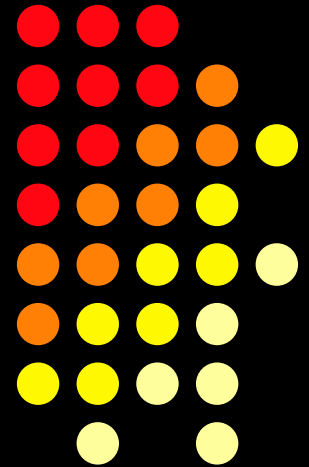


Localization of 4HNE modifications

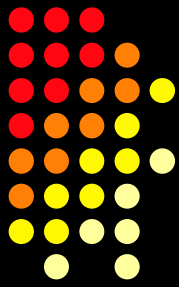
Shannon Eliuk

Sept. 12, 2006

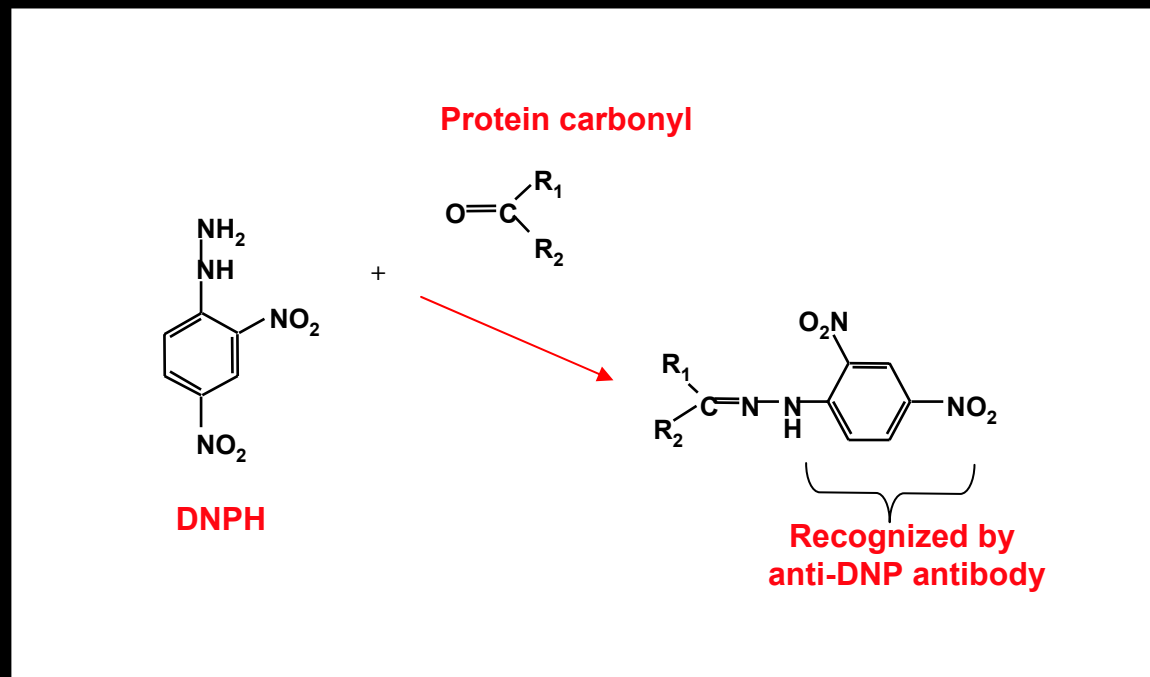
Proteomics Workshop



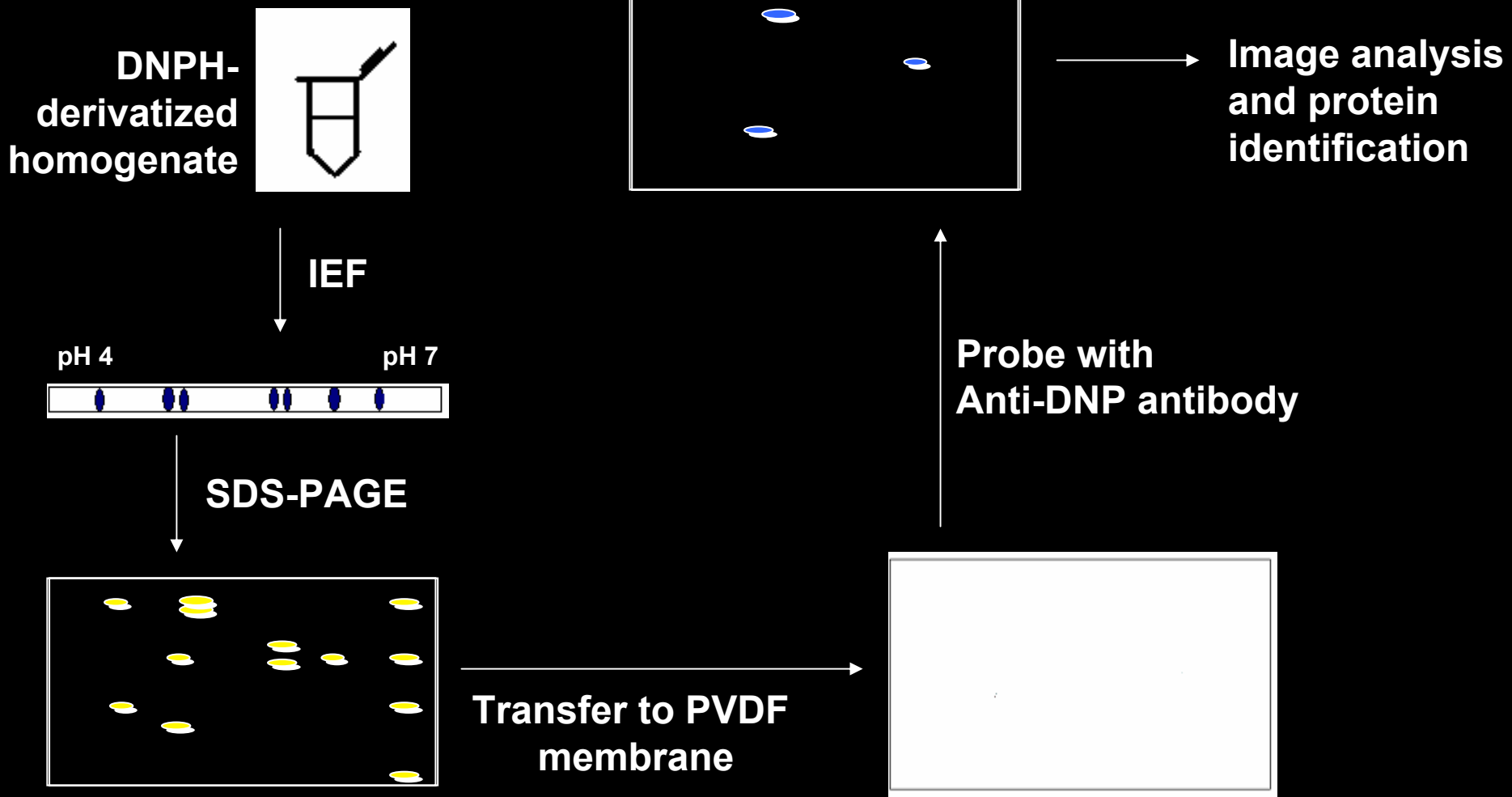
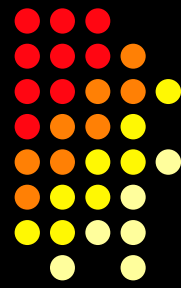
Proteomic Identification of Oxidatively Modified Proteins



