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A recommended and verified procedure for i*n situ* tryptic digestion of formalin-fixed paraffin-embedded tissues for analysis by matrix-assisted laser desorption/ionization imaging mass spectrometry

Audra M. Judd[1,2,*], **Danielle B. Gutierrez**[1,2,*], **Jessica L. Moore**[1,2,‖‖], **Nathan Heath Patterson**[1,2], **Junhai Yang**[1,2,‡‡], **Carrie E. Romer**[1], **Jeremy L. Norris**[1,2,3], **Richard M. Caprioli**[1,2,3,4,5]

[1]Mass Spectrometry Research Center, Vanderbilt University, Nashville TN, 37235

[2]Departments of Biochemistry, Vanderbilt University, Nashville TN, 37235

[3]Departments of Chemistry, Vanderbilt University, Nashville TN, 37235

[4]Departments of Pharmacology, Vanderbilt University, Nashville TN, 37235

[5]Departments of Medicine, Vanderbilt University, Nashville TN, 37235

Abstract

Matrix-assisted laser desorption/ionization imaging mass spectrometry (MALDI IMS) is a molecular imaging technology uniquely capable of untargeted measurement of proteins, lipids, and metabolites while retaining spatial information about their location *in situ*. This powerful combination of capabilities has the potential to bring a wealth of knowledge to the field of molecular histology. Translation of this innovative research tool into clinical laboratories requires the development of reliable sample preparation protocols for the analysis of proteins from formalin-fixed paraffin embedded (FFPE) tissues, the standard preservation process in clinical pathology. Although ideal for stained tissue analysis by microscopy, the FFPE process cross-links, disrupts, or can remove proteins from the tissue, making analysis of the protein content challenging. To date, reported approaches differ widely in process and efficacy. This tutorial presents a strategy, derived from systematic testing and optimization of key parameters, for reproducible *in situ* tryptic digestion of proteins in FFPE tissue and subsequent MALDI IMS analysis. The approach describes a generalized method for FFPE tissues originating from virtually any source.

Correspondence: Dr. Richard M. Caprioli, 9160 MRB III, Department of Biochemistry, Vanderbilt University, Nashville, TN 37232, USA, Phone: (615) 322-4336, Fax: (615) 343-8372, Richard.M.Caprioli@Vanderbilt.edu.

‖‖Current Address: Frontier Diagnostics LLC, Nashville, TN, 37210

‡‡Current Address: AbbVie Inc., North Chicago, IL, 60064

*Co-first authors

Supplemental Information

Supplemental figures include: Figure 1 – Trypsin activity results; Figure 2 – Ion intensity differences due to varying chamber buffer volume; Figure 3 – Humidity for various digestion chamber conditions; Figure 4 – Matrix solvent composition; Figure 5 – Matrix recrystallization. Supplemental methods include: Method 1: H&E staining, Method 2: Trypsin activity assay, Method 3: Determining protein content in tissue to optimize for trypsin concentration; Method 4: Imaging mass spectrometry; Method 5: Tissue microextractions for LC-MS/MS analysis.

Keywords

colon; formalin-fixed paraffin embedded tissue; *in situ* tryptic digestion; MALDI imaging mass spectrometry; molecular histology

Introduction

Matrix-assisted laser desorption/ionization imaging mass spectrometry (MALDI IMS) is an analytical technology for directly determining the spatial distributions of many hundreds of analytes simultaneously in tissue.[1] Molecular information is obtained directly from thin tissue sections such as those used in basic science and clinical laboratories. Samples are prepared for analysis by coating the tissue with MALDI matrix, a low molecular weight organic molecule that aids in the molecular desorption and ionization process after irradiation by the laser within the MALDI source. Since the analysis is accomplished without the use of antibody tags,[1,2] MALDI IMS can be used to characterize a wide range of molecular species in an untargeted manner, significantly increasing insights into the important molecular mechanisms that underpin disease. Since its inception,[2] MALDI IMS has been applied to clinical and biological problems and has been shown to significantly augment traditional histopathology approaches with complementary molecular data. Examples include the analysis of tissue microarrays,[3–6] the determination of diagnostic and prognostic classification markers,[7–15] and the diagnosis of specific disease types when histopathology is indeterminate.[16–20]

The vast majority of clinical biopsy samples are preserved as formalin-fixed paraffin embedded (FFPE) tissue blocks in order to stabilize morphology for follow-up histopathological analysis years or even decades later. Thus, FFPE tissues allow investigators to perform extensive retrospective studies not otherwise possible. However, FFPE preservation presents several challenges for MALDI IMS analysis.[21] First, removal of paraffin is required for analysis, and this typically uses organic solvents that remove endogenous lipids and some proteins. Endogenous peptides[22] and metabolites[4,14] can be imaged without further sample preparation prior to matrix application. However, the intramolecular crosslinks formed during formalin fixation make it difficult to analyze proteins directly. In contrast to fresh frozen tissue, where proteins can be measured directly, with FFPE tissue peptides must serve as surrogates for their precursor proteins. As a result, the second requirement for protein imaging of FFPE tissue is an antigen retrieval step, which increases the accessibility of proteins to endoproteinases during *in situ* protein digestion to release peptides for IMS analysis. The preparation of FFPE tissue for peptide mapping by IMS requires careful optimization of parameters to preserve tissue morphology and analyte localization as well as to obtain abundant peptide signal intensities.

A recent review of on-tissue digestion for IMS applications discusses various methods, instrumentation, and applications.[23] *In situ* enzymatic digestion has been accomplished using robotic spotting technologies to apply trypsin directly onto the tissue section in a manner that minimizes delocalization of analytes.[24–26] Drawbacks of utilizing robotic spotters include low spatial resolution (150–250 μm) and low throughput. Recently, reagent

sprayers have been used to apply proteolytic enzymes and matrix to tissues with increased spatial resolution for fresh frozen and FFPE tissues.[27–34] Spray approaches can differ widely, and no validated/systematic procedures have been reported to ensure high-quality *in situ* digestion of tissues.

In this article, we provide a detailed discussion regarding the key parameters of *in situ* tryptic digestion of protein in FFPE tissue for MALDI IMS analysis. We focus on the application of trypsin by robotic spray technologies that provide for increased spatial resolution and efficiency. The following steps are discussed for an *in situ* digestion MALDI IMS workflow (Figure 1): initial tissue preparation, tissue deparaffinization and antigen retrieval, the application of trypsin by robotic spraying, *in situ* digestion, and matrix application. Important aspects of *in situ* digestion are addressed including the duration of digestion and chamber conditions, along with the effects of matrix solvent composition and matrix recrystallization.

