MALDI and LCMS Protein Biomarkers of Ionizing Radiation

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INTRODUCTION

- The goal of this project was to identify dose-related protein biomarkers of ionizing radiation (IR) with mass spectrometry, specifically, biomarkers of the 2 Gy threshold dose for radiation sickness.
- A civilian nuclear power plant accident or a terrorist nuclear event on U.S. soil could result in a vast number of casualties.
- Some of the victims would be hospitalized because of acute signs and symptoms, such as vomiting, burns and pain requiring immediate care.
- Some victims would appear symptom free, but may have received ≥ 2 Gy; total body radiation (TBI). **They would develop radiation sickness in the next 6-24 hours**.
- Initial mass spectrometry (MS) experiments demonstrated radiation dose-related albumin and other induced proteins in radiosensitive murine buccal mucosa and tongue tissue.

METHODS a

- Forty Swiss Webster mice, 10 control and 30 experimental.
- The anesthetized animals received TBI, with a low linear energy transfer (LET) photon beam (Linac 23 MV linear accelerator) as follows: 1Gy(10 mice), 2Gy(10 mice) and 3Gy(10 mice), in groups of five.
- Sucrose 30%, 5cc was infused (intracardiac/femoral perfusion) under terminal anesthesia.[1] The sucrose cryoprotected the tissue from freezer artifact (disruption of tissue architecture by ice crystals).[2]

1. Eichenbaum KD, Eichenbaum JW, Fadiel A, Miller DC, Demir N, Naftolin F, Stern A, Pevsner PH. *BioTechniques* 2005; **39**: 487.
2. Terracio L, Schwabe KG. *J Histochem Cytochem* 1981; **29**: 1021.

METHODS b

- Fresh paraformaldehyde fixative 3.7% perfusion (3cc) followed the sucrose. The tongue and heart were removed after perfusion.
- The organs were placed in 3.7% paraformaldehyde for one hour (10:1 solution:tissue, V/V) at 4℃. The s hort immersion time fixed the tissue without completely cross-linking all tissue proteins.[3] Cross-linking interferes with both matrix assisted laser desorption ionization (MALDI), MALDI imaging (IMS), and liquid chromatography mass spectrometry (LCMS).
- The tissue was then immersed in sucrose 30% (10:1 solution:tissue, V/V) at 4℃ until equilibration (t issue sinks to the bottom of the sucrose solution). The sucrose immersion provided further cryoprotection.

3. Pevsner PH, Melamed J, Remsen T, Kogus A, Francois F, Kessler P, Stern A, Anand S. *Biomarkers Med* 2009; **3**: 55.

CRYOSECTION PREPARATION

- The samples were placed in a tissue mold filled with tissue freezing medium (TFM). TFM supports the tissue and prevents cutting artifacts.[5]
- The tissue mold was placed on a small rapid-freeze disk in the cryostat.
- The mold was immersed in liquid nitrogen-cooled isopentane which flattened the block and improved tissue sectioning.
- Contiguous one-micrometer (µm) serial sections were obtained for histology, IMS, and protein extraction for LCMS.

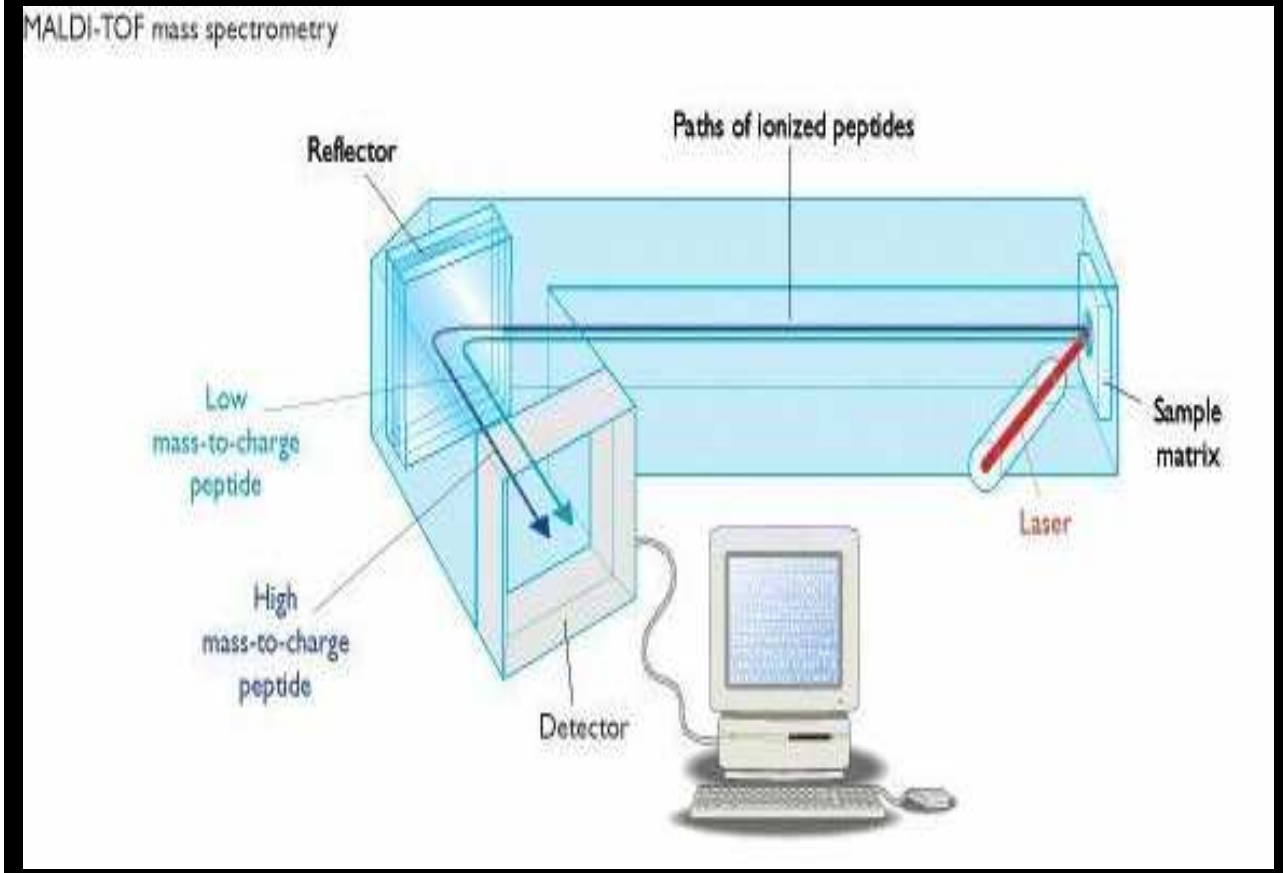
4. Rosene DL, Roy NJ, Davis BJ. *J Histochem Cytochem* 1986; **34**: 1301.
5. Pevsner P, Naftolin F, Vecchione D, Stall B, Miller D, Kessler P, Stern A. Microtubule Associated Proteins (MAP) and Motor Molecules: Direct Tissue MALDI Identification and Imaging. British Mass Spectrometry Society Annual Meeting, Edinburgh, Scotland, September 9-12, 2007

IMS SECTIONS

- A section was applied to a stainless steel (glass slide sized) MALDI conductive plate.
- The plates were immersed in a methanol bath for 15 minutes followed by immersion in a xylene bath for 15 minutes.
- The purpose of methanol and xylene baths was to improve IMS by defatting the tissue, and removing all TFM, a polymer that interferes with MALDI .[6]
- A protein calibrant mixture (insulin, cytochrome C, apomyoglobin, aldolase and BSA) covered with uniform matrix was used for MALDI and IMS calibration.
- Sinapic acid (matrix) was applied uniformly to the tongue tissue for IMS

6. Pevsner PH, Melamed J, Remsen T, Kogus A, Francois F, Kessler P, Stern A, Anand S. *Biomarkers Med* 2009; **3**: 55.
7. Pevsner PH, Naftolin F, Hillman DE, Miller DC, Fadiel A, Kogus A, Stern A, Samuels HH. *Rapid Commun Mass Spectrom* 2007; **21**: 429.

MALDI TOF TOF MASS SPECTROMETER



BAROCYLER EXTRACTION

- A contiguous section was obtained for protein extraction and LCMS analysis for protein identification.
- The section was immersed in 900 µl of ammonium bicarbonate 100 mM in a pressure cycling device (Barocycler) pulse tube.
- The proteins were extracted with 35,000 psi alternating pressure for about 30 seconds.[8]
- The extracted samples were centrifuged for about 5 minutes at 15 G then lyophilized to a volume of 50 µl.