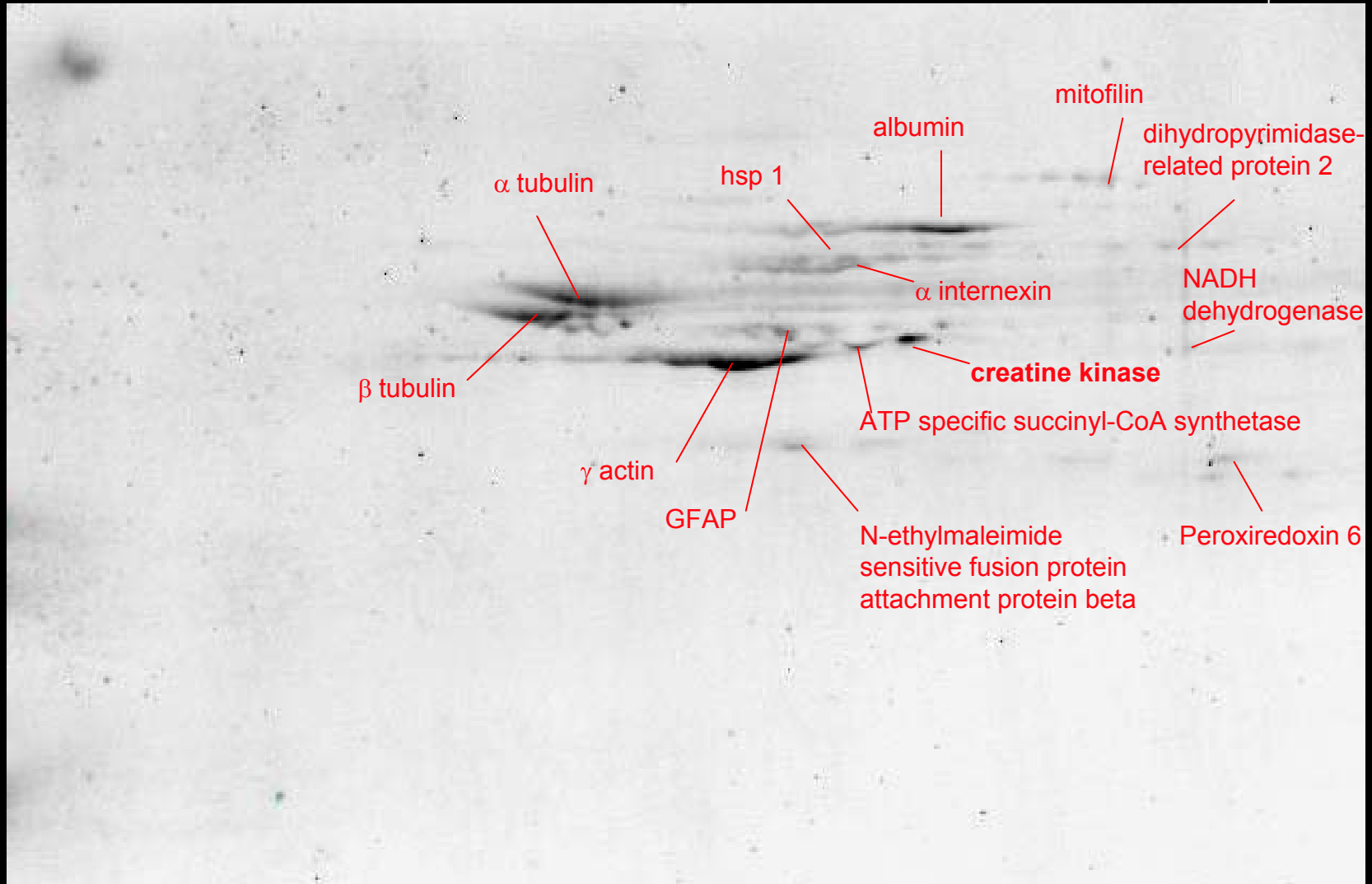
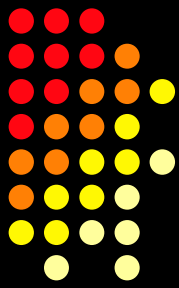
- By use of protein carbonyl derivatization with 2,4-dinitrophenyl hydrazine coupled with 2D Western Blot and mass spectrometry



Investigation of Protein Oxidation: 2D gel electrophoresis and immunoblot



2D Oxyblot showing oxidized proteins in mouse brain





Oxyblot

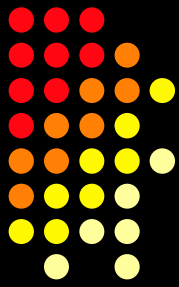
- Pros

- Relatively quick and easy
- Quantitative
- Sensitive
- Gives global picture of proteins that may be oxidatively modified in a biological sample