Initial Tissue Preparation

Protocols for sectioning FFPE blocks are tissue-specific. Workflows should produce sections that maintain tissue morphology. A recommended starting point is to cut sections from FFPE blocks at 4–8 μm thickness and float mount the sections from a 47–50 °C water bath onto conductive ITO coated slides (Delta Technologies).[5] Adjacent tissue sections to those used for MALDI IMS analysis can be collected and stained using Hematoxylin and Eosin (H&E) for inspection by routine microscopy (see Supplemental Method 1).

Step 1: Tissue Deparaffinization and Antigen Retrieval

The first two steps required for peptide analysis by IMS include paraffin removal and antigen retrieval (see Method Box 1). Deparaffinization is typically accomplished by washing in a series of xylenes and graded ethanol.[21] Antigen retrieval is a common step for immunohistochemical analysis of FFPE tissue, as it exposes the epitopes for successful antibody binding. Heat-based protein unfolding also improves the *in-situ* digestion of cross-linked proteins, presumably providing trypsin greater accessibility for protein cleavage, and gives rise to many tryptic peptides (Figure 2A & 2B). Without antigen retrieval, few signals are recorded (Figure 2C & 2D).

Step 2: Trypsin Application to FFPE Tissues

The workflow presented here focuses on the application of trypsin using spray technologies (see Method Box 2). In addition to potentially providing increased spatial resolution compared to robotic spotters, spraying offers the capacity for higher throughput. A comparison of both workflows within our laboratory demonstrated over 3-fold higher throughput using a robotic sprayer approach when compared to previously published approaches using spotters.[5] Although methods for a TM-sprayer (HTX Technologies) are presented in this article, protocols for other spray technologies or spotting technologies can be used effectively if conditions are carefully validated.

Prior to spraying tissues with trypsin, it is important to ensure trypsin will remain active after spraying and for the duration of the digestion. A trypsin activity assay can be performed employing Nα-benzoyl-DL-arginine-p-nitroanilide (BAPNA, Sigma-Aldrich product B3133) as the substrate (Supplemental Figure 1A and Supplemental Method 2). Using our described protocol, results demonstrate that trypsin activity is maintained for up to 4 hours at 37 °C but is reduced by approximately 60% after overnight incubation and by 85–90% after incubation at 55 °C (Supplemental Figure 1B).

For trypsin application, a final on-tissue amount of 3.2 ng/mm^2 is recommended as a starting point (see Optimization Box 1). Experience has shown this concentration produces successful digestions of many tissues. Figures 3 and 4 show the *in situ* digestion of colon tissue as an example. For colon tissue, this amount of trypsin represents an estimated enzyme:protein ratio of 1:23 per square millimeter of tissue. The optimal amount of trypsin per area of tissue required to produce abundant peptides can vary depending on tissue type and protein density within tissue sub-structures. Therefore if 3.2 ng/mm^2 does not produce abundant peptide signal for a specific tissue of interest, a preliminary test will help determine protein content in the tissue in order to adjust the trypsin concentration as needed (Supplemental Method 3). Determination of the best trypsin concentration may also be application specific. One must consider both the efficiency of the digestion along with the optimal result by MALDI IMS to select a final condition. Quantitative or targeted experiments may require optimization of these conditions around specific ions of interest, and digestion of heterogeneous tissues may require control experiments to determine the potential for region-specific differences, as was previously noted in a quantitative assessment using brain tissue.[35]

Step 3: Digestion Conditions

Tissue Hydration.

Previous studies have established a humid digestion chamber environment using a solution of ammonium bicarbonate to create an atmosphere of approximately pH 7.4, a condition beneficial for trypsin activity.[27,36] This results in a higher number of peptide peaks that can be observed by MALDI MS of the tissue. It is important to ensure that digestion conditions don't cause analyte delocalization. Figures 5 and 6 show that 100 μL of ammonium bicarbonate in the described digestion chamber setup (see Method Box 3) facilitate digestion leading to abundant peptide signal and minimal analyte delocalization in imaging experiments (see Optimization Box 2). Supplemental Figure 2 shows differences in MALDI ion peak intensities from tissues digested with varying volumes of digestion chamber buffer. Consistency and reproducibility of humidity in the chamber is essential for generating reproducible images, especially when comparing a series of tissues within an experiment. To further characterize the effect of humidity on *in situ* digestion, hygrometers can be placed in digestion chambers to record changes in humidity over time (Supplemental Figure 3).

Digestion Time.

Digestion time is an essential parameter for obtaining high peptide signal and minimizing delocalization. Longer digestions provide more time for enzymatic cleavage and can thereby

lead to higher peptide signal. However, lengthy reactions can increase the amount of nonspecific cleavage and result in unintended modifications, making it difficult to identify peptides.[37] With on-tissue digestion, longer incubations could lead to increased delocalization of analytes across the tissue surface, especially if the chamber conditions are wet. A digestion time of 2 hours is recommended to produce abundant peptide signals and maintain analyte localization (Figure 7 and Optimization Box 3). For quantitative experiments, consistent proteolytic cleavage is important to reproducibly quantify the peptides of interest. A recent study evaluated the effect of digestion time for on-tissue digestion of frozen brain tissue and demonstrated differences in peptide signal for myelin basic protein between white matter and gray matter and for fully cleaved and miscleaved peptides.[35] Prior to initiating a quantitative, targeted study, it is important to understand peptide dynamics, the effect of different tissue regions on digestion efficiency, and the potential for non-specific cleavage or reaction induced modifications.

Step 4: Matrix Application for MALDI IMS

Matrix Solvent Composition.

Within a specific MALDI IMS application the particular analytes of interest will influence the choice of matrix. Common matrices that are useful across a broad range of analyte classes and molecular weights include 2,5-dihydroxybenzoic acid (DHB) and alpha-cyano-4-hydroxycinnamic acid (CHCA). These matrices have been successful for imaging peptides, proteins, small molecules, and lipids. In addition to the type of matrix, the solvent composition of the matrix solution plays a critical role within the experiment. Analytes that are soluble within the matrix solution will co-crystallize with the matrix, leading to ionization and detection during the IMS experiment. Different analytes or analyte classes can be imaged based on solvent selection. The details of matrix selection have been thoroughly reviewed[1] and a protocol for matrix application is presented in Method Box 4. An important consideration while optimizing solvent composition for spray applications is the localization of analytes. Solutions that are predominantly aqueous, and take longer to dry, may result in analyte delocalization during repeated rounds of matrix deposition onto the tissue. It is critical to select a matrix composition that does not result in analyte delocalization. Use of purified CHCA[38,39] at a concentration of 5 mg/mL in 90% acetonitrile, 0.1% TFA maintains *in situ* analyte spatial distributions (Figure 8, Supplemental Figure 4) and is therefore recommended for peptide imaging of FFPE tissue (see Optimization Box 4).