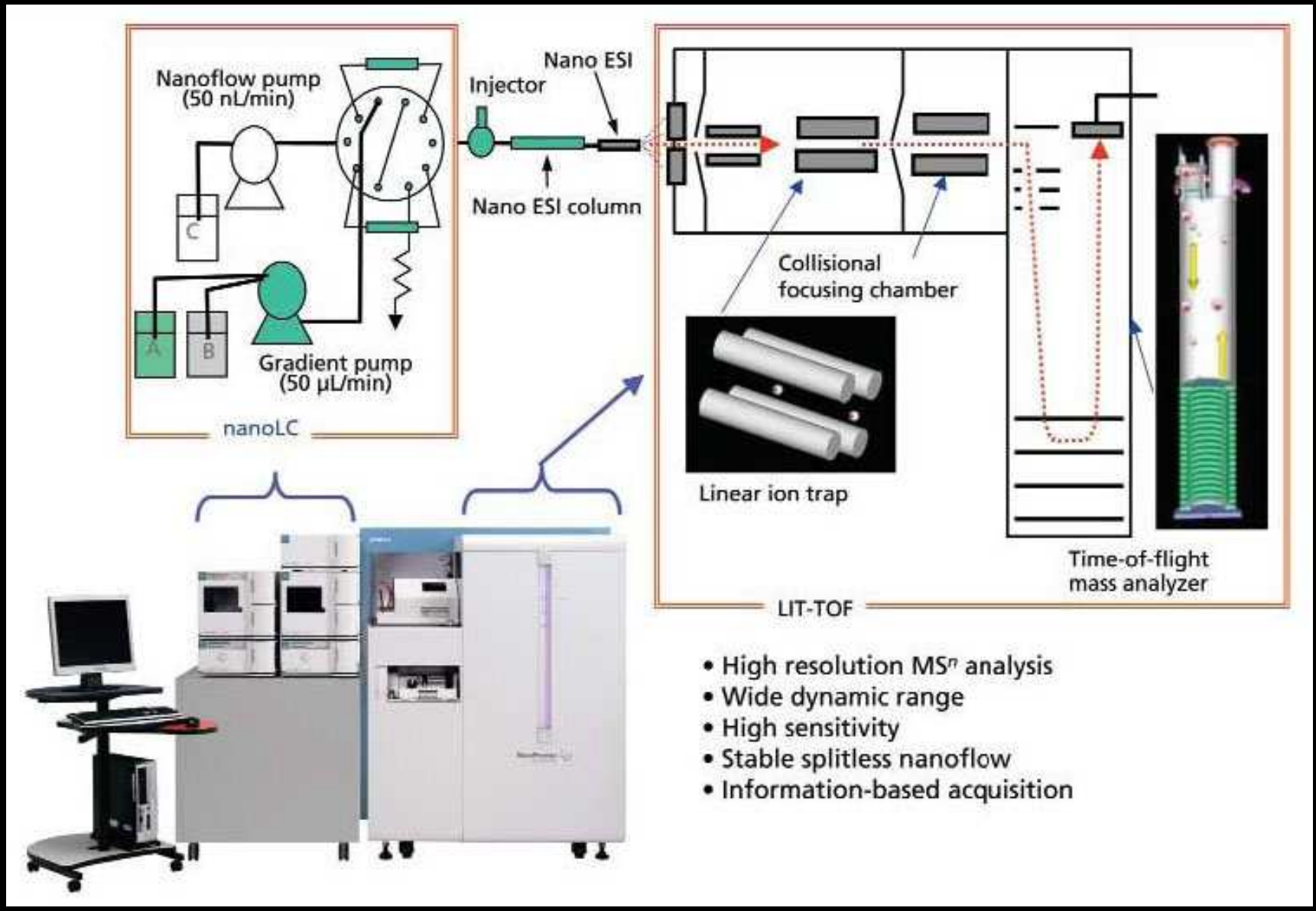
8. Pevsner P, Vecchione D, Remsen T, Kessler P, Momeni M, Duddempudi S, Francois F, Stern A, Anand S. Imaging MALDI vs Histologic Imaging of Cancer of the Colon- A new Diagnostic Paradigm. Biomarkers World Congress, Philadelphia, PA, May 19-21, 2008

BAROCYLER

TRYPSIN DIGEST

- Trypsin, 1 µl (1 µg/µl), was added to the 50 µl sample and maintained at 37℃ overnight.
- The trypsin digest was analyzed with LCMS for protein biomarker identification.

Nanoflow LCMS



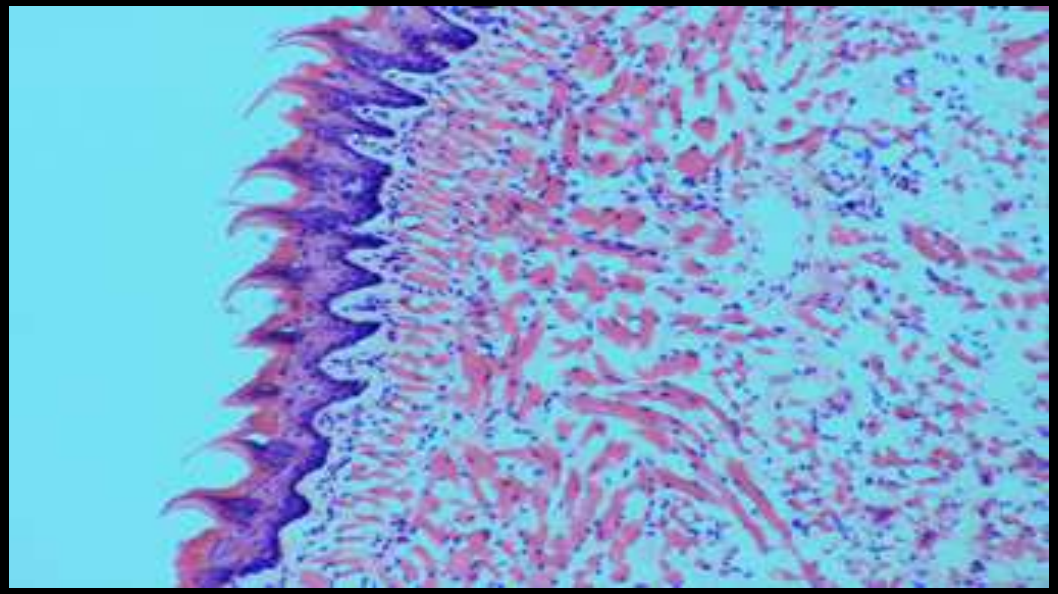
IMS & LCMS

- IMS(images) were obtained from tissue sections of normal, 1Gy, 2Gy and 3Gy irradiated tongue.
- LCMS spectra were obtained from normal, 1, 2 and 3 Gy irradiated tongue samples; normal and 2 Gy irradiated heart samples.

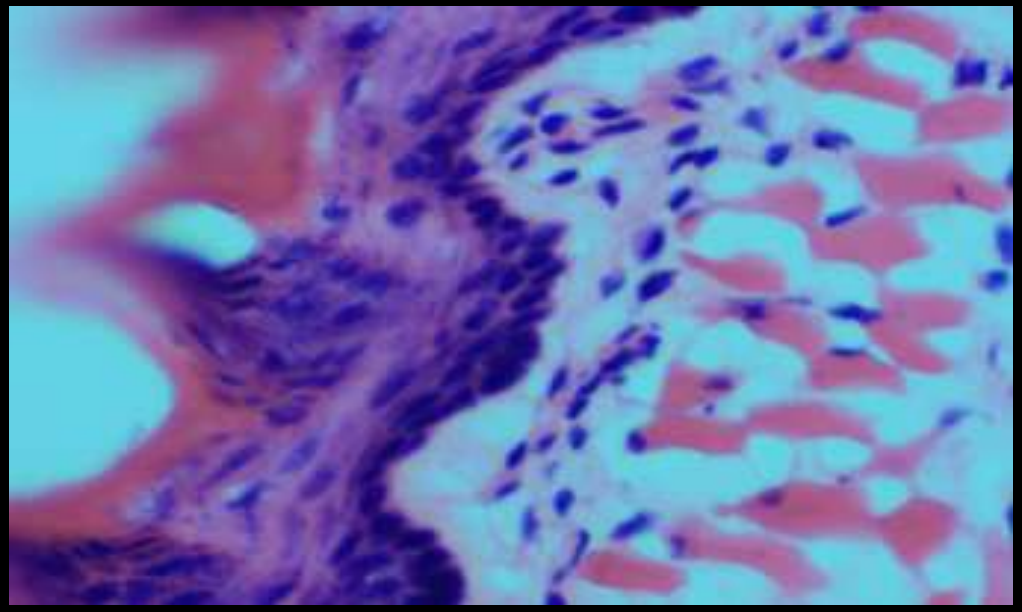
RESULTS

- Hematoxylin & eosin (H&E) were used to stain tissue sections post 1Gy TBI.
- The samples demonstrated progressive increase in tissue destruction. The corresponding IMS tissue change was the identification of albumin(not seen in the tissue IMS of the normal control).
- At 2 Gy there was increased peripheral tissue damage of the spicules on H&E, and a corresponding increase in peripheral albumin in the IMS images.
- At 3 Gy the peripheral tissue damage of the spicules and the central damage of the tongue was severe on the H&E sections. The albumin was now virtually absent in the periphery and concentrated in the center of the IMS image.