- Cons

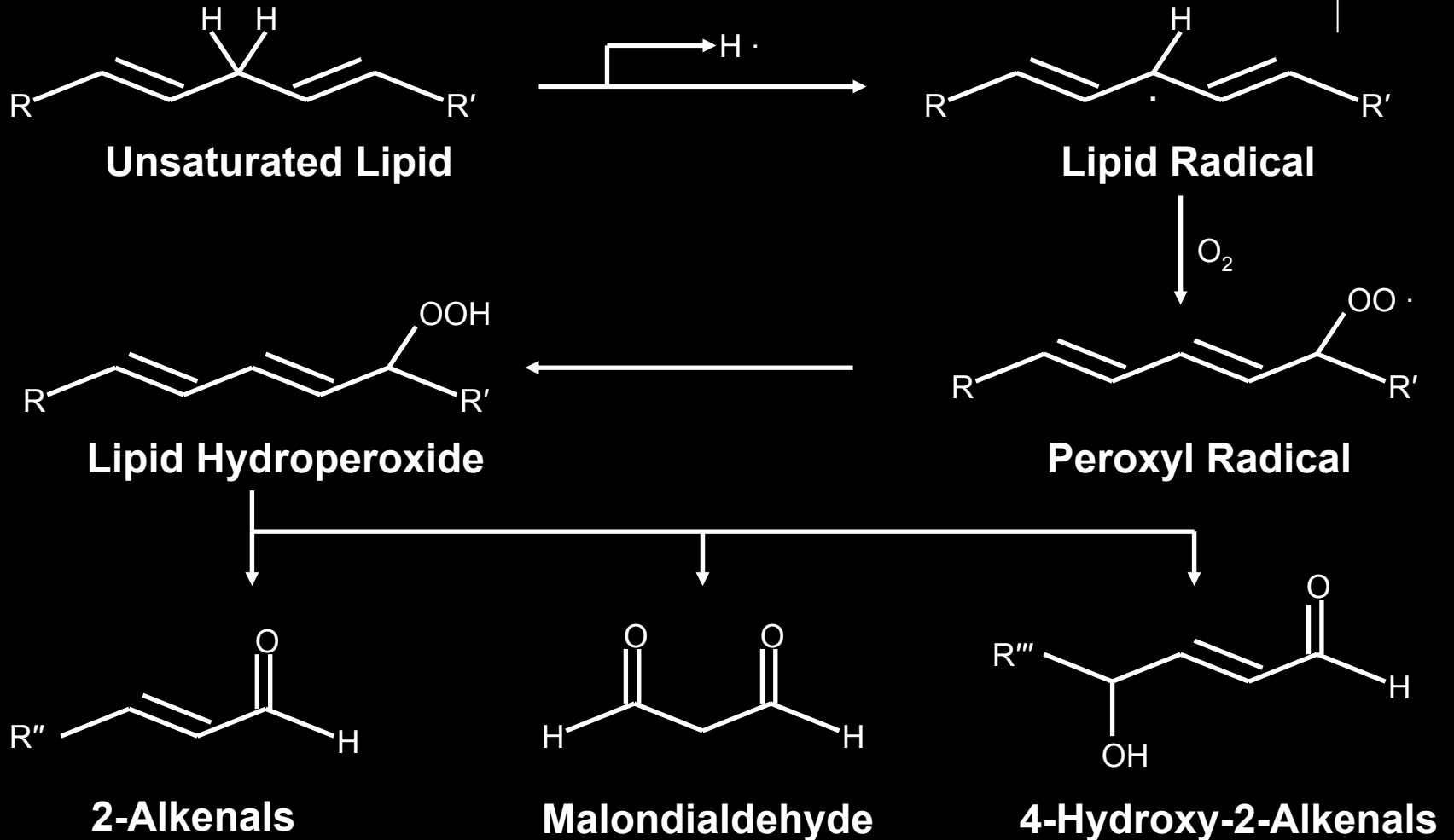
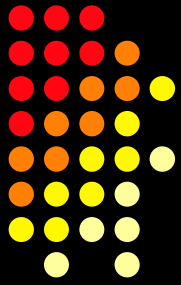
- Identification of modified protein requires subsequent MS analysis
- Identification of modified protein is not conclusive
- Does not elucidate site or chemistry of modification

Method for localizing sites of post-translational modification

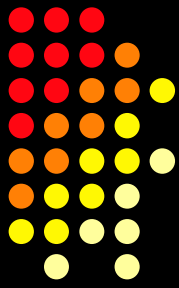


- Use an *in vitro* approach to develop and optimize method for modification localization
- Use *in vitro* 4-hydroxy-2-nonenal modification of creatine kinase as a model; this protein has been shown qualitatively to be oxidatively modified *in vivo*.
- Develop a direct infusion LTQ-FT-ICR MS and MS/MS method for PTM identification
 - Goal: to decrease analysis time from more commonly used liquid chromatography methods

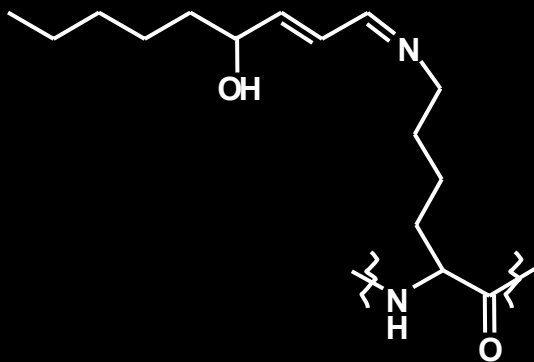
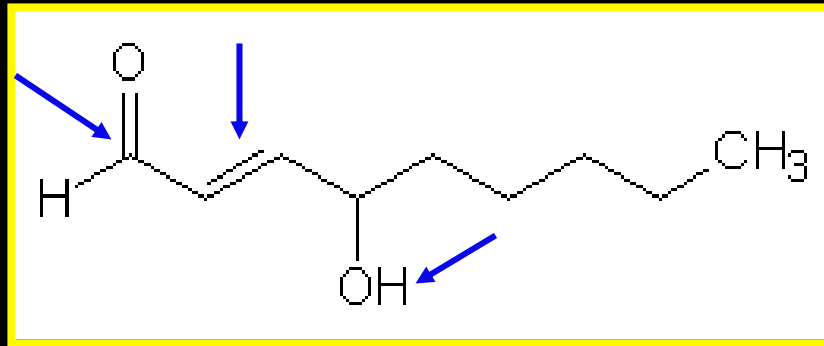
Lipid Peroxidation



4-hydroxy-2-nonenal (4HNE)

