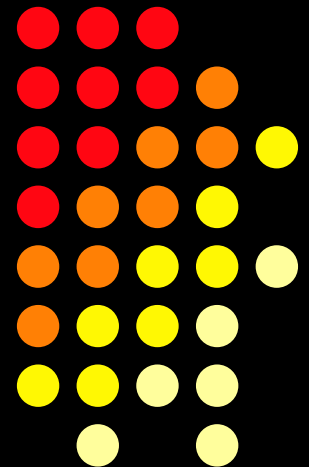


# Localization of 4HNE modifications

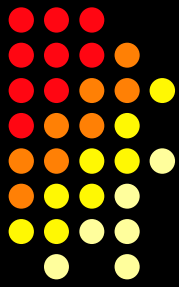
Shannon Eliuk

Sept. 12, 2006

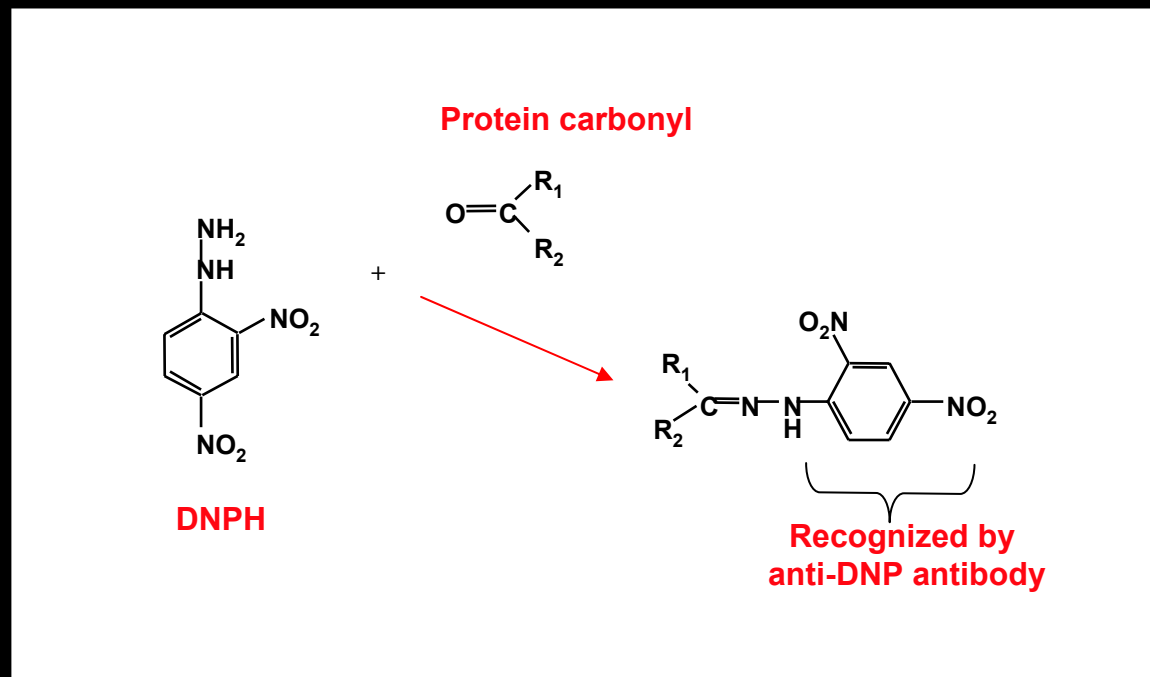
Proteomics Workshop



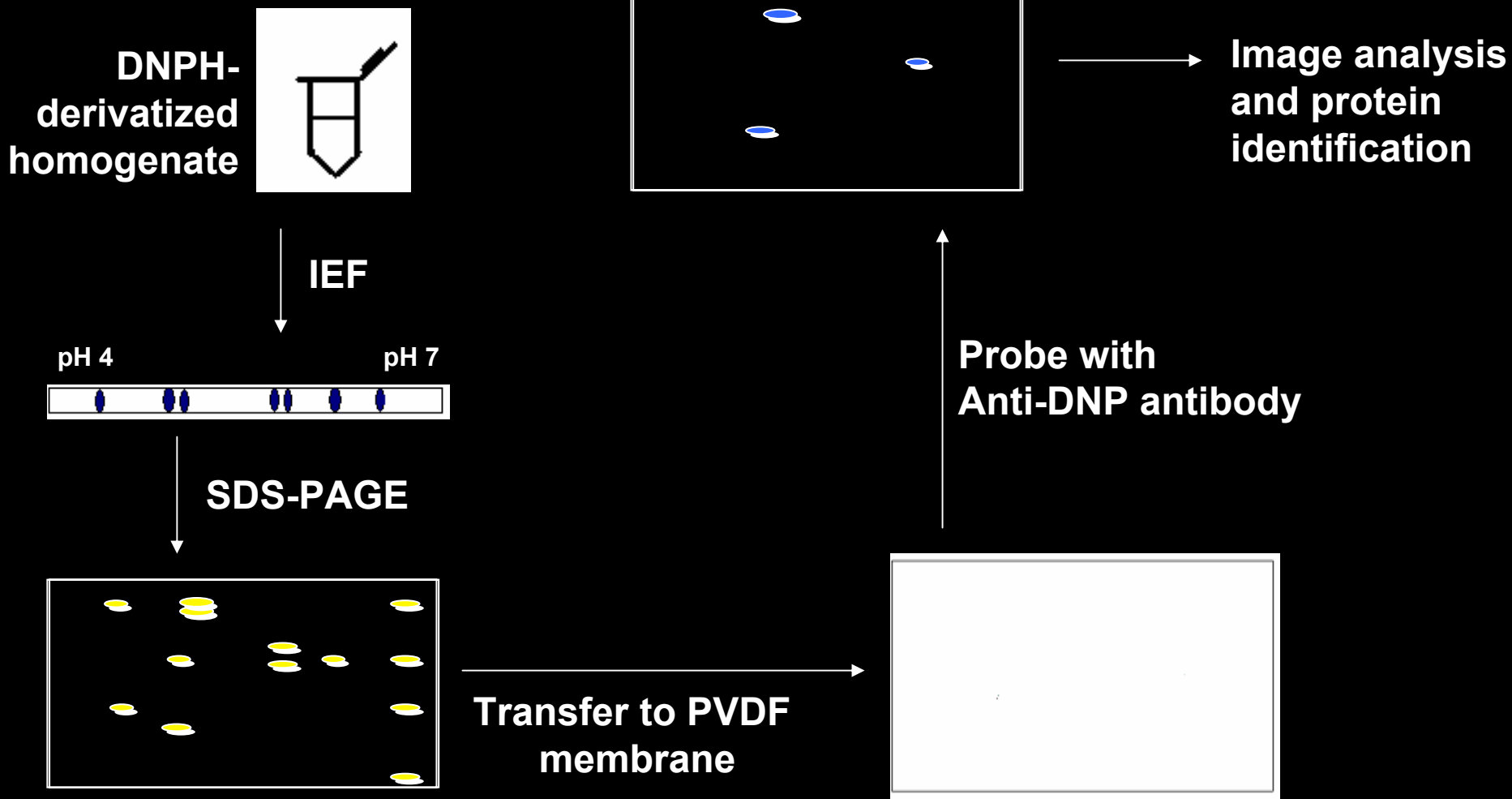
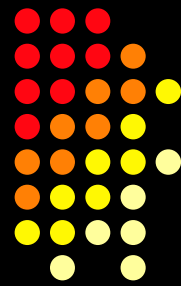
# Proteomic Identification of Oxidatively Modified Proteins