H&E OF NORMAL MURINE TONGUE

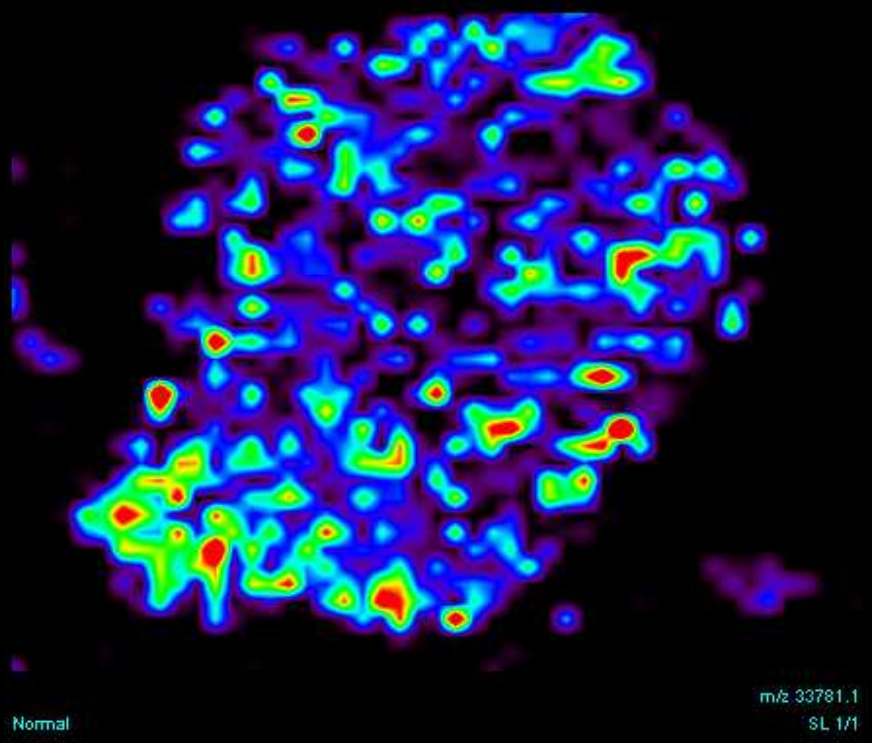


Histopathology of normal murine tongue 10X, longitudinal section. Note the well defined epithelial cornified spicule layer and basal cells.



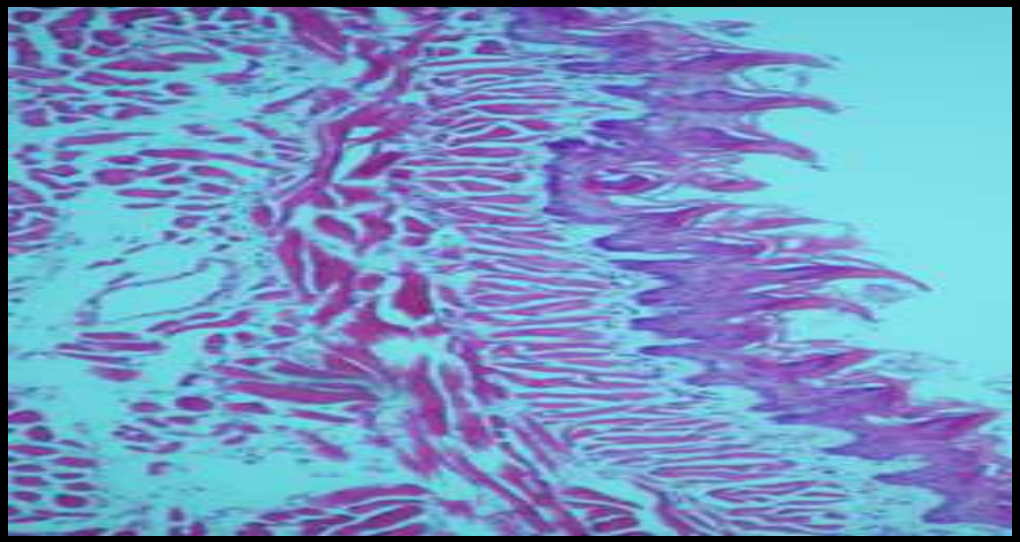
Histopathology of normal murine tongue, 63X longitudinal section. Note the well defined epithelial cornified spicule layer and basal cells.

Normal 33781.1 Da Double Charge Albumin

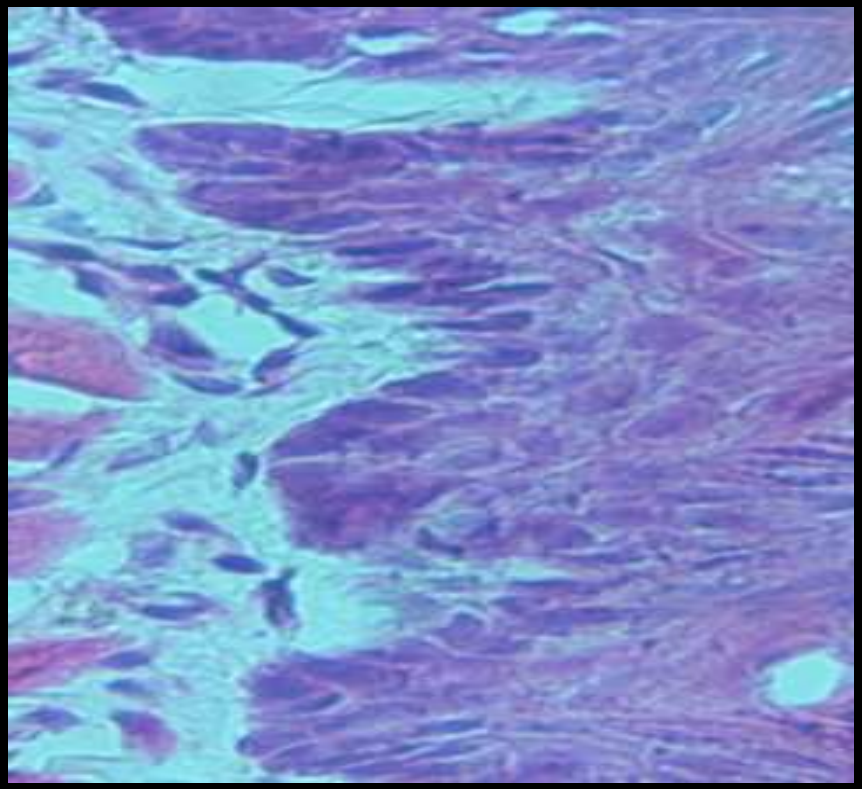


IMS of normal murine tongue, longitudinal section. Note scattered foci of residual Albumin (red) within tongue vessels.

MURINE TONGUE H&E SECTION OF 1Gy TBI

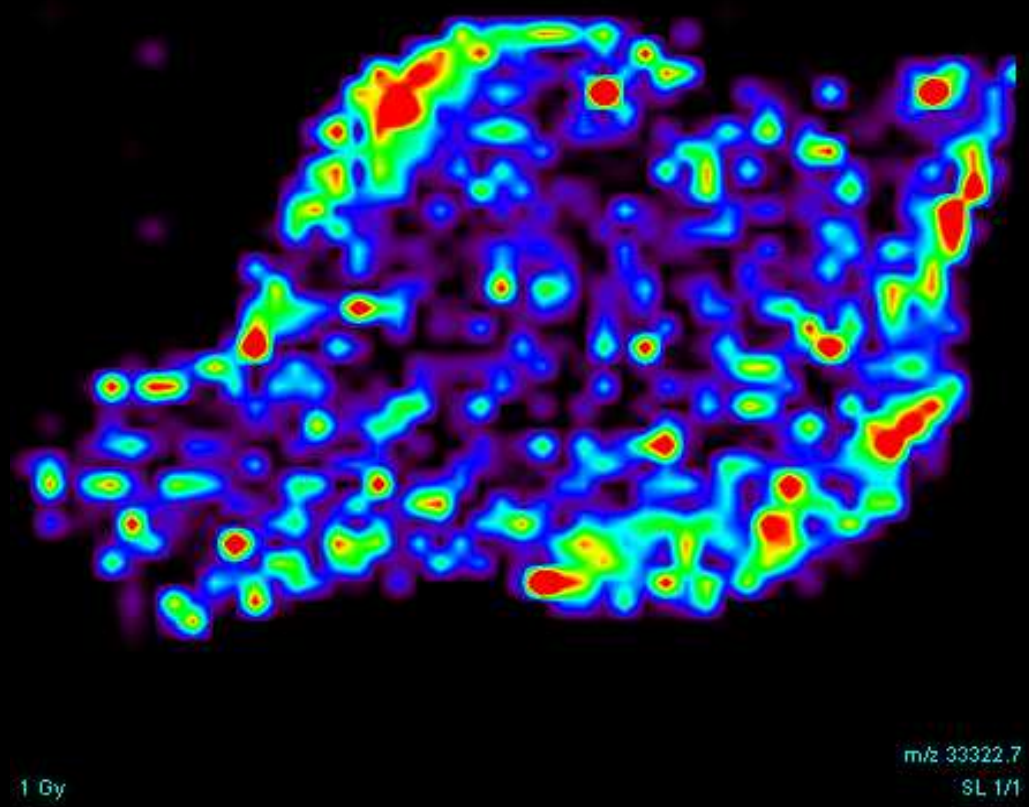


H&E section 10X one hour post 1 Gy TBI. Note destructive changes in the cornified spicules and edema in the basal layers