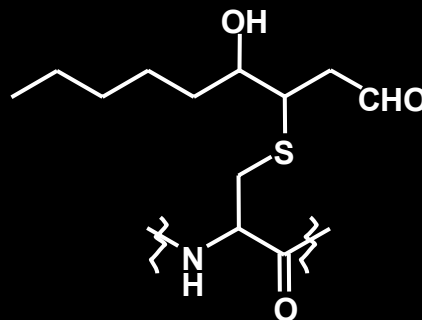


A reactive aldehyde formed as a result of lipid peroxidation



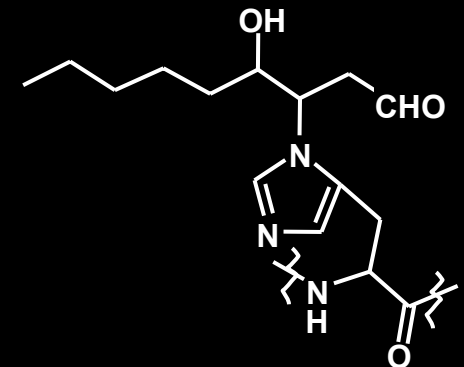
4HNE-Modified Lysine

Schiff Base Adduct



4HNE-Modified Cysteine

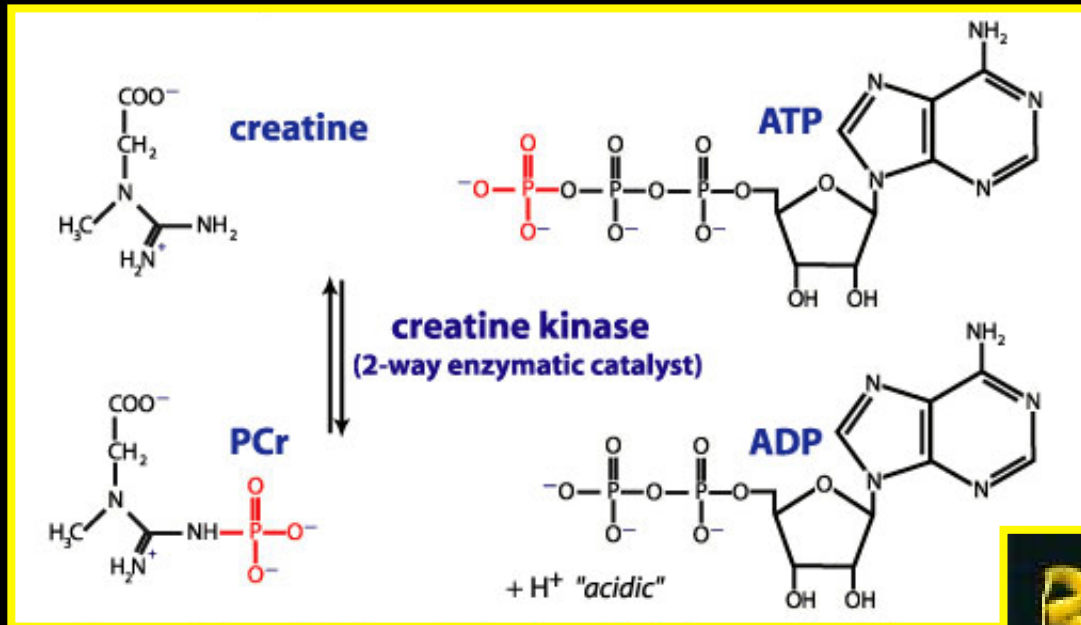
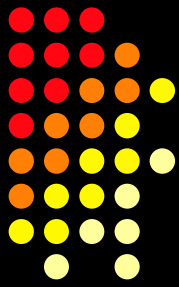
Michael Adduct



4HNE-Modified Histidine

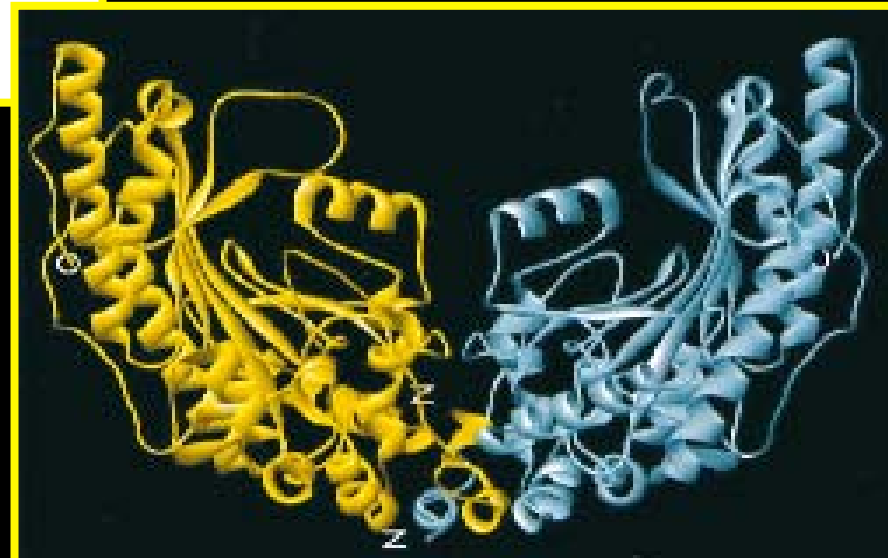
Michael Adduct

Creatine Kinase

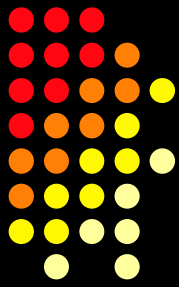


Creatine kinase isoforms
CK-BB, CK-MM, CK-MB, Mi-CK

<http://www.creatinemonohydrate.net/illustrations/reactions.html>



CK: Important Amino Acids



MPFSNSHNAL KLRFP AEDEF PDL SAHNNHM AKVLTPELYA ELRAKSTPSG
FTLDDVIQTG VDNPGHPYIM TVGCVAGDEE SYEVFKDLFD PIIEDRHGGY
KPSDEHKTDL NPDNLQGGDD LDPNYVLSSR **V**RTGRSIRGF CLPPHC SRGE
RRAIEKLAVE ALSSLDGDLA GRYYALKSMT EAEQQQLIDD **H**FLFDKPVSP
LLLASGMARD WPDARGIWHN DNKTFLVWVN **E**EDHL**R**VISM QKGGNMKEVF
TRFCTGLTQI ETLFKSKDYE FMWNP HLG YI L**T**CPSNLGTG L**R**AGV**H**IKLP
NLGKHEKFSE VLKRLRLQ**K**R GTGGVD TAAV GGVFDVSNAD RLG FSEVELV
QMVVDGVKLL IEMEQRLEQG QAIDDLMPAQ K

Nucleotide binding site

H296, H191, D335, R292, I188, 323-332 (flexible loop for binding ADP)

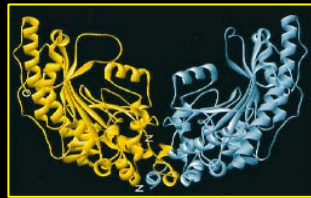
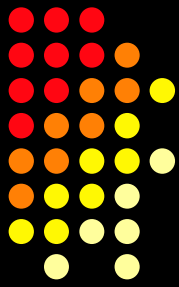
Creatine Binding Site

E232, G65, I69, C283, 60-70 (Creatine binding pocket), H66 (required for catalytic reaction)

Nucleotide phosphate binding site

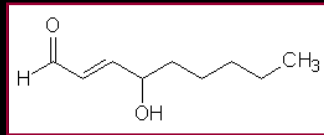
A concentration of +ve charges chiefly 5 highly conserved R residues (130, 132, 236, 292, 320)
(Lahiri et al., 2002)

4HNE Modification of CK



CKB

+



4HNE

Incubate at 37C
2 hrs

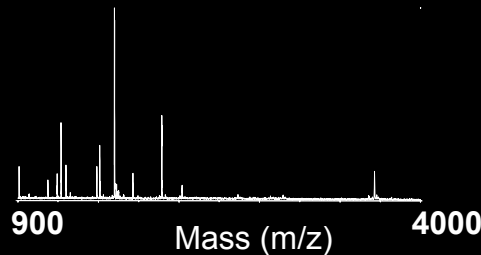


4HNE modified CKB

Digest overnight
(Trypsin/Chymotrypsin)