Matrix Recrystallization

The matrix solvent facilitates mixing of soluble analytes within the tissue and matrix crystals and thereby plays an essential role in signal detection from the tissue.[1] Matrix compositions with a high percentage of organic solvent will evaporate faster than more aqueous solutions, which is advantageous for maintaining analyte localization but may reduce the mixing time of native analytes with the matrix and result in reduced ion signal. For protein analysis, rehydration of tissues after matrix application has been shown to improve signal.[40] Given the variety of tissue environments, the utility of matrix recrystallization will be tissue specific; colon tissue images had higher signal intensity

without tissue rehydration (Supplemental Figure 5). For recrystallization of CHCA matrix, it is recommended to heat slides for 2 minutes at 85 °C and then incubate the slides at 85 °C for three minutes in a sealed chamber containing 50 μL of 50 mM acetic acid.

Method Summary

Application of the above outlined workflow provided an optimized method for peptide imaging of colon and several additional tissues (see Method Box 5). Inclusion of an antigen retrieval step proved to be necessary and yielded robust peptide signal from these tissue sections. Optimal digestion was performed using a trypsin concentration of 3.2 ng/mm^2 for 2 h at 37 °C with 100 μL of 100 mM ammonium bicarbonate in the digestion chamber. The best matrix for maintaining analyte localization was 5 mg/mL CHCA in 90% acetonitrile, 0.1% TFA. Matrix recrystallization did not improve results for colon tissue, but did for other tissues.

Conclusions

This tutorial article provides a strategy for performing *in situ* digestion and MALDI peptide imaging of FFPE tissue specimens. This optimized strategy was established by testing various parameters through tissue microextractions followed by LC-MS/MS analysis and coupling these results to spatially informative imaging data. Some parameters defined in this recommended workflow are generally applicable across a wide range of FFPE tissue types (*e.g.*, the need for an antigen retrieval step prior to digestion), while others (*e.g.*, matrix composition and recrystallization) can be varied based on tissue type. Despite potential differences in individual parameters for different tissues and experimental scenarios, it is essential to minimize analyte delocalization that can occur during digestion and matrix application.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Method Box 1:

Paraffin removal and antigen retrieval.

To remove paraffin, slides were sequentially washed in xylene for 3 min (x2), 100% ethanol for 1 min (x2), 95% ethanol for 1 min, 70% ethanol for 1 min, and Milli-Q purified water for 3 min (x2), followed by drying for 10 min. Antigen retrieval was performed in a decloaking chamber (Biocare Medical) using 10 mM Tris buffer, pH 9. Samples were heated to 95 °C for 20 min with a 10 sec cool down at 90 °C. Slides were removed and cooled in the buffer at room temperature for 20 min and then buffer exchanged by replacing half of the solution with Milli-Q water four times and finally the entire solution with Milli-Q water. Slides were air dried and stored in a dry box over desiccant.

Optimization Box 1:

In situ digestion by different trypsin concentrations.

A comparison of three different trypsin concentrations (0.64, 3.2, and 24 ng/mm^2, final) shows a trend in which more peptides and proteins were identified with the use of 3.2 ng/mm^2 trypsin (Figure 3A and B). Analysis of peptide peak intensities from MALDI imaging data shows that the 3.2 ng/mm^2 concentration produced more peaks of higher intensity than other concentrations (Figure 3C).

Method Box 2:

Spray application of trypsin.

A solution of 0.074 μg/μL of trypsin in 100 mM ammonium bicarbonate, 9% acetonitrile was loaded into a 0.5 mL Hamilton™ syringe connected to the TM Sprayer nozzle through Upchurch PEEK™ tubing (0.005 in I.D.). The sprayer parameters were programmed as previously described.[27] The nozzle was set to 30 °C and was 40 mm from the surface of the slides. Nitrogen was used as a carrier gas at 9.5 psi. Trypsin was delivered from the syringe to the sprayer at a flow rate of 8 μL/min. A total of 8 passes were completed at a rate of 750 mm/min in a criss-cross pattern, with 2 mm track spacing and no drying time.

Optimization Box 2:

Analysis of various digestion chamber buffer volumes.

The use of 100 μL of ammonium bicarbonate resulted in MALDI images with the highest ion signal and minimal delocalization compared to the use of higher volumes (500 and 1000 μL) or no buffer (Figure 5A–D). LC-MS/MS analysis supported these findings (Figure 5E–G).

Method Box 3:

Digestion chamber setup.

Digestion chambers were prepared using 92 × 16 mm plastic Petri dishes with a 5 × 5 cm WypAll™ square placed in the bottom of each dish and 100 μL of 100 mM ammonium bicarbonate placed on the sides of the WypAll™. A single slide was placed into each prepared Petri dish, sealed using Petri-Seal™ and parafilm, and incubated at 37 °C to facilitate digestion.

Optimization Box 3:

Digestion time.

To establish an optimal length of time for *in situ* digestion, colon tissue was digested overnight (16–18 h) or for 2 h. Qualitative analysis of MALDI ion images showed similar signal intensity for both conditions (Figure 7A). Likewise, a quantitative comparison of ion peak intensities from MALDI images showed similar intensities for most ions. Overnight digestion produced significantly higher intensity signals for some ions, mainly those below *m/z* 2,000 (Figure 7B). LC-MS/MS analysis of microextractions identified a similar number of proteins and peptides from the 2 h and overnight digestions, and the two conditions shared 58 overlapping proteins, 77% of the total identified across the two groups (Figure 7C–E). Overnight digestion did not provide an advantage over the 2 h digestion, and the lengthier incubation period increases the chances for analyte delocalization. In addition, it was shown that trypsin activity decreases after 4 hours (see Supplemental Figure 1). Therefore, a 2 h digestion is recommended to produce abundant peptide signals and maintain analyte localization.

Method Box 4:

Matrix application.

Matrix was deposited onto digested colon tissue using a robotic sprayer. The nozzle of the TM Sprayer was aligned 40 mm from the surface of the slide, heated to 85 °C, and the nitrogen carrier gas was set to 10 psi. Purified CHCA at a concentration of 5 mg/mL in 90% acetonitrile, 0.1% trifluoroacetic acid (TFA) was loaded onto the 5 mL loop of a standard HPLC pump, and 90% acetonitrile was used as a pushing solvent at a flow rate of 0.1 mL/min. Matrix was sprayed onto tissue sections for a total of 8 passes at a rate of 700 mm/min in a crisscross pattern, with 2 mm track spacing and no drying time. Samples were stored over desiccant and out of light until analysis.