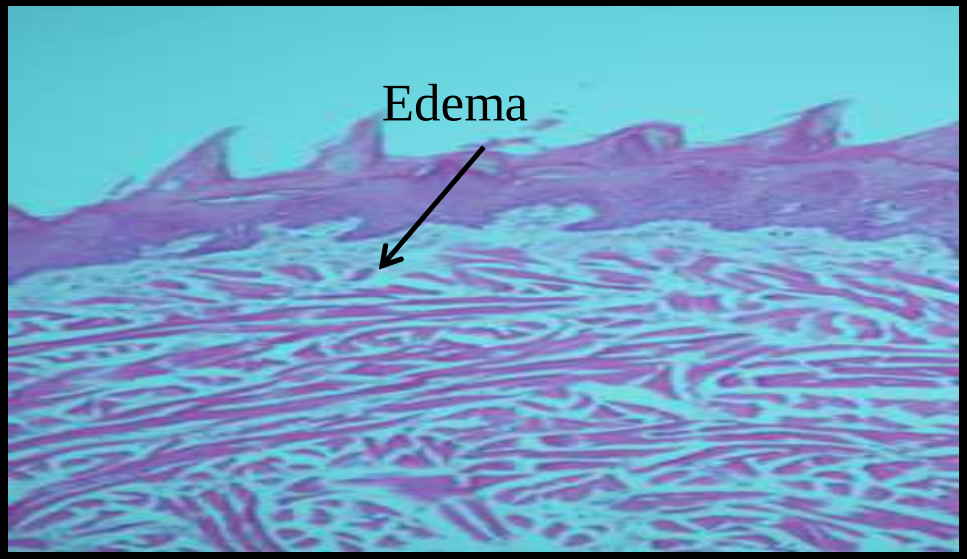
H&E section 63X one hour Post 1 Gy. Note swelling of basal cells and edema of sub-basal layer

1Gy 33,322.7 Da Double Charge Albumin

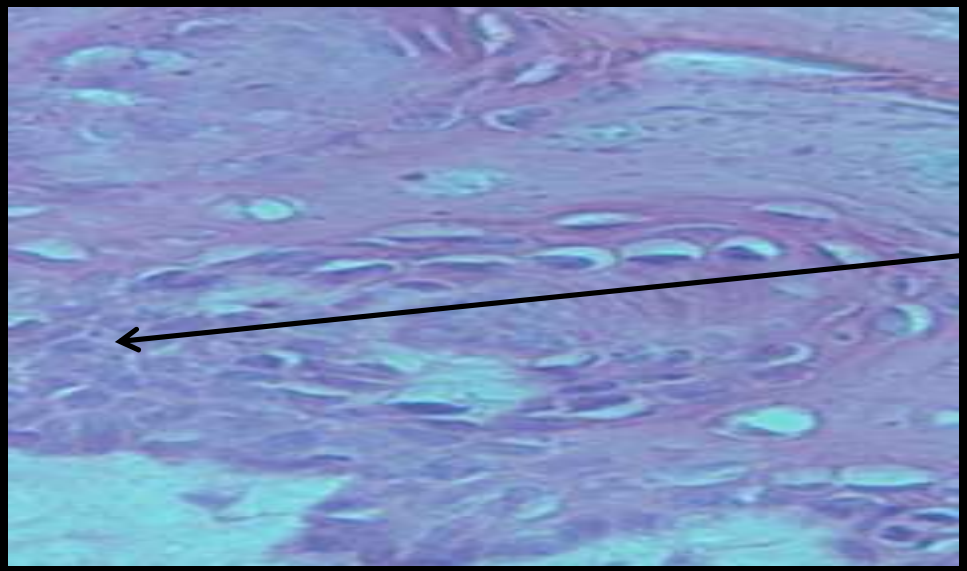


MALDI image of murine tongue one hour post 1 Gy TBI, longitudinal section. Note peripheral foci of Albumin

MURINE TONGUE H&E SECTION OF 2Gy TBI

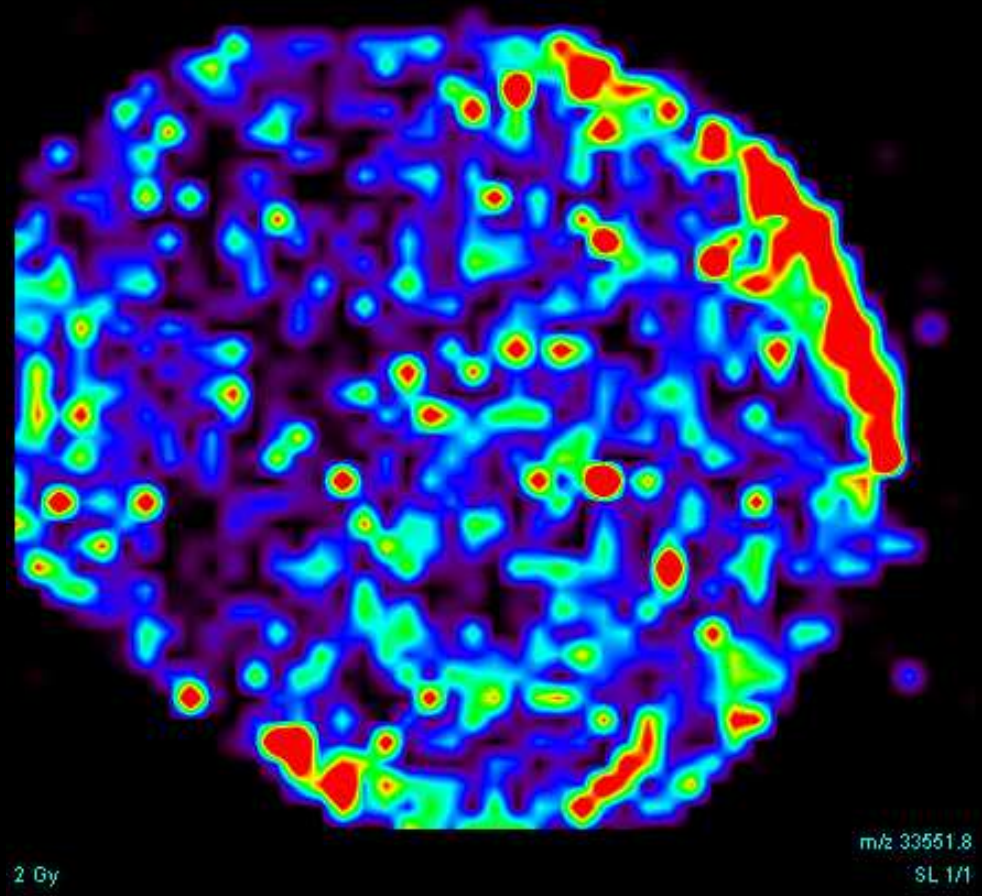


H&E section 10X of murine tongue one hour post 2 Gy TBI. Note progressive disruption of the cornified spicule layer, and increased edema of the sub-basement membrane layer



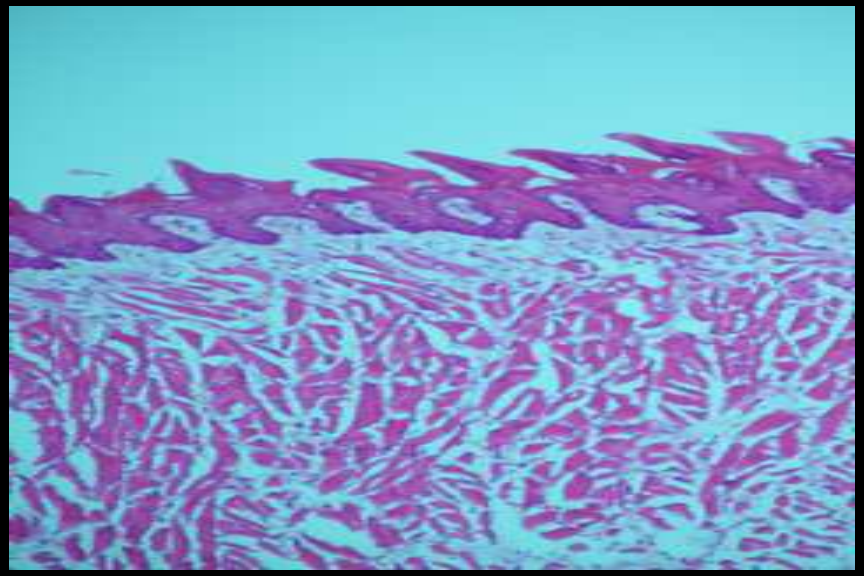
Post 2 Gy H&E section 63X. Note marked “smudging” and edema of the basal cells

2Gy 33,551.1 Da Double Charge Albumin

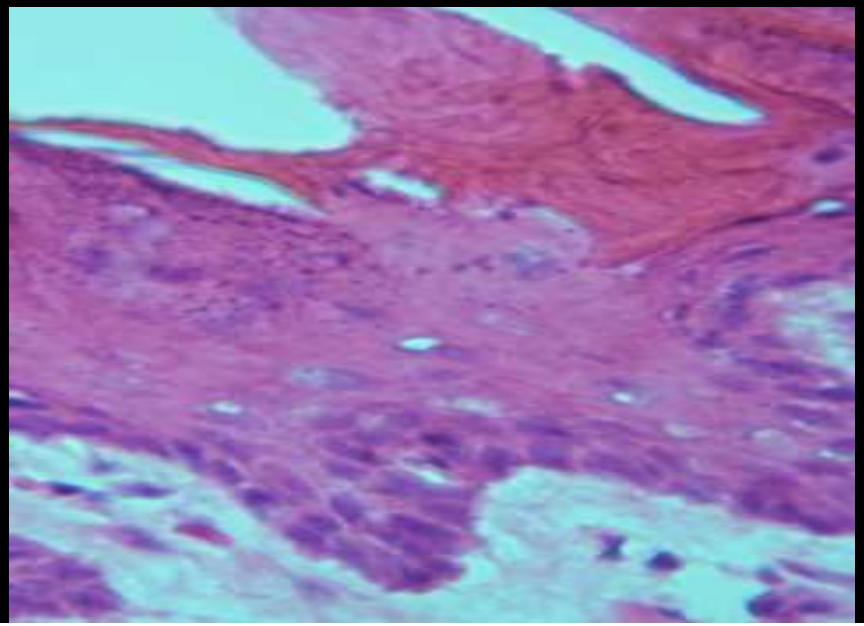


IMS, note marked increase in peripheral foci of Albumin compared to the post 1 Gy IMS (Red).