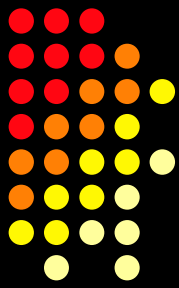


**Mass
Spectrometry**



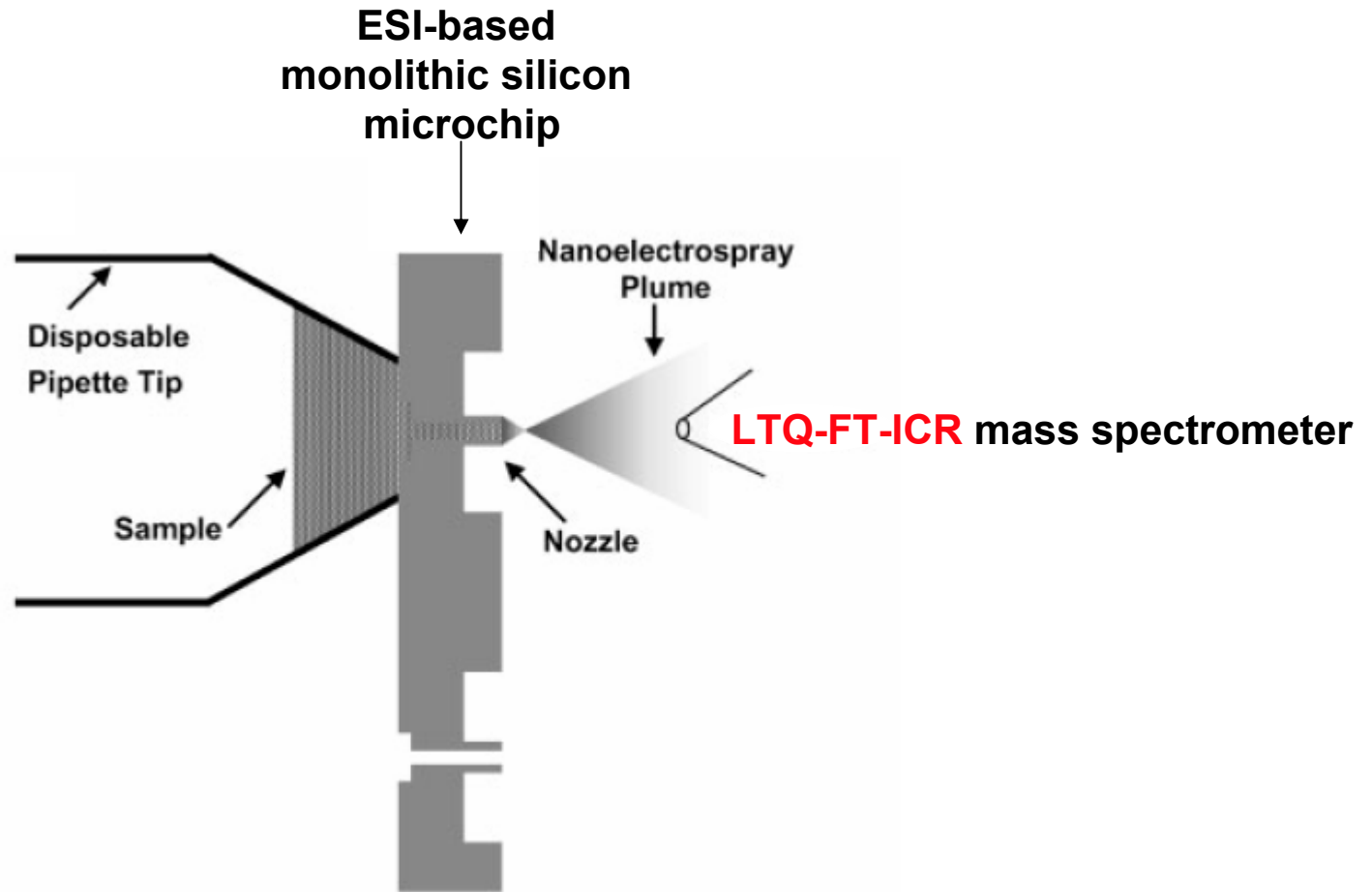
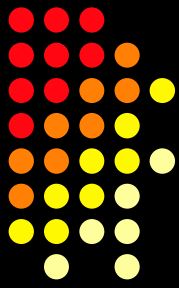
**MEASURE
FUNCTIONAL
CONSEQUENCES**

Direct Infusion by use of the TriVersa NanoMate (Advion)

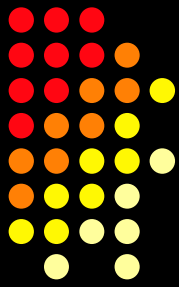


- Fully automated sample handling
- Chip-Based ionization and direct infusion
- Stable, reproducible spray
- No carryover between samples

Chip-based direct infusion



(Zhang et al., 2003)



What are we looking for?

Masses of known peptides

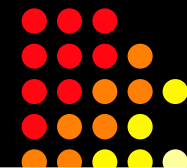
- Each amino acid has a known and unique mass and thus each peptide (string of amino acids) also has a known and unique mass

Masses of known peptides + 4HNE

4HNE modification mass shift

Michael Adduct	156.1150
Schiff Base Adduct	138.1045
2-Pentylpyrrole Adduct	120.0939