Optimization Box 4:

Matrix application.

To optimize matrix application for MALDI peptide imaging, 3 different matrix compositions were tested: 5 mg/mL CHCA in 50%, 70%, or 90% acetonitrile, 0.1% TFA. The matrix solutions containing 50% and 70% acetonitrile, 0.1% TFA resulted in analyte delocalization across the tissue, while the use of 90% acetonitrile, 0.1% TFA maintained *in situ* analyte spatial distributions (Figure 8). This was supported by comparing the spatial distribution of several ion images (Figure 8A & 8B) to histological features in an H&E stained serial section (Figure 8C). Supplemental Figure 4 shows the result of different matrix conditions on individual ion signals.

Method Box 5:

Framework for optimization.

This method represents a recommended starting point and has been applied successfully to many tissue types; however, some tissues may require optimization and ultimately different conditions. Method optimization should implement a two-tiered approach consisting of liquid chromatography tandem mass spectrometry (LC-MS/MS) and MALDI IMS (Supplemental Methods 4 & 5). Following on-tissue digestion, and prior to matrix application for MALDI IMS analysis, tryptic peptides can be microextracted from tissues as previously described[42] for downstream LC-MS/MS analysis. This strategy provides a framework for testing *in situ* tryptic digestion parameters and evaluating 1) the efficacy of *in situ* protein digestion and 2) the resulting MALDI IMS peptide signal and analyte localization.

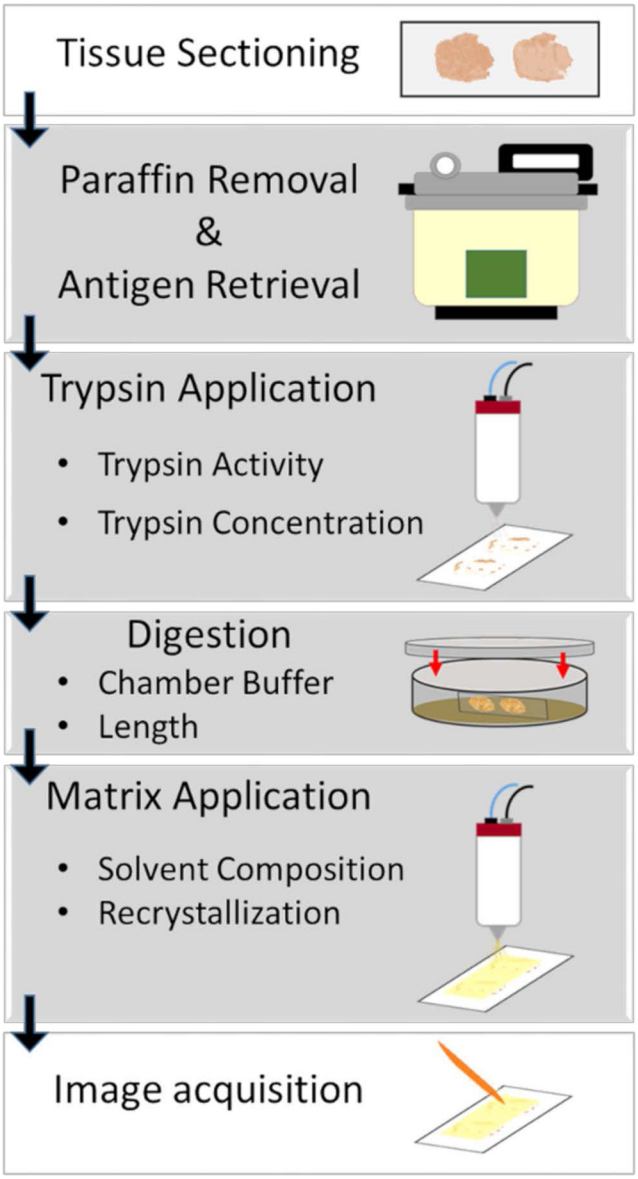

Figure 1. Workflow for *in situ* digestion using robotic spray application of trypsin for MALDI IMS peptide analysis of FFPE tissues.

Steps that were optimized within the framework developed herein are highlighted in gray, with tested parameters listed.

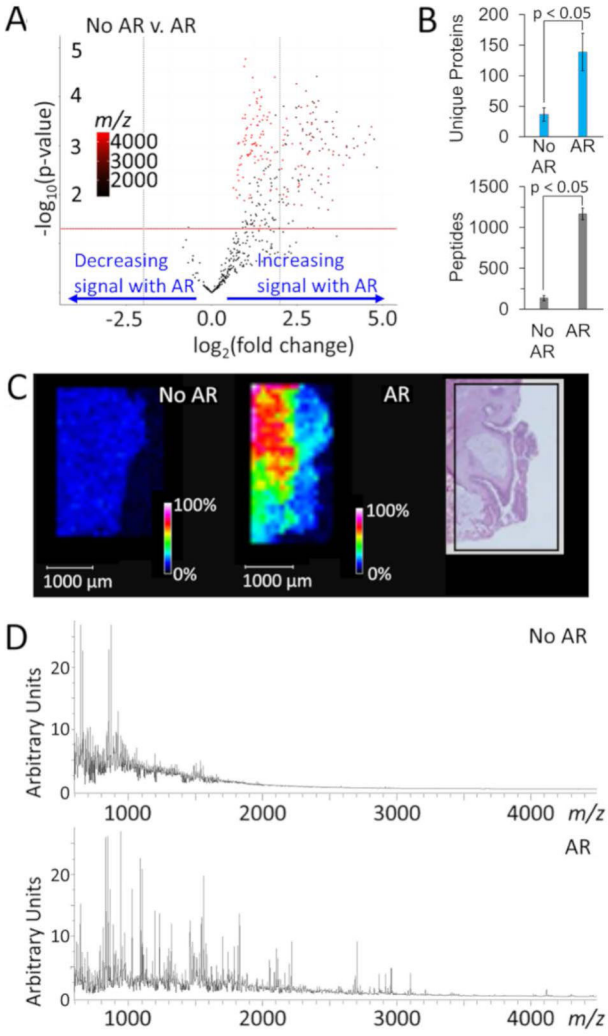

Figure 2. Antigen retrieval.