MURINE TONGUE H&E SECTION OF 3GY TBI

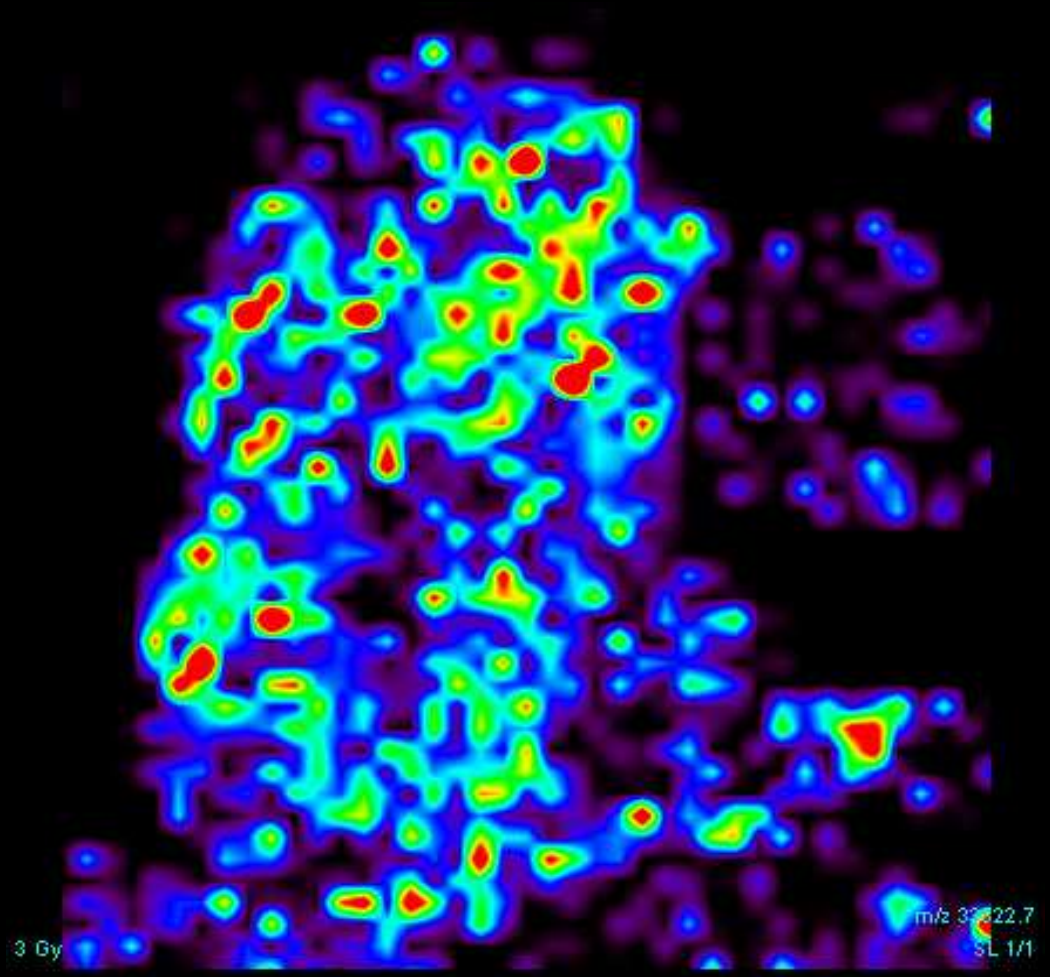


H&E section 10X of murine tongue one hour post 3 Gy TBI. Note progressive destructive changes and virtual complete loss of the cornified spicule layer corresponding to the loss of peripheral Albumin foci in the MALDI image; increased edema and damaged architecture of the center of the tongue, compared to the 2 Gy H&E section.



Post 3 Gy H&E section 63X. Note severe damage at the base of the spicules, chromatin debris, and edema compared to the 2 Gy H&E.

3Gy 33,322.7 Da Double Charge Albumin

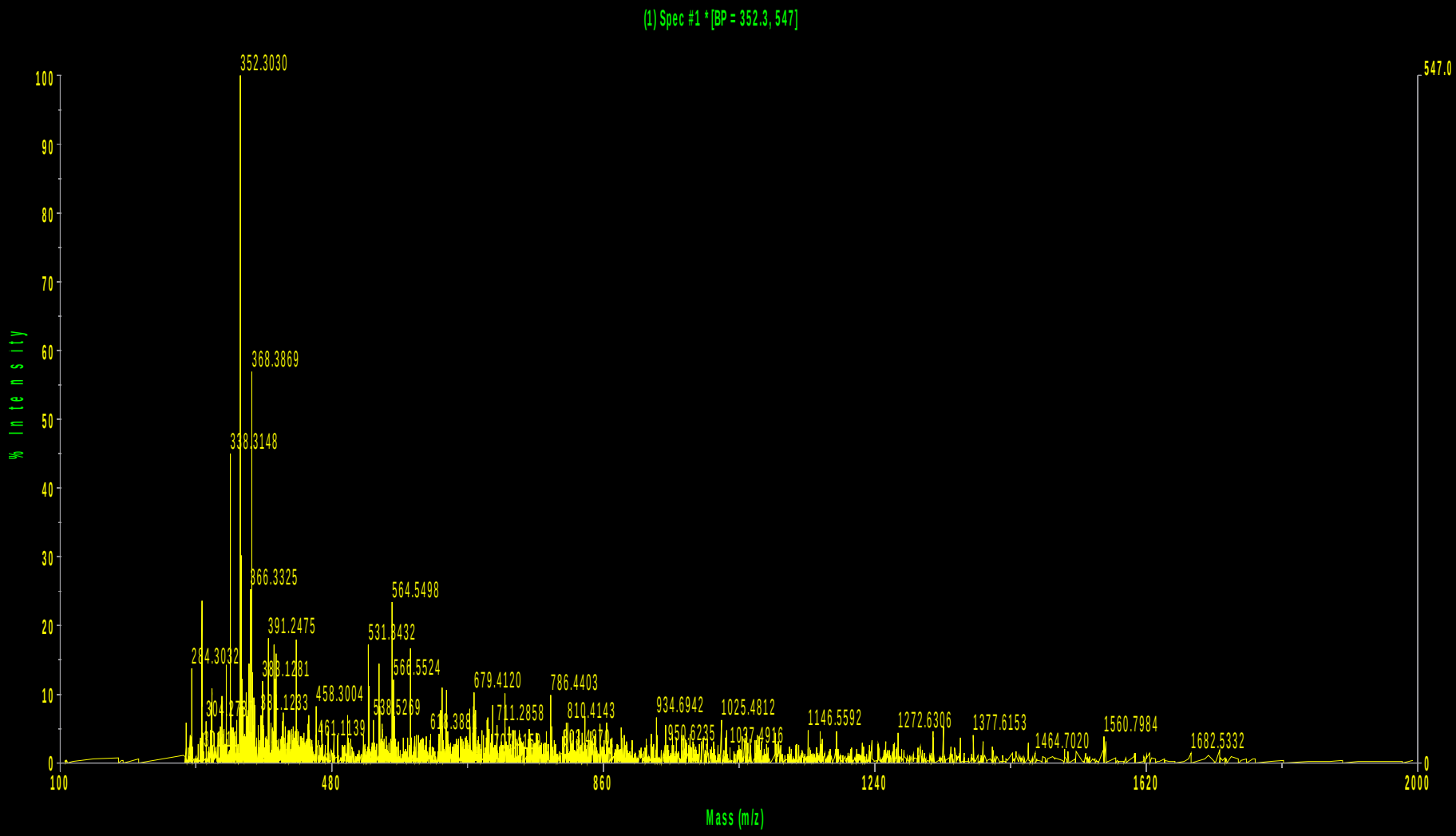


IMS of murine tongue one hour post 3 Gy TBI. Note loss of peripheral albumin and increase in central foci of Albumin. This is due to tissue loss at the periphery and increased destruction of the center of the tongue. Albumin (Red) compare with the post 2 Gy image