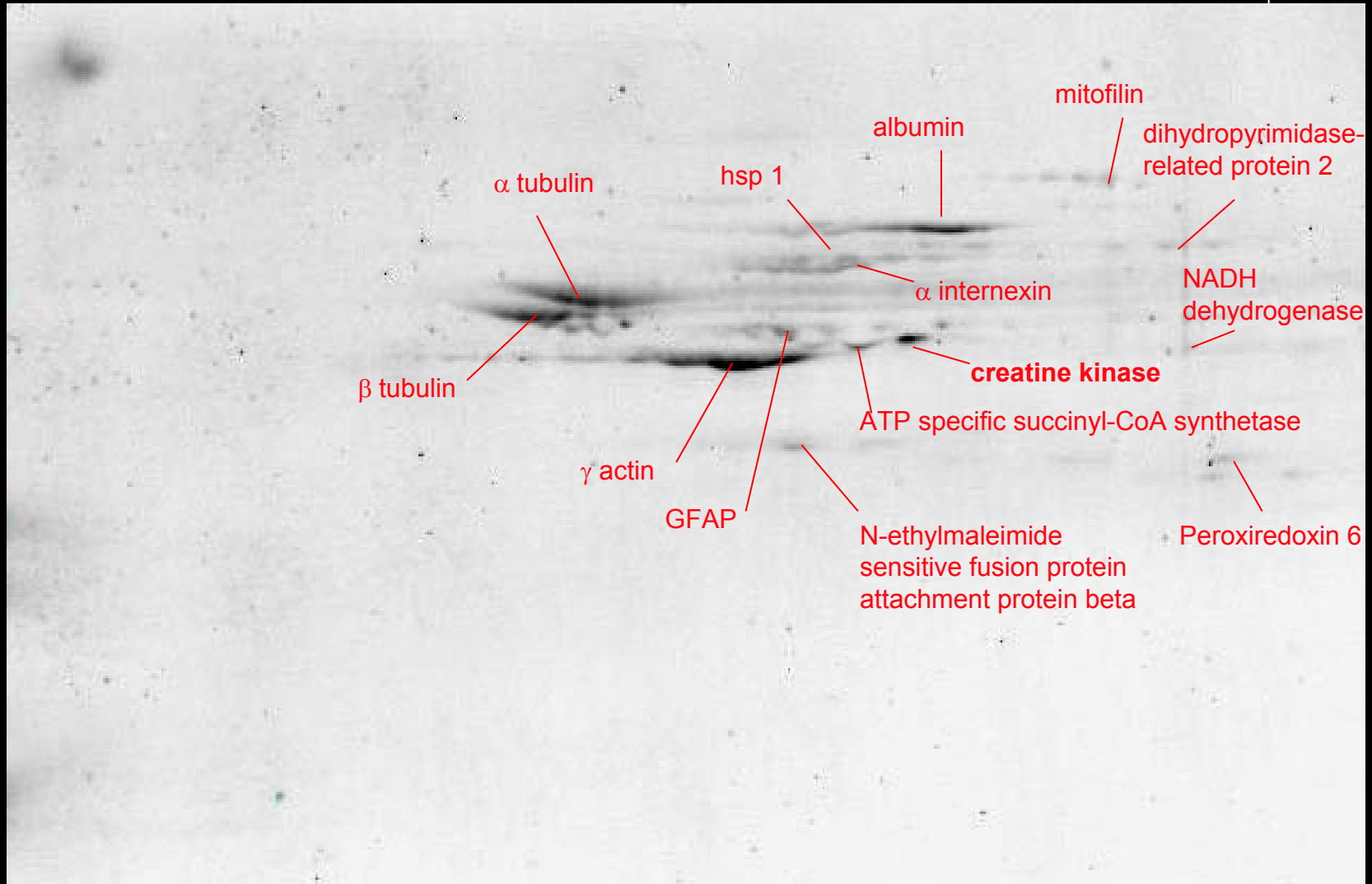
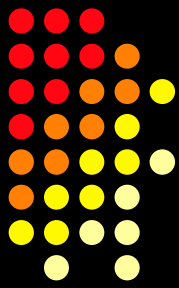
- By use of protein carbonyl derivatization with 2,4-dinitrophenyl hydrazine coupled with 2D Western Blot and mass spectrometry