FT-ICR MS Full Scan Spectra

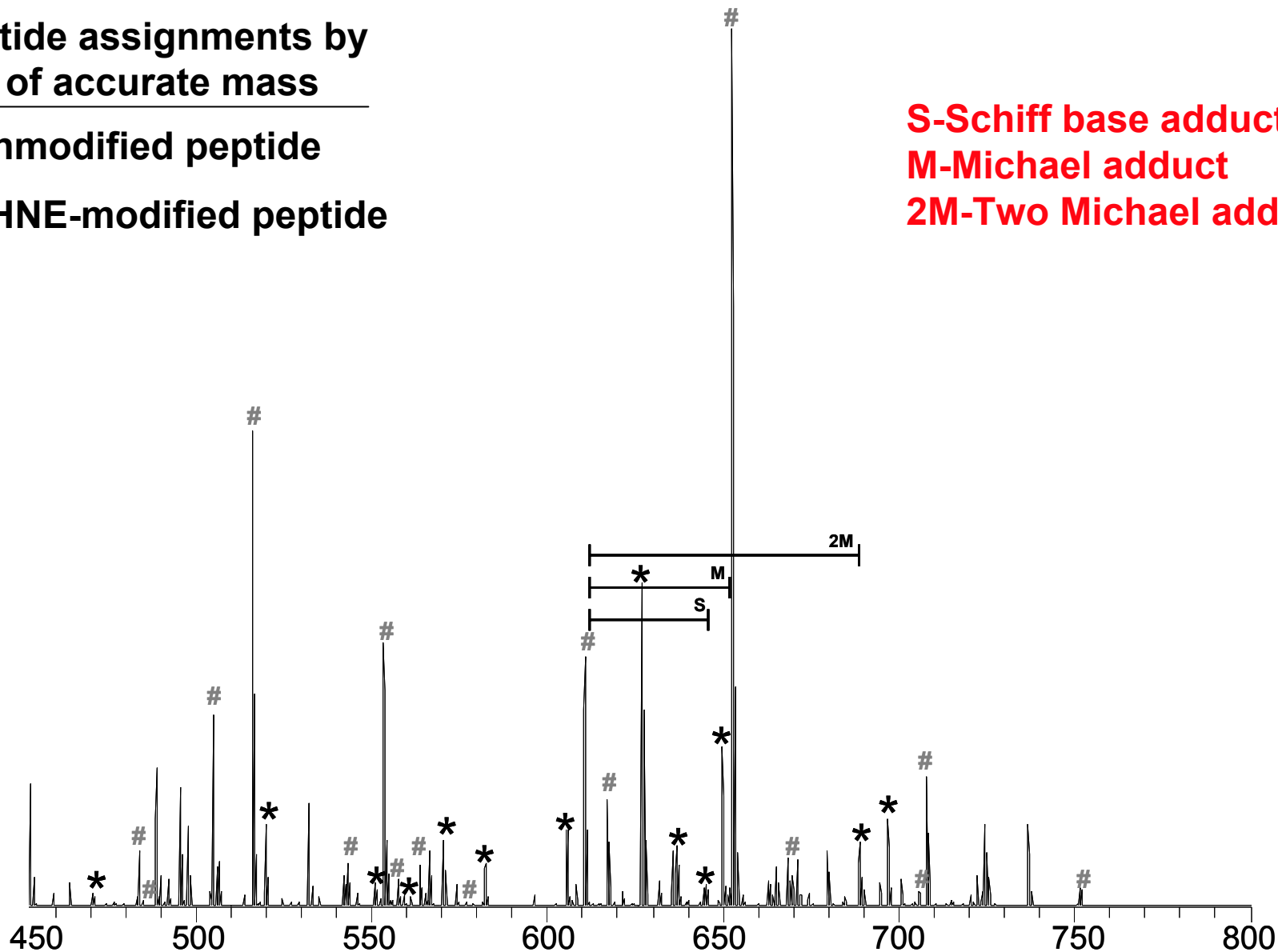


Peptide assignments by
use of accurate mass

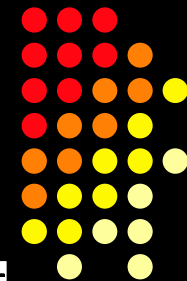
#-Unmodified peptide

*-4HNE-modified peptide

S-Schiff base adduct
M-Michael adduct
2M-Two Michael adducts



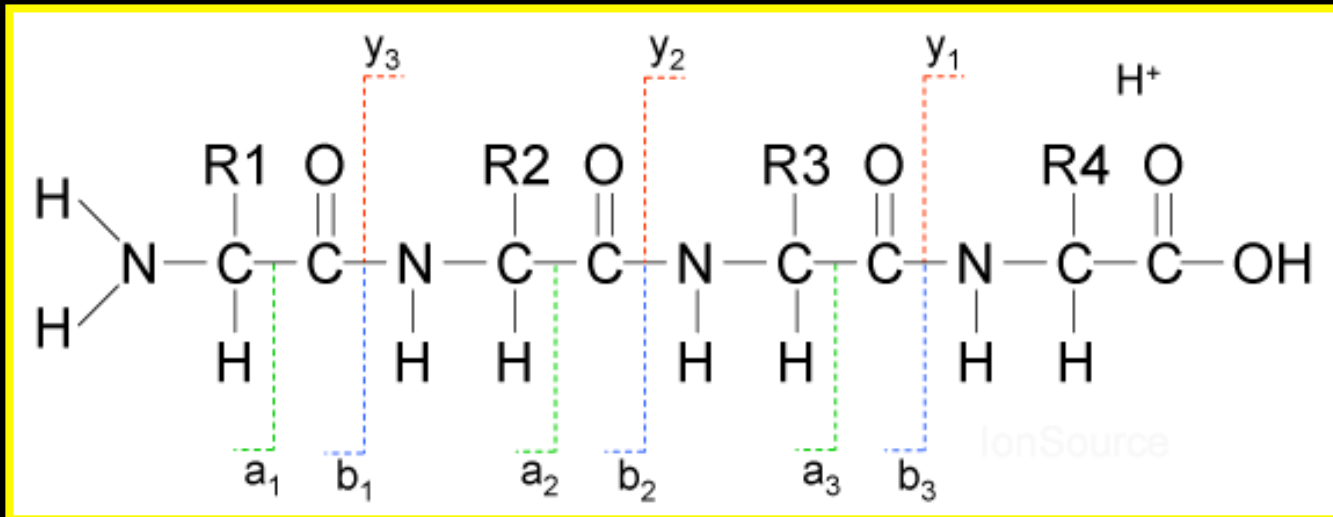
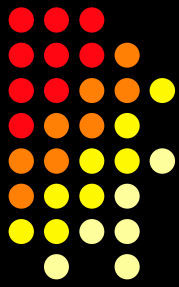
Accuracy of FT-ICR MS



Peptide	Sequence	Modified amino acid	Adduct Form	Observed Mass	Theoretical Mass	Error (ppm)
PFSNSHNAL	2-10	H ⁷	Schiff base	1124.5726	1124.5735	0.83
PFSNSHNAL	2-10	H ⁷	Michael	1142.5837	1142.5841	0.33
PFSNSHNALK	2-11	H ⁷	Michael	1270.6791	1270.6790	-0.03
PFSNSHNALK	2-11	H ⁷	Schiff base	1252.6672	1252.6685	1.03
LRFPAEDEFPDLSAHNNHMAK	12-32	H ²⁶	Michael	2595.2647	2595.2660	0.50
LRFPAEDEFPDLSAHNNHMAK	12-32	H ²⁶ and H ²⁹	Michael	2751.3777	2751.3811	1.22
RFPAEDEFPDLSAHNNHMAKVL	13-34	H ²⁶ and H ²⁹	Michael	2850.4474	2850.4495	0.73
TLDDVIQTGVDPNGHPY	52-68	H ⁶⁶	Michael	1996.9845	1996.9862	0.87
KDLFDPIIEDRHGGY	86-100	K ⁸⁶ and H ⁹⁷	Michael	2087.1067	2087.1059	-0.37
KDLFDPIIEDRHGGY	86-100	H ⁹⁷	Michael	1930.9894	1930.9909	0.77
HGGYKPSDEHK	97-107	H ⁹⁷	Michael	1410.6996	1410.7012	1.13
HGGYKPSDEHK	97-107	H ⁹⁷ and K ¹⁰¹	Michael	1566.8153	1566.8162	0.63
CLPPHCSRGERAI	141-154	C ¹⁴¹ and C ¹⁴⁵	Michael	1907.0282	1907.0248	-1.79
ALKSMTEAEQQQLIDHFLF	175-194	H ¹⁹¹	Michael	2520.2640	2520.2691	2.00
GIWHNDNK	212-222	H ²¹⁹	Schiff base	1121.5713	1121.5738	2.21
GIWHNDNK	213-223	H ²¹⁹	Michael	1139.5837	1139.5844	0.62
HNDNKTF	219-225	H ²¹⁹	Michael	1031.5149	1031.5156	0.68
TFLVWVNEEDHLR	224-236	H ²³⁴	Michael	1813.9453	1813.9483	1.66
VNEEDHLRVISM	229-240	H ²³⁴	Michael	1597.8248	1597.8254	0.39
VNEEDHLRVI	230-239	H ²³⁴	Schiff base	1361.7414	1361.7424	0.71
VNEEDHLRVI	230-239	H ²³⁴	Michael	1379.7525	1379.7529	0.29
FCTGLTQIETLFK	253-265	C ²⁵⁴	Michael	1656.8922	1656.8917	-0.31
WNPHLGY	273-279	H ²⁷⁶	Michael	1042.5355	1042.5356	0.18
ILTCPSNL	280-287	C ²⁸³	Michael	1016.5682	1016.5697	1.41
RAGVHIKLPNLGKHEKF	292-308	H ²⁹⁶	Michael	2100.2460	2100.2440	-0.91
AGVHIK	293-298	H ²⁹⁶	Schiff base	762.4873	762.4872	-0.07
LPNLGKHEK	299-307	H ³⁰⁵	Michael	1191.7091	1191.7096	0.37