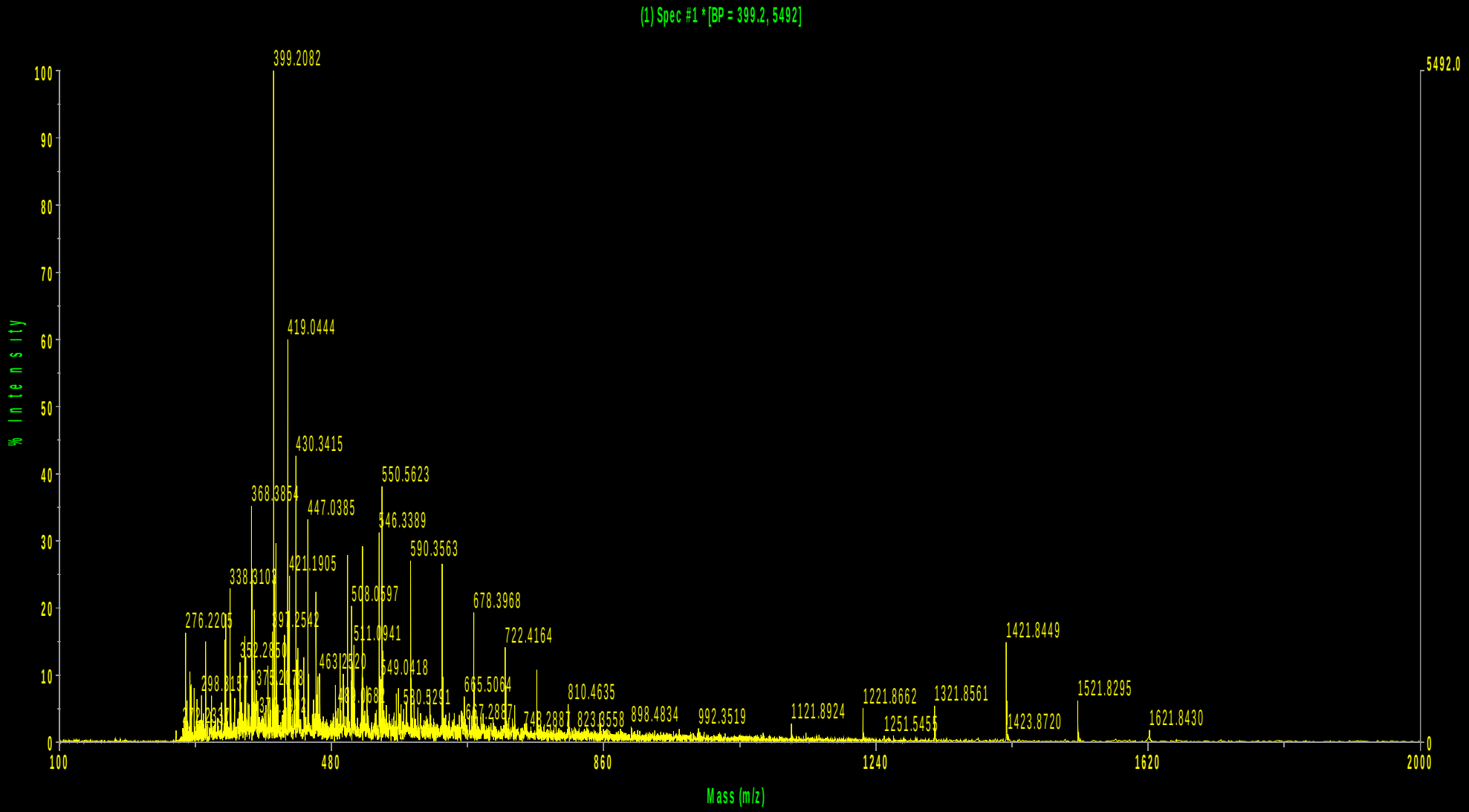
RESULTS

- IMS tongue
  - Albumin was demonstrated in the 1Gy, 2Gy and 3Gy sections.
  - Hemoglobin Subunit α in the IMS post 1 Gy.
  - Fatty Acid-Binding Protein Adipocyte in the IMS image post 2 Gy.
  - Hemoglobin α Chains in the IMS image post 3 Gy.
- LCMS tongue
  - Albumin was demonstrated in the contiguous tissue section extracts of 1Gy, 2Gy and 3Gy.
- LCMS heart
  - Protein identified in the normal and post 2 Gy cardiac tissue was identical, Actin, alpha cardiac muscle 1 OS=Mus musculus

