Pivot table showing number of significantly upregulated signals observed across time points across all five mice. (B) Pivot table showing number of significantly downregulated signals observed across time points across all five mice. (C) Top ten *m/z* values according to average p-values when comparing weeks one and seven.

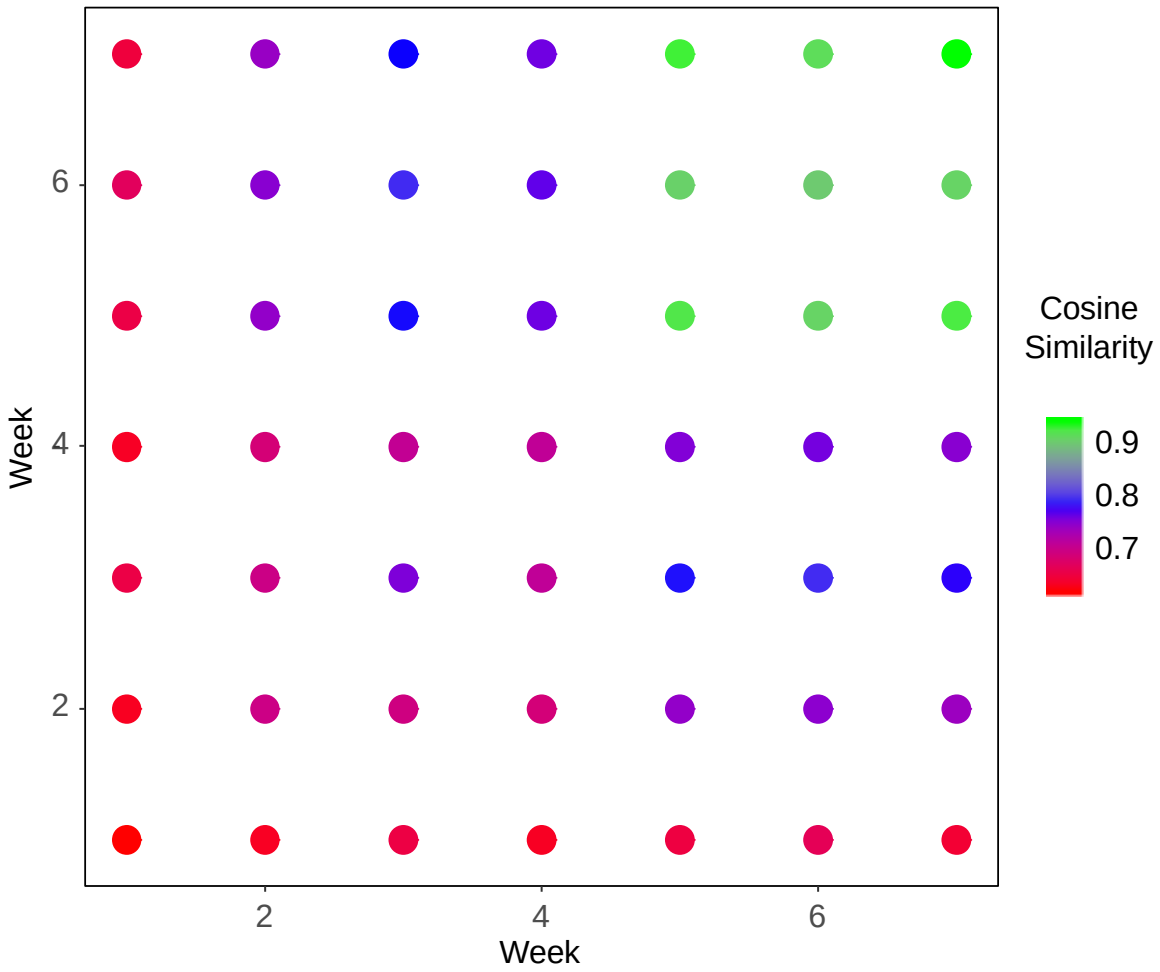

Figure 4: Dot matrix plot comparing spectral similarity between time points. High cosine scores (green) indicates that the global profiles are highly similar between intersecting time point (i.e. weeks six and seven) while low cosine scores (red) indicates less similarity in features between the spectra of the respective time points.