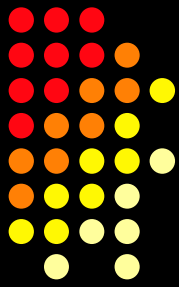


# Investigation of Protein Oxidation: 2D gel electrophoresis and immunoblot



# 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

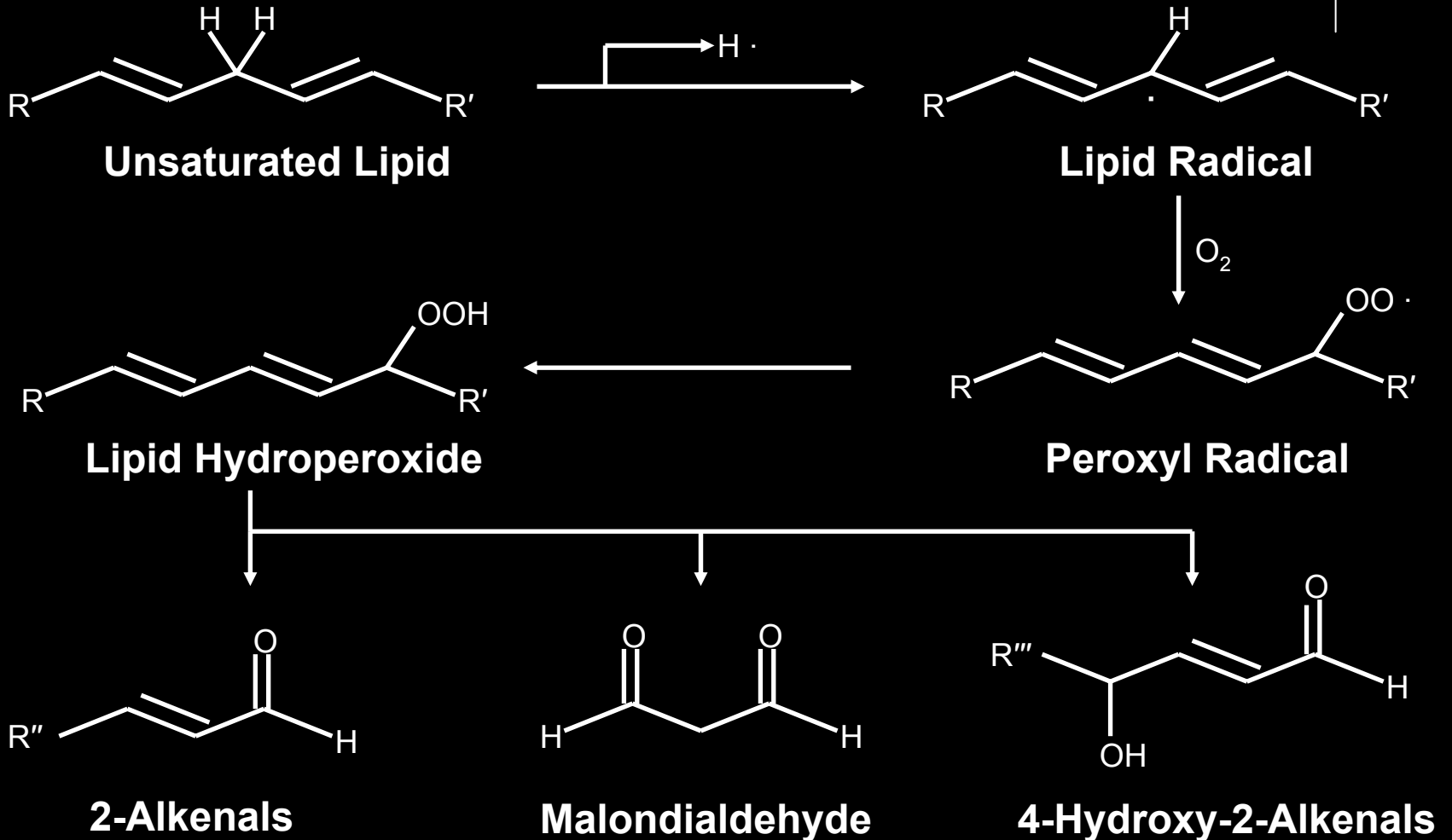
- Identification of modified protein requires subsequent MS analysis
- Identification of modified protein is not conclusive
- Does not elucidate site or chemistry of modification

# 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

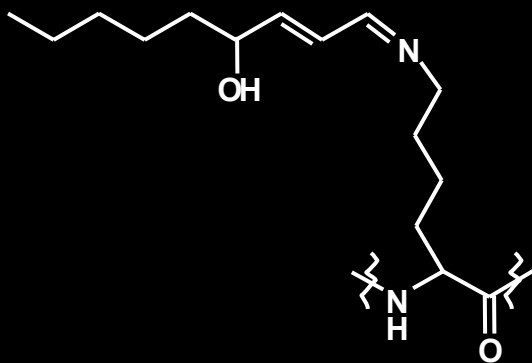
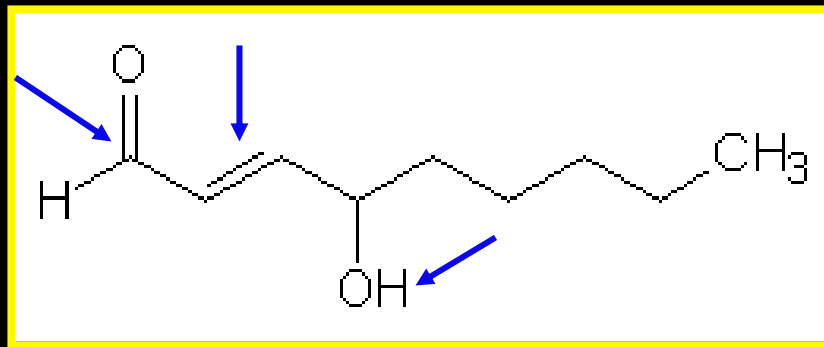
# Lipid Peroxidation