Fragmentation by CID

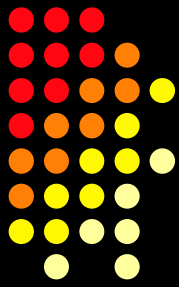
(Collision Induced Dissociation)



<http://www.ionsource.com/tutorial/DeNovo/nomenclature.htm>

The most common fragments observed with ion trap, triple quadrupole, and QTOF mass spectrometers

CID Peptide Fragmentation



TFLVWVNEEDHLR

Inert Gas
(He)

	102	T	FLVWVNEEDHLR	1556	
	249	TF	LVWVNEEDHLR	1409	
	362	TFL	VWVNEEDHLR	1296	
N-terminal	461	TFLV	WVNEEDHLR	1197	
b ions	647	TFLVW	VNEEDHLR	1911	C-terminal
m/z	746	TFLVWV	NEEDHLR	912	y ions
	860	TFLVWVN	EEDHLR	798	m/z
	989	TFLVWVNE	EDHLR	669	
	118	TFLVWVNEE	DHLR	540	
	1233	TFLVWVNEED	HLR	425	
	1370	TFLVWVNEEDH	LR	288	
	1483	TFLVWVNEEDHL	R	175	

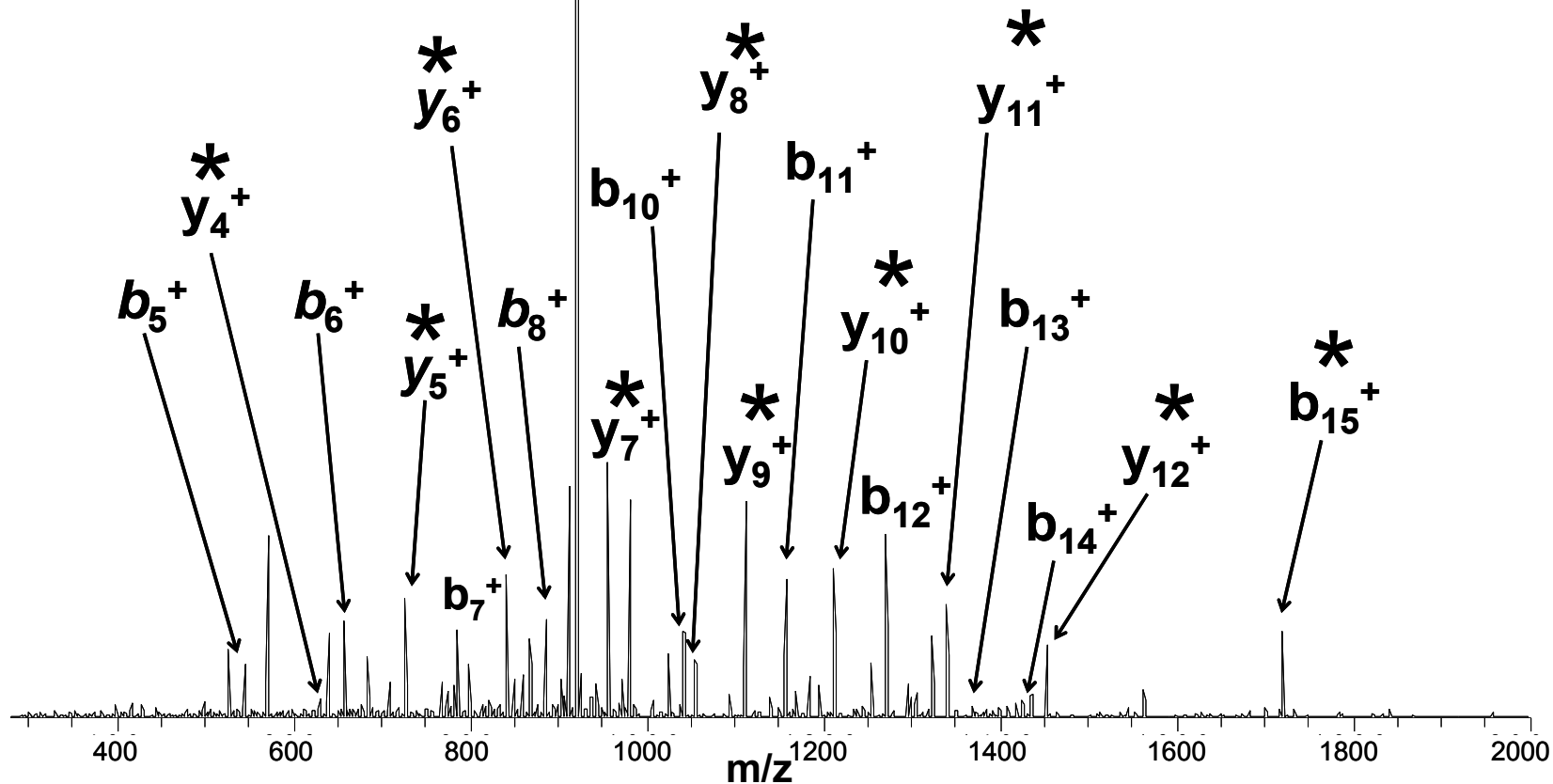
The ion's mass which would be affected by a modified H?