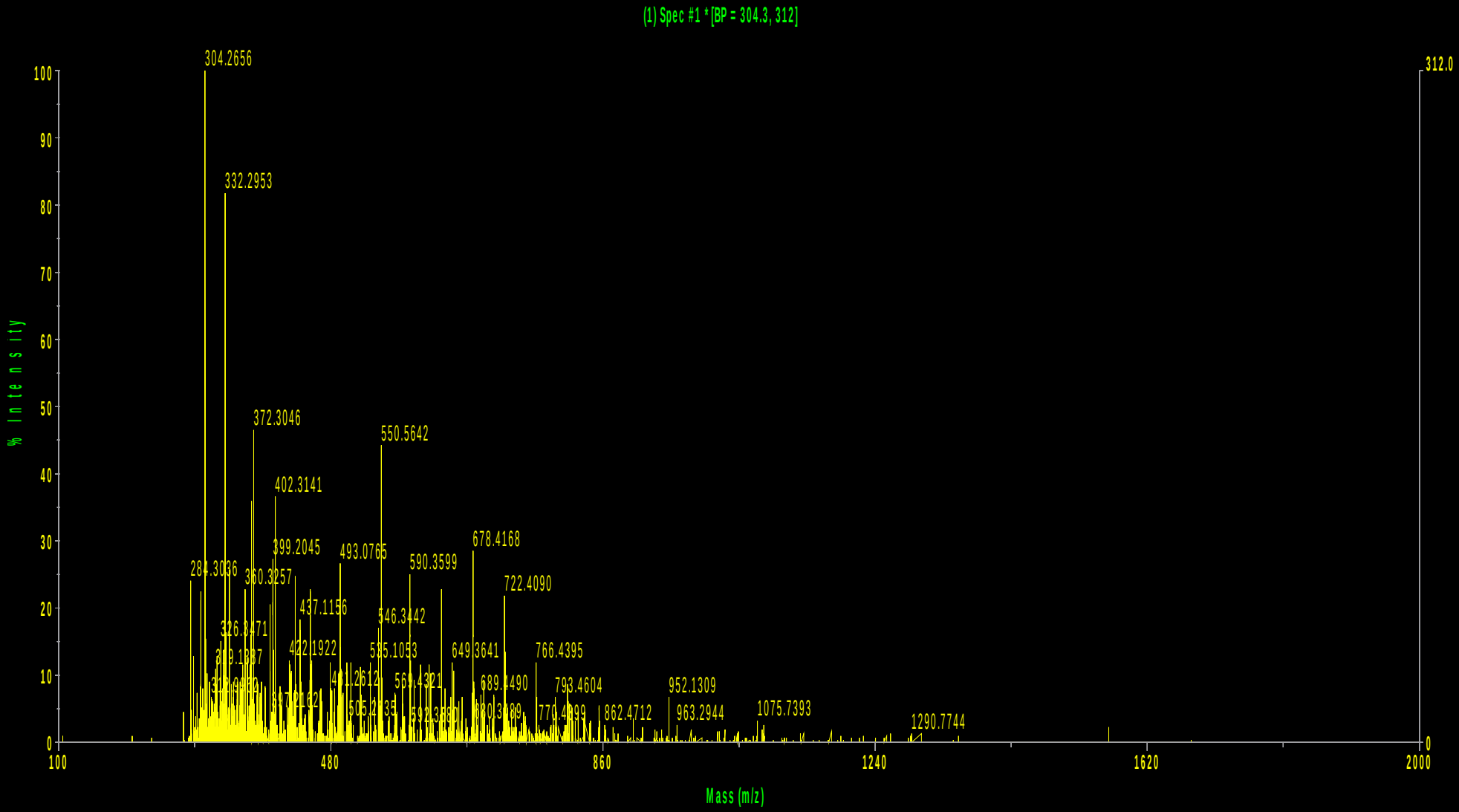
LCMS spectrum tongue normal



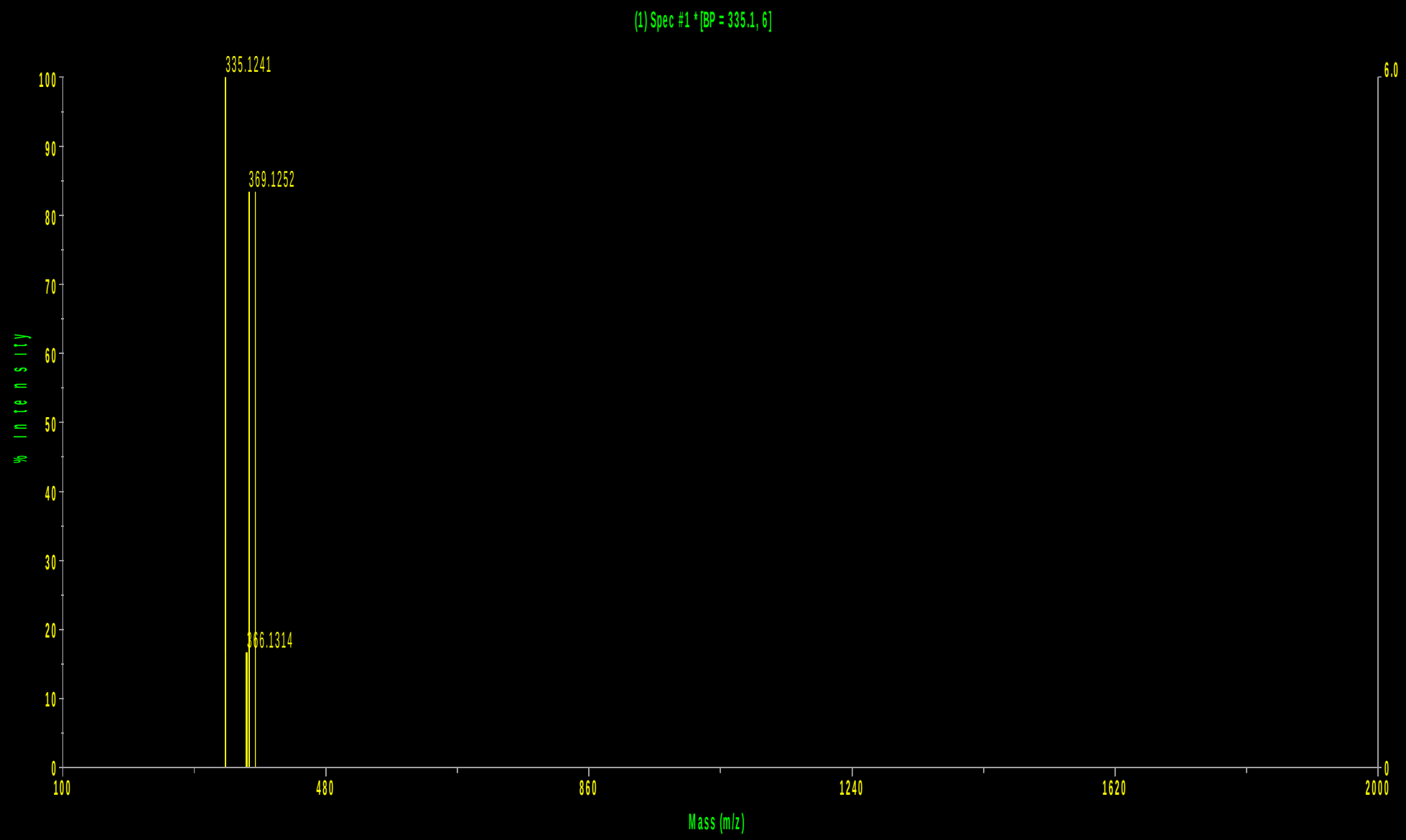
LCMS spectrum tongue post 1 Gy



LCMS spectrum tongue post 2 Gy

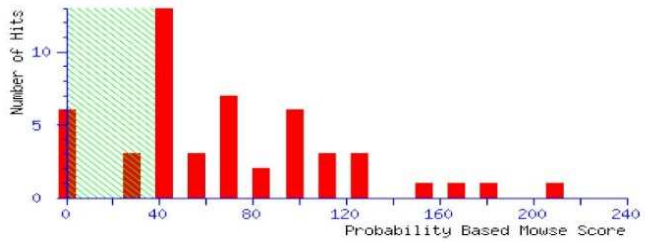


LCMS spectrum tongue 3 Gy



Mascot search of proteins in normal murine tongue

Peptide Summary Report (../data/20090408/FtiAifcTh.dat)



Peptide Summary Report

Format As	Peptide Summary		Help
	Significance threshold p< 0.05	Max. number of hits AUTO	
	Standard scoring ○ MudPIT scoring ◉	Ions score or expect cut-off 0	Show sub-sets 0
	Show pop-ups ◉ Suppress pop-ups ○	Sort unassigned Decreasing Score	Require bold red ☐

No albumin was identified.

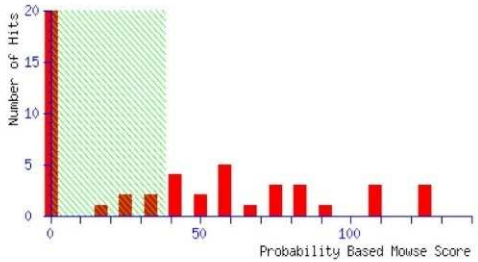
Mascot search of proteins in 1GY murine tongue

Peptide Summary Report (../data/20090408/FtiAifuwe.dat)

I59009	epidermal keratin subunit II - mouse
HAMS	hemoglobin alpha chains - mouse
K2C4_MOUSE	Keratin, type II cytoskeletal 4 (Cytokeratin-4) (CK-4) (Keratin-4) (K4) (Cytoskeletal 57 kDa keratin).- Mus musculus (Mouse).
Q6IME9_MOUSE	Type-II keratin Kb35.- Mus musculus (Mouse).
K2C8_MOUSE	Keratin, type II cytoskeletal 8 (Cytokeratin-8) (CK-8) (Keratin-8) (K8) (Cytokeratin endo A).- Mus musculus (Mouse).
Q3TV03_MOUSE	Adult male stomach cDNA, RIKEN full-length enriched library, clone:2210414C06 product:albumin 1, full insert sequence.- Mus musculus (Mouse).
Q9Z1R9_MOUSE	Trypsinogen 16 (Protease, serine, 1).- Mus musculus (Mouse).
JQ0028	cytokeratin 19 - mouse
Q2M1G8_MOUSE	2410039E07Rik protein (Fragment).- Mus musculus (Mouse).
AAH11074	BC011074 NID: - Mus musculus

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 38 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



Peptide Summary Report

Format As | Peptide Summary | Help

Albumin appears at the end of the list and corresponds to the findings in the MALDI image one-hour post 1 Gy. The 1 Gy IR induced proteins are listed in Table 1.

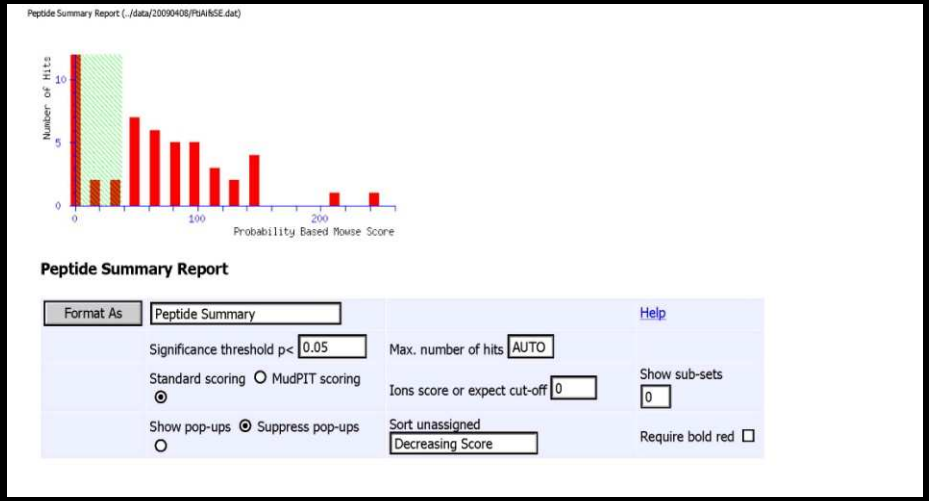
Mascot search of proteins in 2GY murine tongue

Peptide Summary Report (../data/20090408/FtiAi5SE.dat)