# 4-hydroxy-2-nonenal (4HNE)

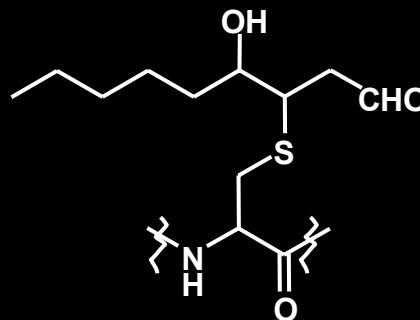


A reactive aldehyde formed as a result of lipid peroxidation



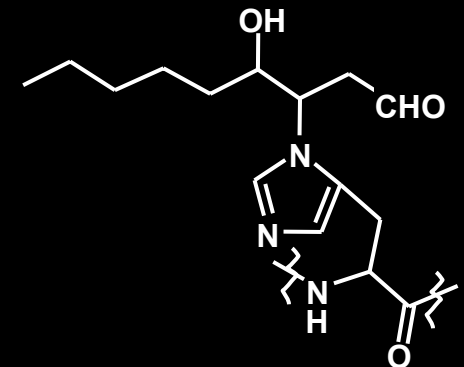
4HNE-Modified Lysine

**Schiff Base** Adduct



4HNE-Modified Cysteine

**Michael** Adduct

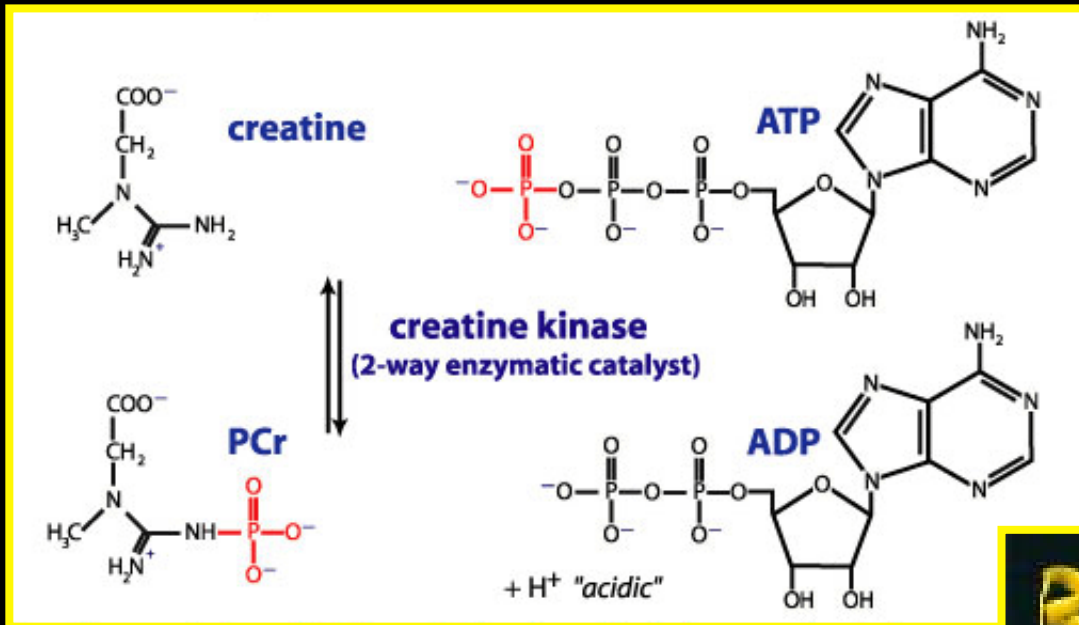
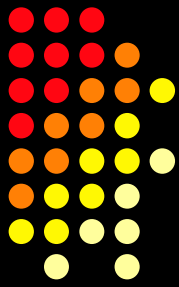


4HNE-Modified Histidine

**Michael** Adduct

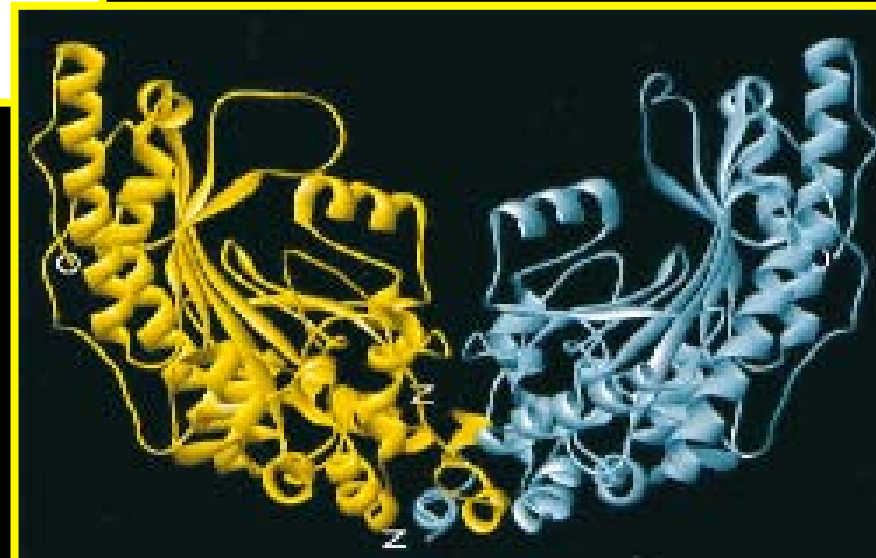


# Creatine Kinase

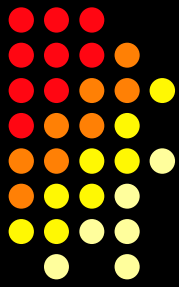


Creatine kinase isoforms  
CK-BB, CK-MM, CK-MB, Mi-CK

<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 VRTGRSIRGF CLPPHC SRGE  
RRAIEKLAVE ALSSLDGDLA GRYYALKSMT EAEQQQLIDD HFLFDKPVSP  
LLLASGMARD WPDARGIWHN DNKTFLVWVN EEDHLRVISM QKGGNMKEVF  
TRFCTGLTQI ETLFKSKDYE FMWNP HLG YI LTCPSNLGTG LRAGVHIKLP  
NLGKHEKFSE VLKRLRLQKR GTGGVD TAAV GGVFDVSNAD RLG FSEVELV  
QMVVDG VKLL IEMEQRLEQG QAIDDLMPAQ K