A) Differences in MALDI ion peak intensities from tissue processed with or without antigen retrieval (AR). Ions above the red dotted line have signal intensities that are significantly different between conditions ($p<0.05$). All data were derived from three biological replicates (with three technical replicates each). B) The number of identifications determined by LC-MS/MS analysis of microextractions (reverse hits and proteins identified by fewer than 2 peptides were not counted). No AR: 37 ± 11 proteins and 139 ± 31 peptides; AR: 137 ± 32 proteins and 1,167 ± 71 peptides. Means between AR and No AR were found to be significantly different using an unpaired t-test in GraphPad Prism version 5.04. Data were derived from 3 technical replicates of one patient sample. C) MALDI IMS of *m/z* 1459.7 ± 0.2 from tissue treated without (left) or with antigen retrieval (middle). H&E stained FFPE colon tissue (right); the black box shows the region imaged. D) MALDI IMS summary spectra showing average tissue signal without (top) and with (bottom) antigen retrieval. Sample preparation: tissues were sprayed with trypsin (0.64 ng/mm^2 final) and digested at 37 °C overnight in a chamber containing 100 µL of 100 mM ammonium bicarbonate. Microextractions were collected and then 5 mg/mL CHCA in 90% acetonitrile, 0.1% TFA

was sprayed onto the tissues. Samples were rehydrated with 50 μL of 50 mM acetic acid at 85 °C for 3 min.

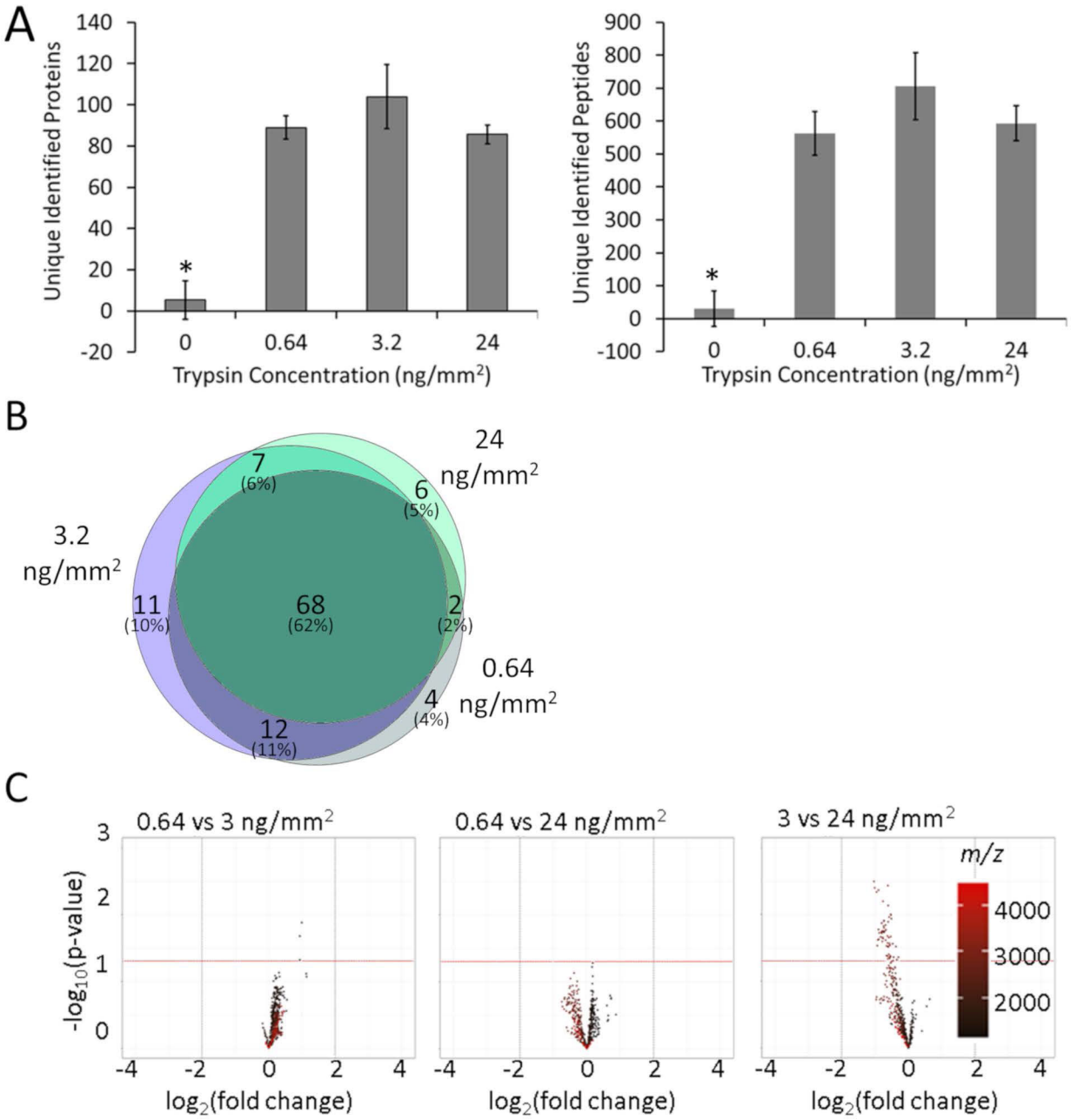

Figure 3. Optimization of trypsin concentration – maximizing peptide signal.

A) LC-MS/MS analysis of microextractions from tissues digested with varying amounts of trypsin. Means were compared using one-way ANOVA in GraphPad Prism version 5.04. Changes in proteomics results produced by altering trypsin concentration from 0.64–24 ng/mm^2 were not statistically significant ($p<0.05$) when compared to one another, but control experiments with no trypsin added (0 ng/mm^2) resulted in a statically significant ($p<0.05$) diminishment of peptide and proteins detected (asterisk). Error bars represent standard deviation from three technical replicates of one patient sample. B) The overlap of all proteins identified by LC-MS/MS in at least two out of three technical replicates per condition. This plot was made using EulerAPE_3.0.0.[41] C) Differences in MALDI ion

peak intensities from tissue digested with various concentrations of trypsin. Ions above the red dotted line have signal intensities that are significantly different between conditions ($p<0.05$). Data were derived from three technical replicates from each of three patient samples. Sample Preparation: tissues were processed with antigen retrieval and, after trypsin deposition, were digested overnight at 37 °C in a chamber containing 100 μL of 100 mM ammonium bicarbonate. Microextractions were collected and then 5 mg/mL CHCA in 90% acetonitrile, 0.1% TFA was sprayed onto the tissue. Samples were rehydrated with 50 μL of 50 mM acetic acid at 85°C.