Q32P04_MOUSE	Krt2-5 protein (Fragment).- Mus musculus (Mouse).
CAA33084	MMHFABP NID: - Mus musculus
Q80VP7_MOUSE	Hypothetical protein MGC54654.- Mus musculus (Mouse).
Q8BIS2_MOUSE	10 days neonate skin cDNA, RIKEN full-length enriched library, clone:4732456N10 product:similar to KERATIN, TYPE II CYTOSKELETAL 6D (CYTOKERATIN 6D) (CK 6D) (K6D KERATIN).- Mus musculus (Mouse).
Q6NXH9_MOUSE	Type II keratin Kb36.- Mus musculus (Mouse).
K1C15_MOUSE	Keratin, type I cytoskeletal 15 (Cytokeratin-15) (CK-15) (Keratin-15) (K15).- Mus musculus (Mouse).
FABPH_MOUSE	Fatty acid-binding protein, heart (H-FABP) (Heart-type fatty acid- binding protein) (Mammary-derived growth inhibitor) (MDGI).- Mus musculus (Mouse).
Q3UV17_MOUSE	Adult female vagina cDNA, RIKEN full-length enriched library, clone:9930024P18 product:similar to Keratin 2p.- Mus musculus (Mouse).
K2C4_MOUSE	Keratin, type II cytoskeletal 4 (Cytokeratin-4) (CK-4) (Keratin-4) (K4) (Cytoskeletal 57 kDa keratin).- Mus musculus (Mouse).
Q6IME9_MOUSE	Type-II keratin Kb35.- Mus musculus (Mouse).
Q6IFT3_MOUSE	Keratin Kb40.- Mus musculus (Mouse).
Q8VCW2_MOUSE	RIKEN cDNA 4631426H08.- Mus musculus (Mouse).
Q2M1G8_MOUSE	2410039E07Rik protein (Fragment).- Mus musculus (Mouse).
FABPA_MOUSE	Fatty acid-binding protein, adipocyte (AFABP) (Adipocyte lipid-binding protein) (ALBP) (A-FABP) (P2 adipocyte protein) (Myelin P2 protein homolog) (3T3-L1 lipid-binding protein) (422 protein) (P15).- Mus musculus (Mouse).
I59009	epidermal keratin subunit II - mouse
Q3TVO3_MOUSE	Adult male stomach cDNA, RIKEN full-length enriched library, clone:2210414C06 product:albumin 1, full insert sequence.- Mus musculus (Mouse).
DEMSMC	malate dehydrogenase (EC 1.1.1.37), cytosolic - mouse
JQ0028	cytokeratin 19 - mouse
Q3UDM1_MOUSE	Bone marrow macrophage cDNA, RIKEN full-length enriched library, clone: G530119K21 product:ATP-binding cassette, sub-family C (CFTR/MRP), member 1, full insert sequence. (Fragment).- Mus musculus (Mouse).
Q3UBW7_MOUSE	Bone marrow macrophage cDNA, RIKEN full-length enriched library, clone: I830015F18 product:transferrin, full insert sequence.- Mus musculus (Mouse).

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 38 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



Albumin appeared in the list as Q3TVO3_mouse. Albumin and the proteins post 2 Gy listed in Table 1 are IR dose related biomarkers.

Mascot search of proteins in 3GY murine tongue

Peptide Summary Report (../data/20090408/FtiAifsEL.dat)

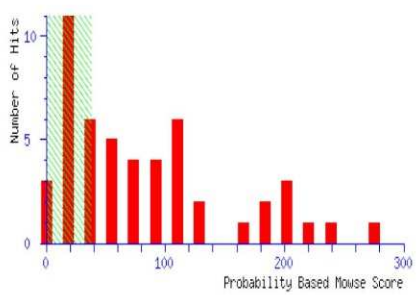
Mascot Search Results

User	: NYUSOM
Email	: seagea01@nyumc.org
Search title	:
MS data file	: E:\20090324_LCMS_Samples\16_001_CID.mgf
Database	: MSDB 20060831 (3239079 sequences; 1079594700 residues)
Taxonomy	: Mus musculus (house mouse) (90914 sequences)
Timestamp	: 8 Apr 2009 at 14:05:43 GMT
Protein hits	: HAMS hemoglobin alpha chains - mouse
	13 days embryo liver cDNA, RIKEN full-length enriched library,
	Q9CY12_MOUSE clone:2510039D09 product:hemoglobin, beta adult major chain, full insert
	sequence.- Mus musculus (Mouse).
	10 days neonate skin cDNA, RIKEN full-length enriched library,
	Q8BGZ7_MOUSE clone:4732475I03 product:CYTOKERATIN homolog (10 days neonate skin cDNA, RIKEN
	full-length enriched library, clone:4732468K03 product:CYTOKERATIN TYPE II
	homolog) (6 days neonate skin cD
	Q32P04_MOUSE Krt2-5 protein (Fragment).- Mus musculus (Mouse).
	Q3TV03_MOUSE Adult male stomach cDNA, RIKEN full-length enriched library, clone:2210414C06
	product:albumin 1, full insert sequence.- Mus musculus (Mouse).
	KRMSE1 keratin, 59K type I cytoskeletal - mouse
	K1C15_MOUSE Keratin, type I cytoskeletal 15 (Cytokeratin-15) (CK-15) (Keratin-15) (K15).-
	Mus musculus (Mouse).
	10 days neonate skin cDNA, RIKEN full-length enriched library,
	Q8BIS2_MOUSE clone:4732456N10 product:similar to KERATIN, TYPE II CYTOSKELETAL 6D
	(CYTOKERATIN 6D) (CK 6D) (K6D KERATIN).- Mus musculus (Mouse).

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 38 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

Peptide Summary Report (../data/20090408/FtiAifsee.dat)



Peptide Summary Report

Format As	Peptide Summary		Help
	Significance threshold p< 0.05	Max. number of hits AUTO	
	Standard scoring ○ MudPIT scoring ◉	Ions score or expect cut-off 0	Show sub-sets 0
	Show pop-ups ◉ Suppress pop-ups ○	Sort unassigned Decreasing Score	Require bold red ☐

Mascot search of proteins in murine tongue one hour post 3 Gy TBI. Albumin appeared in the list as Q3TVO3_mouse. Albumin and the other proteins identified post 3 Gy listed in Table 1 are IR dose related biomarkers.

Normal Mouse Tongue

Q9CXH5 – Mus :hemoglobin, beta adult major chain

PO1942 – Mus: hemoglobin subunit alpha Hemoglobin alpha chain Hba-a1 - HAMS

Q9JJ20 – Mus : 14-3-3 protein sigma; Sfn MGI 1891831 Synonyms: Mme1 EMBL AAF36093.1

Q64475 Mus: histone H2B type 1-B (h2B-143)H2B1B_.

Q9Z1R9 Mus: - trypsinogen 16

BAC33789 –Mus: Sgol1 shugoshin-like 1 [(S. pombe) (AK049517.1)]

Mouse Tongue 1hr post 1 GY

Q3TV03 - Mus: albumin 1 - full insert sequence.

Q9CXH5 – Mus :hemoglobin, beta adult major chain

PO1942 – Mus: hemoglobin subunit alpha Hemoglobin alpha chain Hba-a1 - HAMS

Q9Z1R9 - Mus: - trypsinogen 16

Mouse Tongue 1hr post 2GY

Q3TV03 - Mus: albumin 1 - full insert sequence.

PO1942 – Mus: hemoglobin subunit alpha Hemoglobin alpha chain Hba-a1 - HAMS

Q9CY54 - Mus: hemoglobin, beta adult major chain Symbol: Hbb-b1

Q9CY06 - MUS:hemoglobin, beta adult major chain

P02088 – Mus: HBMS Hemoglobin subunit beta

Q3UJH8 –Mus: glutamate oxaloacetate transaminase 1 EC=2.6.1.1

P11404 Mus: fatty acid-binding protein, heart FABPH.

P04117 – Mus: FABPA Fatty acid-binding protein, adipocyte (AFABP) also (Adipocyte lipid-binding protein) (ALBP)

S02654; S00195 Mus: malate dehydrogenase DEMSMC - (EC 1.1.1.37), cytosolic – mouse, L-lactate dehydrogenase

Q3UBW7 – Mus: transferrin

Mouse Tongue 1hr post 3GY

Q3TV03 - Mus: albumin 1 - full insert sequence.

PO1942 – Mus: hemoglobin subunit alpha Hemoglobin alpha chain Hba-a1 - HAMS

Q9CY12 – Mus: hemoglobin, beta adult major chain

Q3USS4 – Mus: glial fibrillary acidic protein

Q3TWV0 – Mus: vimentin
JC4030 – Mus: DnaJ-like protein MTJ1

Q9QZ05 (E2AK4) Mus: eukaryotic translation initiation factor 2-alpha kinase 4 EC=2.7.11.1

DISCUSSION a

- The goal of this project was to determine dose-related IR induced protein.
- Initial MALDI and LCMS, experiments in mice demonstrated radiation dose related albumin and induced proteins in radiosensitive buccal mucosa and tongue tissue.[8]
- For this study, the initial experiments were expanded to include evaluation of induced protein changes in the tongue and heart of mice exposed to 1-3 Gy IR.
- Heart tissue was chosen to illustrate that total body radiation affected other organs which were less radiosensitive than the tongue.

8. Pevsner P, Formenti S, Remsen T, Kruppa G, Kessler P, Rothschild G, Ghiso J, Melamed J, Rosenstein B, Schneider R, Naftolin F, Stern A. 2008; .

DISCUSSION b

- Histologic examination of the murine tongue post 1, 2, and 3 Gy demonstrated

  -progressive epithelial keratin spicule destruction with swelling of the epithelial basal cell layer.

  -progressive loss of the integrity of arteriole endothelium with opening of the tight junctions between endothelial cells and leaking of serum (albumin) first into the periphery and then into the center of the tongue

  -increasing edema is a function of arteriole integrity failure.

  -loss of peripheral structure.

  -disorganization of the central anatomy of the tongue.
- This correlated with serial IMS sections that demonstrated the progressive increase in albumin and other proteins that were found at increasing IR doses.

CONCLUSION

- MALDI, IMS, and LCMS identified IR induced dose-related proteins.
- The identification of these IR dose-related biomarkers is novel.
- These protein biomarkers of IR could distinguish the 2 Gy threshold dose level for radiation sickness.
- This study is proof of principle that MALDI and LCMS can identify dose-related protein biomarkers of IR that can be used in tongue test strips for nuclear disasters.

THANK YOU