## Nucleotide binding site

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

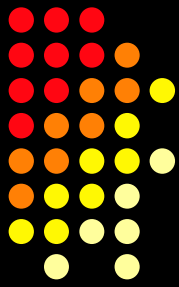
## Creatine Binding Site

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

## Nucleotide phosphate binding site

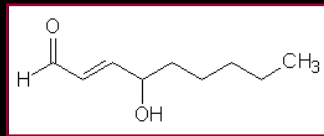
A concentration of +ve charges chiefly 5 highly conserved R residues (130, 132, 236, 292, 320)  
(Lahiri et al., 2002)

# 4HNE Modification of CK



**CKB**

+



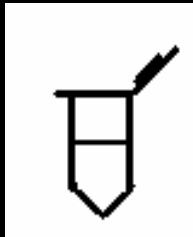
**4HNE**

Incubate at 37C  
2 hrs

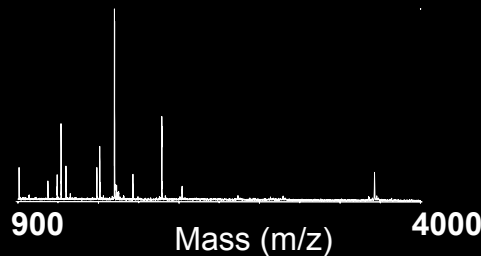


**4HNE modified CKB**

Digest overnight  
(Trypsin/Chymotrypsin)

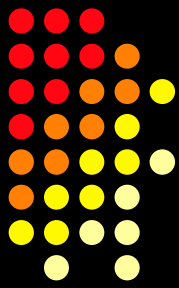


**Mass  
Spectrometry**



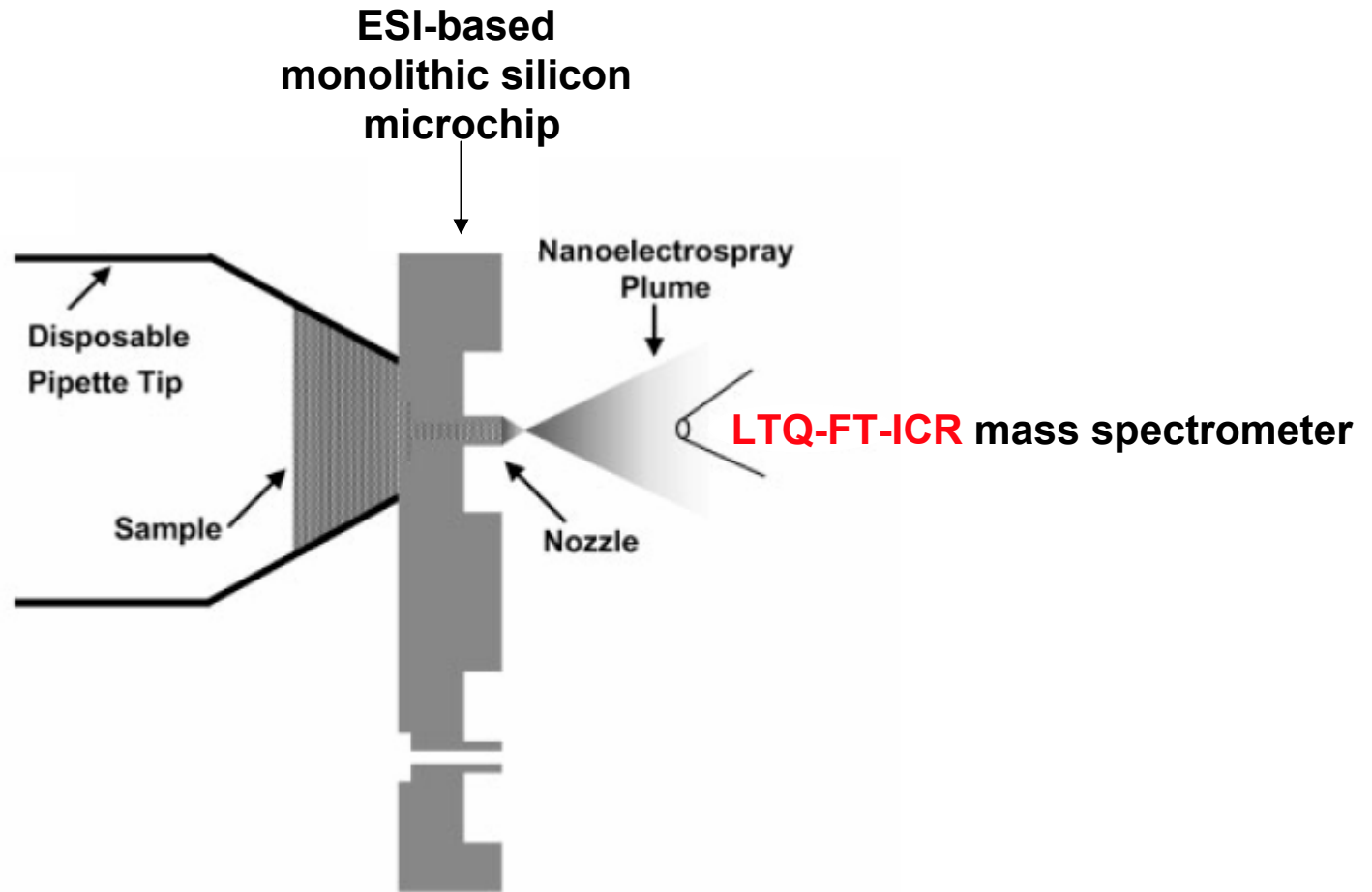
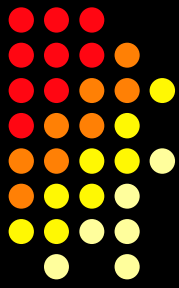
**MEASURE  
FUNCTIONAL  
CONSEQUENCES**