4HNE Michael Adduction of H⁶⁶

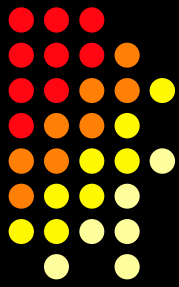


LTDQ-MS/MS

T L D D V I Q T G V D N P G H P Y
b₅ b₆ b₇ b₈ b₁₀ b₁₁ b₁₂ b₁₃ b₁₄ b₁₅
y₁₂ y₁₁ y₁₀ y₉ y₈ y₇ y₆ y₅ y₄ y₃

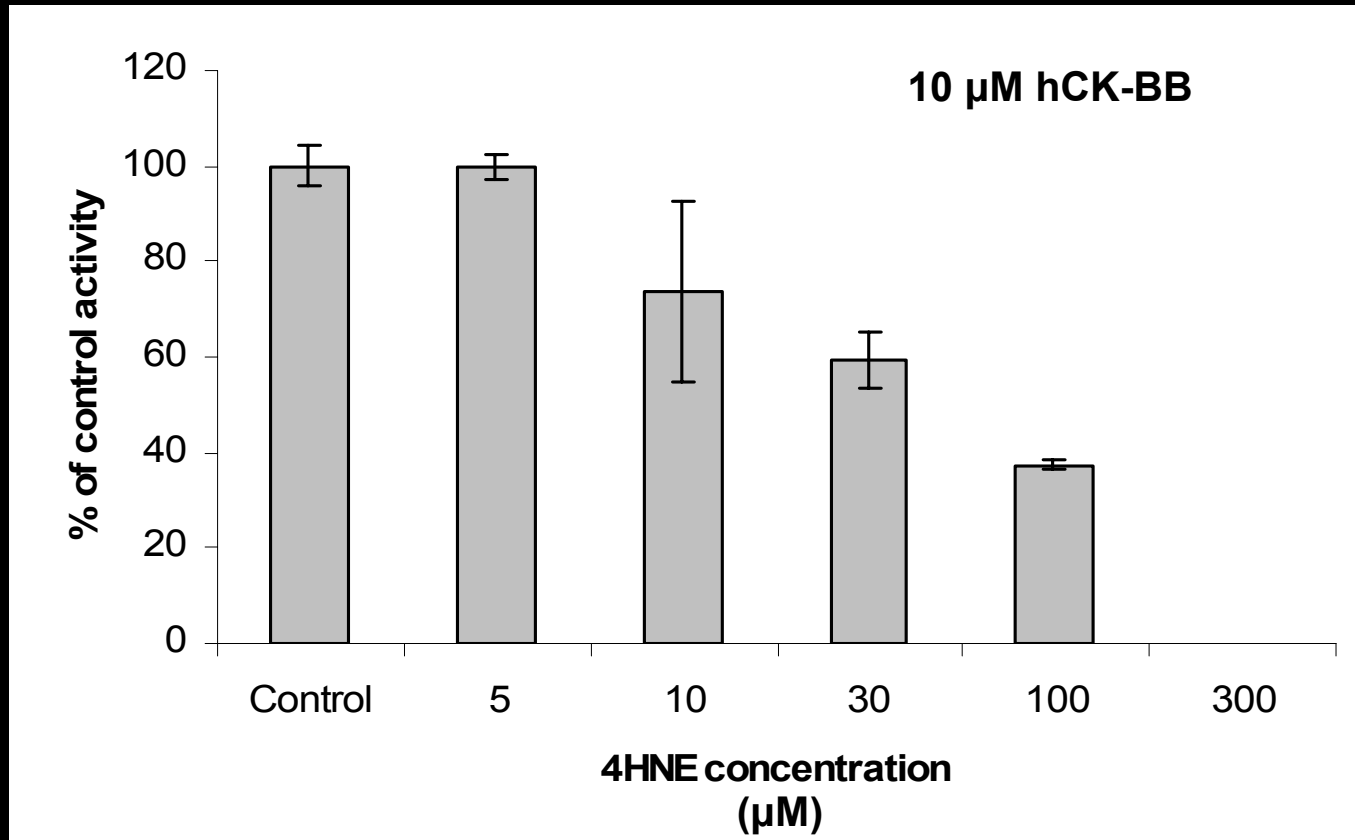
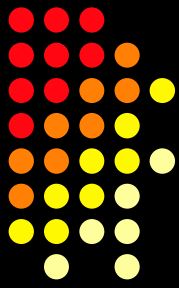


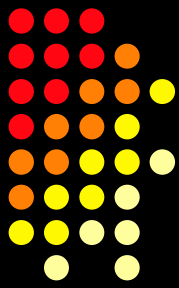
Modifications mapped at different 4HNE concentrations



Modified Amino Acid	Concentration of 4HNE (μM)					
	5000*	300*	100*^	30^	10^	5^
H ⁷	M*,S*	M*,S*	M*^,S*^	S^		
H ²⁶	M*	M	M^	M		
H ²⁹	M*	M*				
K ⁴⁵	M					
H ⁶⁶	M*	M				
K ⁸⁶	M	M				
H ⁹⁷	M*	M*	M^			
K ¹⁰¹	M*	M				
K ¹⁷⁷	M*					
C ¹⁴¹	M*	M	M*^	M^		
C ¹⁴⁵	M*	M	M^	M^		
H ¹⁹¹	M*	M	M^			
H ²¹⁹	M*,S	M*,S	M^			
H ²³⁴	M*,S*	M*,S*	S			
K ²⁴⁷	M,S					
C ²⁵⁴	M,S*	M,S	M^,S^	M^,S^	<u>M^</u>	<u>M^</u>
H ²⁷⁶	M*	M*				
C ²⁸³	M*	M	M*^	M^	M^	
H ²⁹⁶	M,S	M,S	S^			
H ³⁰⁵	M	M				
K ³¹³	M					
K ³⁵⁸	M					
K ³⁸¹	M					

CK-BB activity reduced by HNE

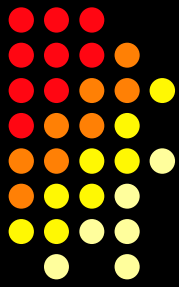




Key Points to Remember

- Oxyblots are useful but have limitations to be aware of
- 4HNE adducted amino acids
 - C, H, K, and R
- 4HNE adducts
 - Michael (156.1150)
 - Schiff Base (138.1045)
 - 2-pentylpyrrole (120.0939)
- Direct infusion with the NanoMate can be used for analysis of low volume samples

Acknowledgements



Kim Lab:

- Dr. Helen Kim
- Laura Chamlee
- Sai Sai Dong
- Patti Hall

Barnes Lab:

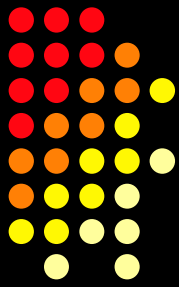
- Dr. Stephen Barnes
- Tracy D'Alessandro
- Erin Shonsey

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- **Dr. Matthew Renfrow**
- Monica Stinnett

UAB CCC Mass Spectrometry/ Proteomics Shared Facility:

- Marion Kirk



Questions/Comments?