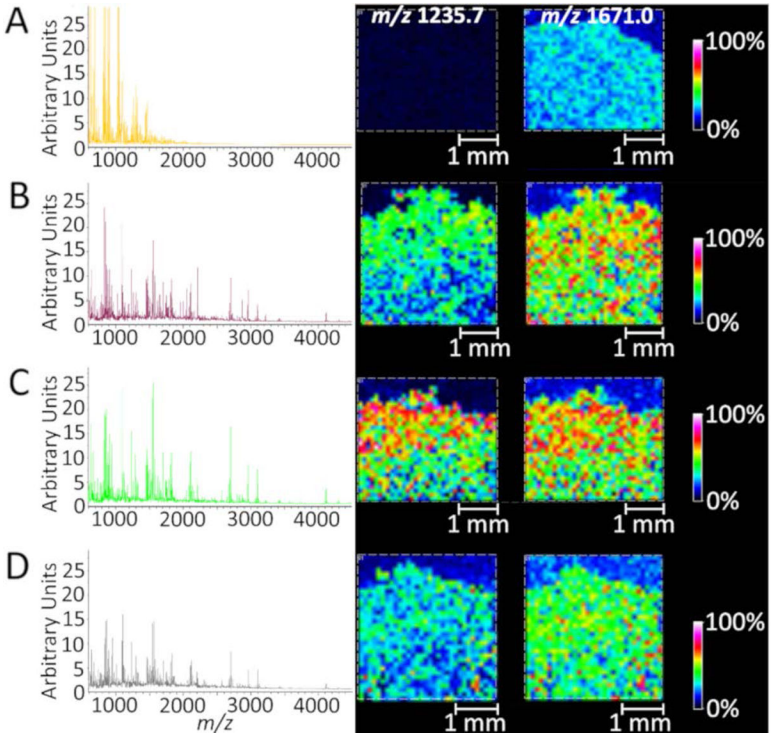

Figure 4. Optimization of trypsin concentration-IMS performance.

A-D) MALDI summary spectra (left) and two representative ion images (right) when A) 0, B) 0.64, C) 3.2, or D) 24 ng/mm^2 of trypsin were used for digestion. Ion images represent: *m/z* 1235.7 ± 0.3 (left) and *m/z* 1671.0 ± 0.5 (right). Sample preparation: tissues were processed with antigen retrieval and, after trypsin deposition, were digested overnight at 37 °C in a chamber containing 100 μL of 100 mM ammonium bicarbonate. 5 mg/mL CHCA in 90% acetonitrile, 0.1% TFA was sprayed onto the tissue. Samples were rehydrated with 50 μL of 50 mM acetic acid at 85°C.

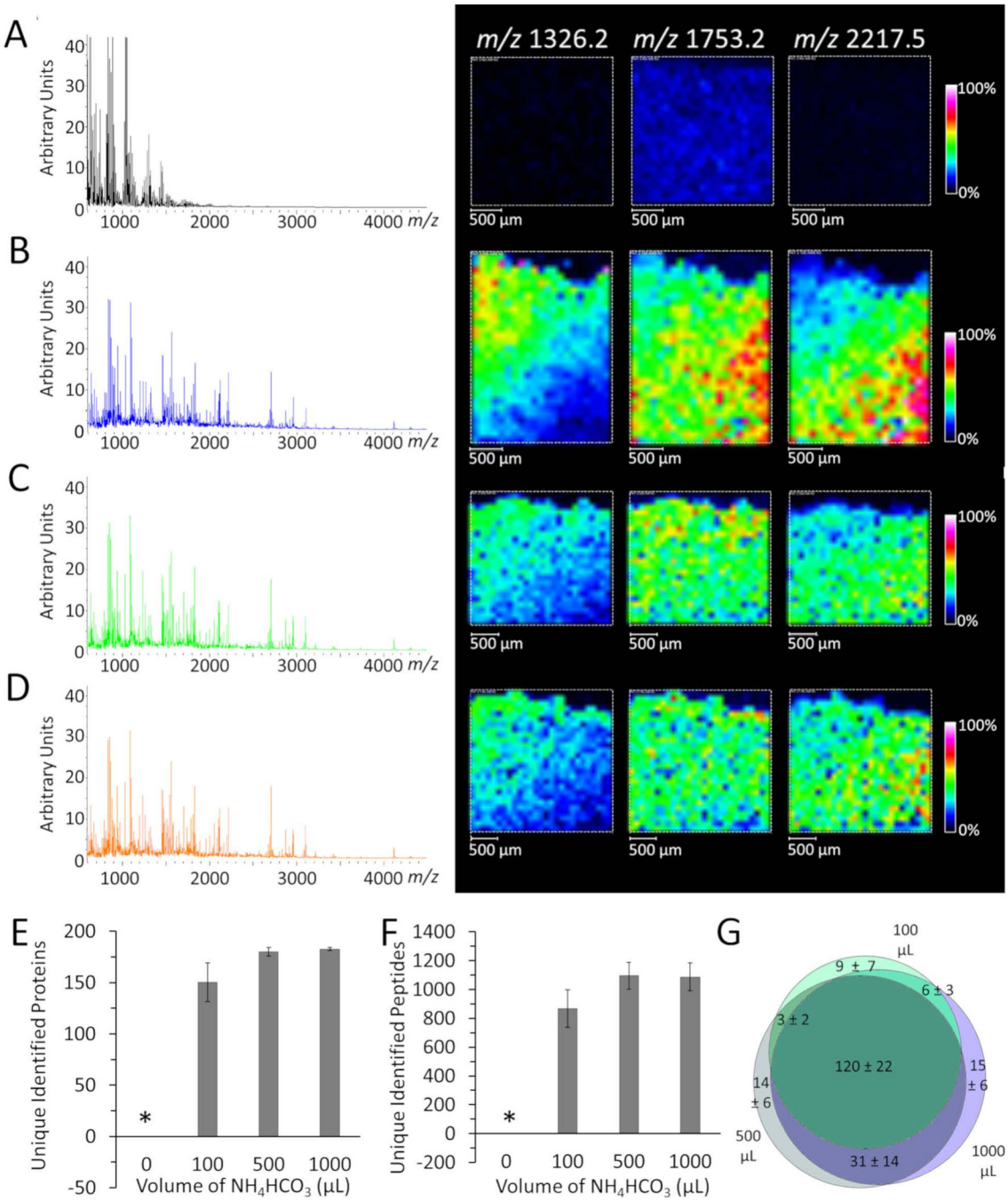

Figure 5. Digestion chamber buffer volume.