# Direct Infusion by use of the TriVersa NanoMate (Advion)

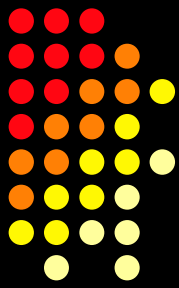


- Fully automated sample handling
- Chip-Based ionization and direct infusion
- Stable, reproducible spray
- No carryover between samples

# Chip-based direct infusion



(Zhang et al., 2003)



# What are we looking for?

## Masses of known peptides

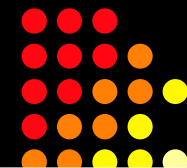
- Each amino acid has a known and unique mass and thus each peptide (string of amino acids) also has a known and unique mass

## 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

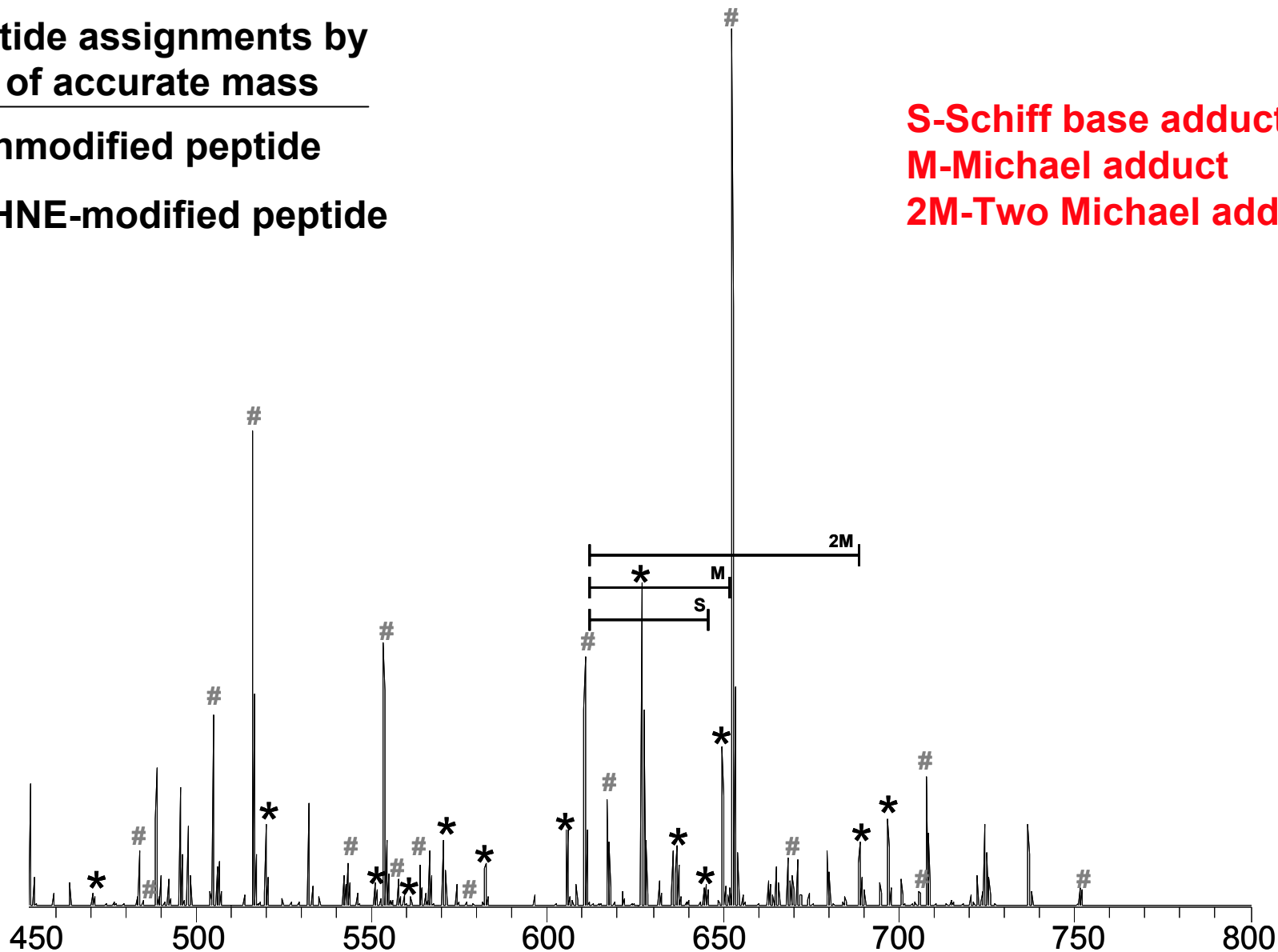


Peptide assignments by  
use of accurate mass

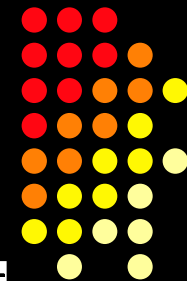
#-Unmodified peptide

\*-4HNE-modified peptide

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



# Accuracy of FT-ICR MS

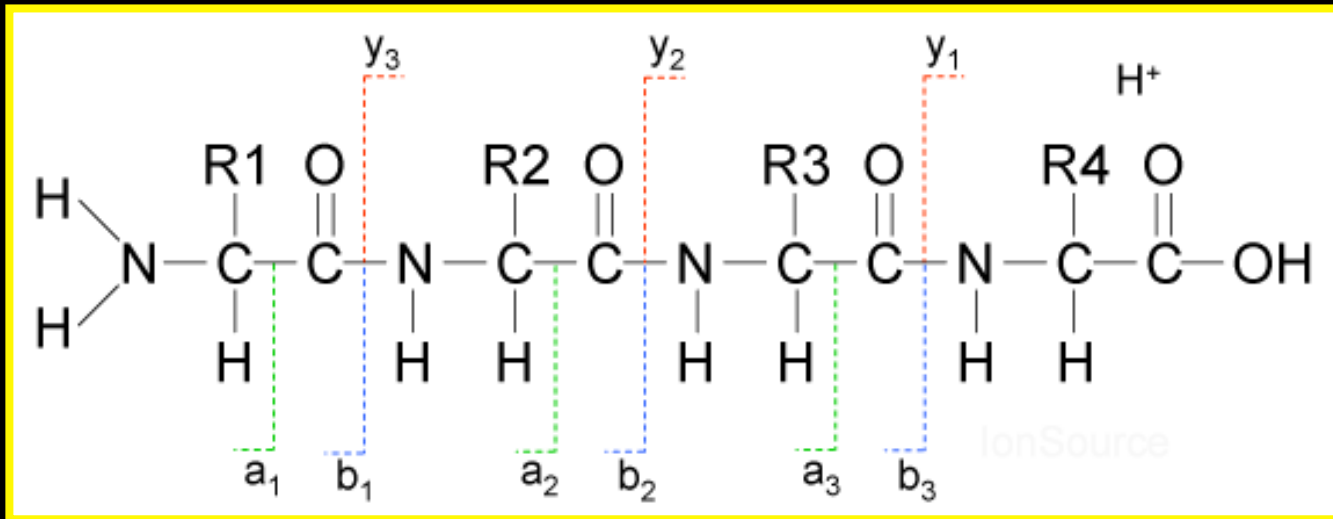
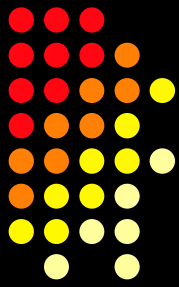


Peptide	Sequence	Modified amino acid	Adduct Form	Observed Mass	Theoretical Mass	Error (ppm)
PFSNSHNAL	2-10	H <sup>7</sup>	Schiff base	1124.5726	1124.5735	0.83
PFSNSHNAL	2-10	H <sup>7</sup>	Michael	1142.5837	1142.5841	0.33
PFSNSHNALK	2-11	H <sup>7</sup>	Michael	1270.6791	1270.6790	-0.03
PFSNSHNALK	2-11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



# Fragmentation by CID

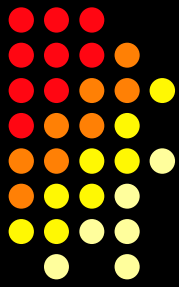
(Collision Induced Dissociation)



<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	

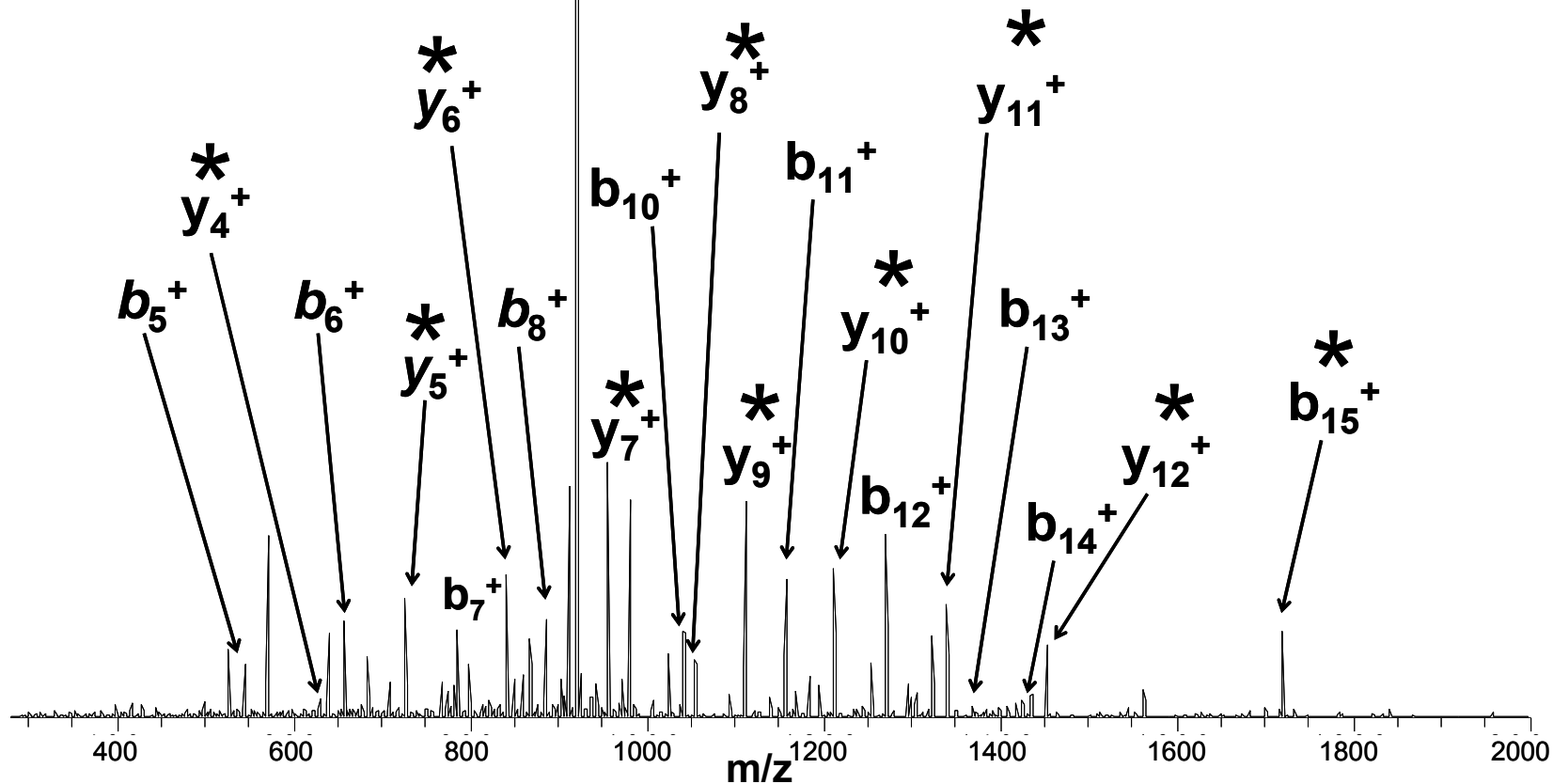
The ion's mass which would be affected by a modified H?