A-D) MALDI summary spectra (left) when A) 0, B) 100, C) 500, or D) 1000 µL of 100 mM ammonium bicarbonate were placed in the digestion chamber and three representative ion images: *m/z* 1326.2 ± 0.2 (left), *m/z* 1753.2 ± 0.2 (center), and *m/z* 2271.5 ± 0.2 (right), E-F) The number of E) proteins and F) peptides identified from tissue microextractions after tissues were digested in a chamber containing various amounts of ammonium bicarbonate. Data represent three technical replicates taken from each of three biological replicates. All concentrations were significantly different from 0 µL of 100 mM ammonium bicarbonate

(asterisks, p value <0.05) but not different from each other. Error bars represent the standard error among the technical replicate averages from each of three patient samples. G) The overlap of proteins identified by LC-MS/MS in at least two out of three technical replicates per condition. Areas and values represent the average of three patient samples. This plot was made using EulerAPE_3.0.0.[41] Sample preparation: tissues were antigen retrieved, sprayed with trypsin (0.64 ng/mm^2, final), and digested. Microextractions were collected, and then 5 mg/mL CHCA in 90% acetonitrile, 0.1% TFA was sprayed onto the tissues. Samples were rehydrated with 50 μL of 50 mM acetic acid at 85 °C.

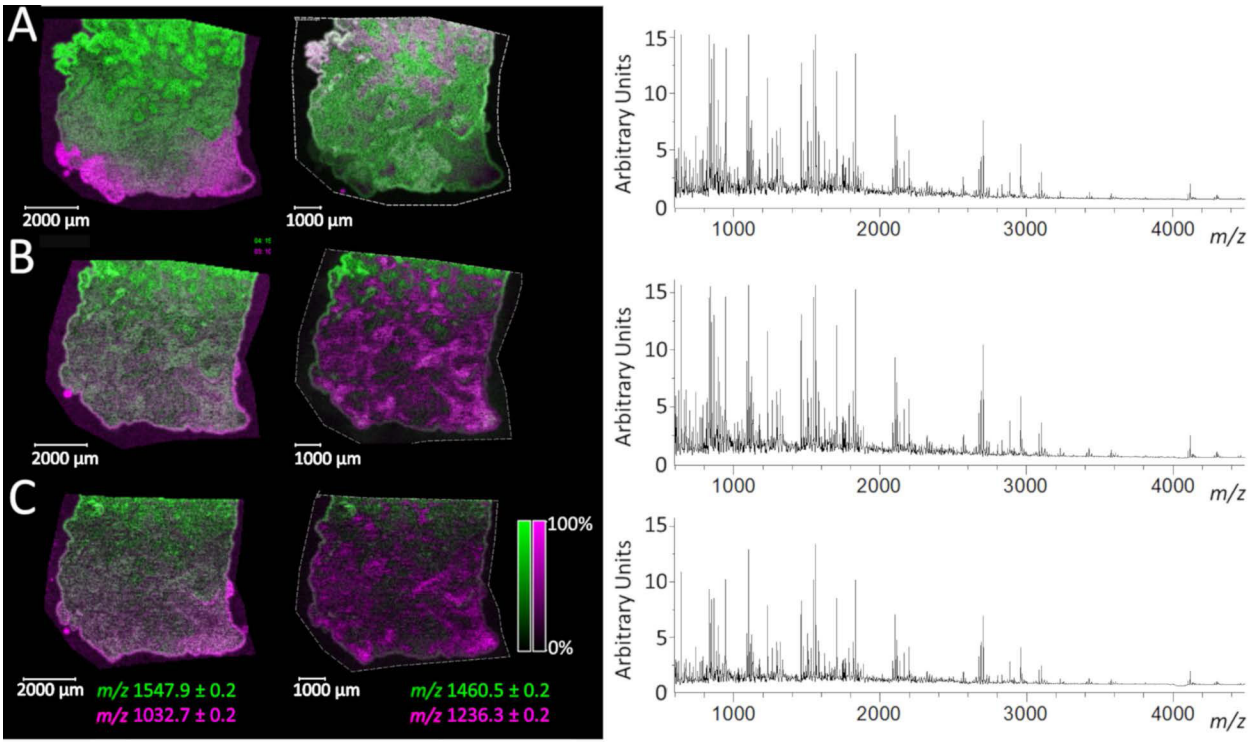

Figure 6. Maintaining analyte localization during digestion.

Ion images and average mass spectra resulting from overnight digestion with various amounts of ammonium bicarbonate in the digestion chamber: A) 100, B) 500, and C) 1000 µL. Left side ion images: *m/z* 1547.9 ± 0.2 (green), *m/z* 1032.7 ± 0.2 (magenta). Right side ion images: *m/z* 1460.5 ± 0.2 (green), *m/z* 1236.3 ± 0.2 (magenta). Sample preparation: tissues were antigen retrieved, sprayed with trypsin (3.2 ng/mm^2, final), and digested overnight. 5 mg/mL CHCA in 90% acetonitrile, 0.1% TFA was sprayed onto tissues. No rehydration was performed.

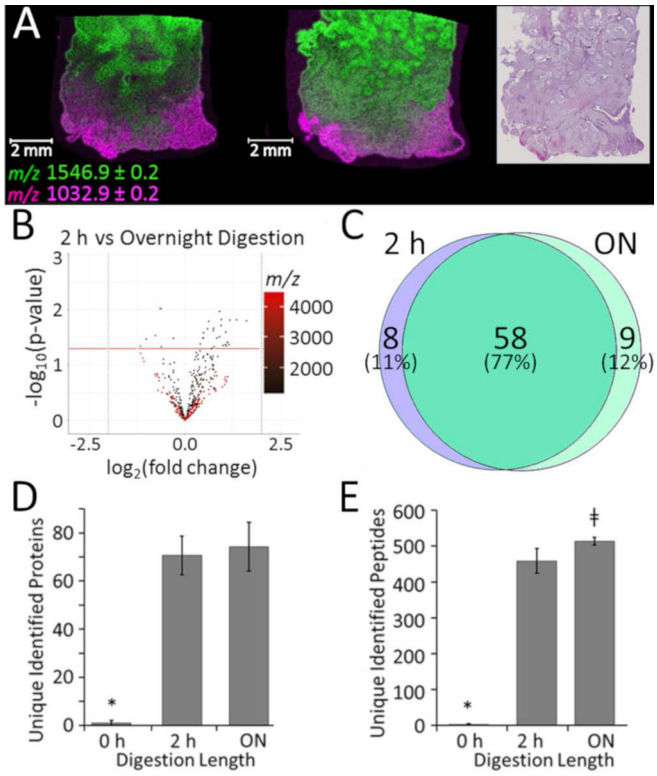

Figure 7. Digestion length.