# 4HNE Michael Adduction of H<sup>66</sup>

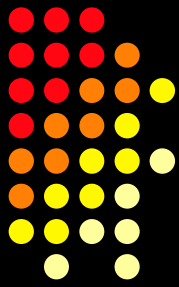


LTDQ-MS/MS

T L D D V I Q T G V D N P G H P Y  
b<sub>5</sub> b<sub>6</sub> b<sub>7</sub> b<sub>8</sub> b<sub>10</sub> b<sub>11</sub> b<sub>12</sub> b<sub>13</sub> b<sub>14</sub> b<sub>15</sub>  
y<sub>12</sub> y<sub>11</sub> y<sub>10</sub> y<sub>9</sub> y<sub>8</sub> y<sub>7</sub> y<sub>6</sub> y<sub>5</sub> y<sub>4</sub> y<sub>3</sub>

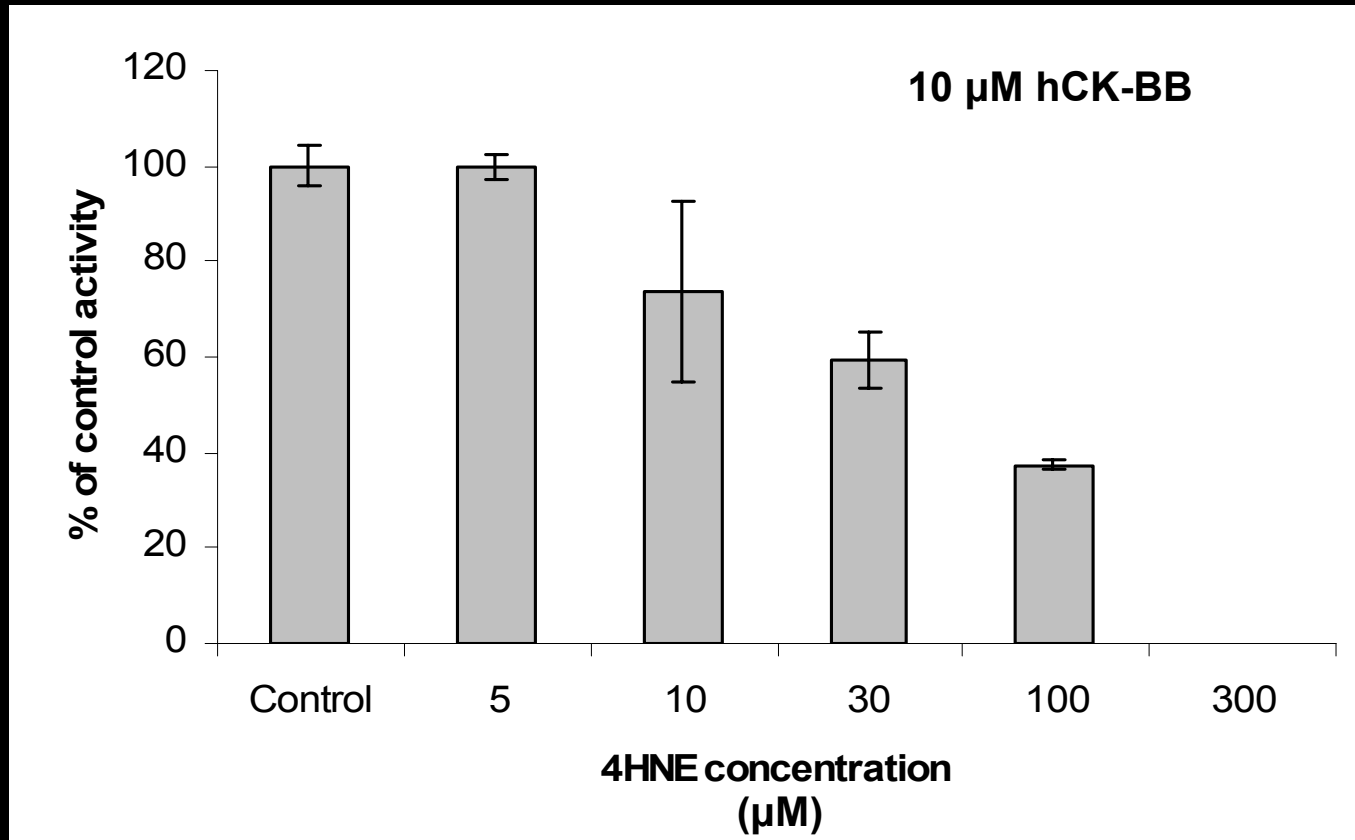
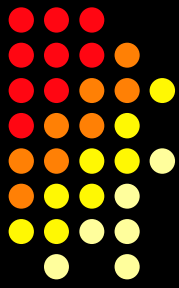


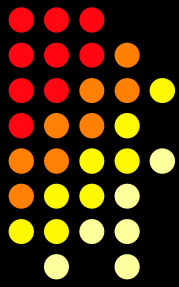
# Modifications mapped at different 4HNE concentrations



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

# CK-BB activity reduced by HNE

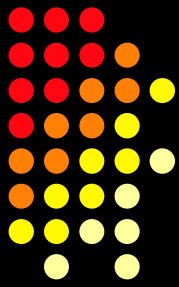




# Key Points to Remember

- 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:

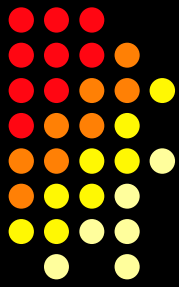
- Dr. Stephen Barnes
- Tracy D'Alessandro
- Erin Shonsey

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

## UAB CCC Mass Spectrometry/ Proteomics Shared Facility:

- Marion Kirk



**Questions/Comments?**