A) MALDI ion images of *m/z* 1546.9 ± 0.2 and *m/z* 1032.9 ± 0.2 from FFPE colon tissue digested for 2 h (left) or overnight (16–18 h, right). B) Differences in MALDI ion peak intensities from tissues digested for 2 h or overnight. Ions above the red dotted line have signal intensities that are significantly different between conditions ($p<0.05$). Data were combined from three technical replicates taken from each of three biological replicates. C-E) LC-MS/MS analysis of microextractions from colon tissues digested for 2 h or overnight (ON, 16–18 h). C) The overlap of all proteins identified by LC-MS/MS in at least two out of three technical replicates per condition. This plot was made using The Venn Diagram Plotter provided by PNNL (https://omics.pnl.gov/software/venn-diagram-plotter). D-E) The number of proteins and peptides identified from each condition. Means were compared by one-way ANOVA using GraphPad Prism version 5.04. All groups were significantly different from 0 h (*, p value <0.05) but not different from each other. The number of peptides identified using overnight digestion was statistically greater than using the 2 h digestion (ǂ, p value <0.05). Data are from three technical replicates. Sample preparation: tissues were antigen retrieved, sprayed with trypsin (3.2 ng/mm^2 final for panel A; 0.64 ng/mm^2 final for panels B-E), and digested in a chamber containing 100 μl of 100 mM ammonium bicarbonate. Microextractions were collected and 5 mg/mL CHCA in 90% acetonitrile, 0.1% TFA was sprayed onto the tissues. Either no rehydration was performed (panel A) or samples were rehydrated with 50 μL of 50 mM acetic acid at 85 °C (panels B-E).

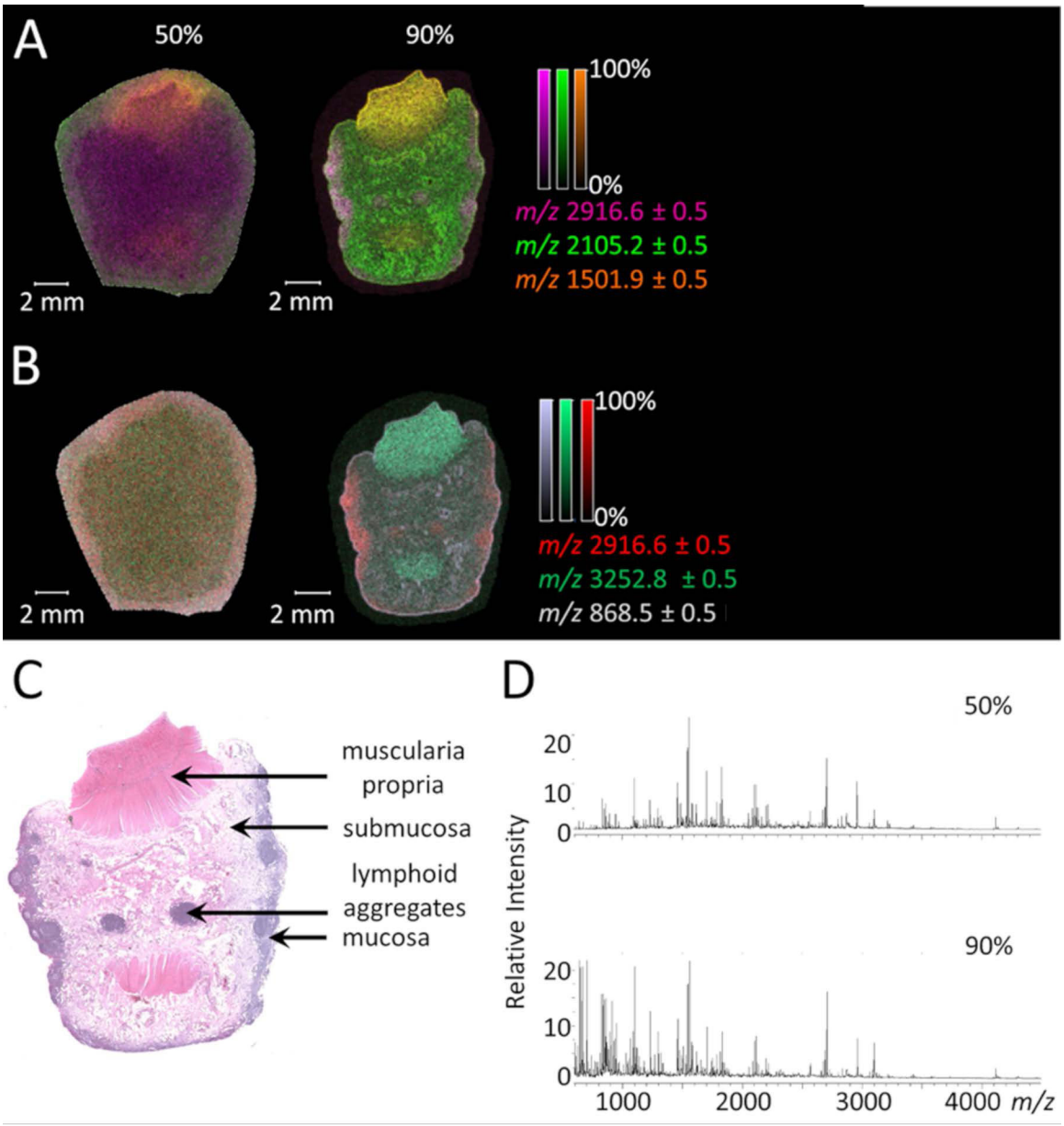

Figure 8. Matrix solution composition.

A) Ion images showing the distributions of *m/z* 2916.6 ± 0.5 (magenta), *m/z* 2105.2 ± 0.5 (green), and *m/z* 1501.9 ± 0.5 (orange) or B) *m/z* 2916.6 ± 0.5 (red), *m/z* 3252.8 ± 0.5 (green), and *m/z* 868.5 ± 0.5 (gray) using a matrix solution of 50% acetontrile, 0.1% TFA (left) or 90% acetonitrile, 0.1% TFA (right). C) H&E stained serial section denoting histological features. D) Average spectra from images obtained with 50% acetonitrile, 0.1% TFA (top) or 90% acetonitrile, 0.1% TFA (bottom). Sample preparation: Formalin-fixed paraffin embedded human colon tissues were antigen retrieved, sprayed with trypsin (3.2 ng/mm^2, final), and digested overnight in a chamber containing 100 μL of 100 mM ammonium bicarbonate. Samples did not undergo rehydration.