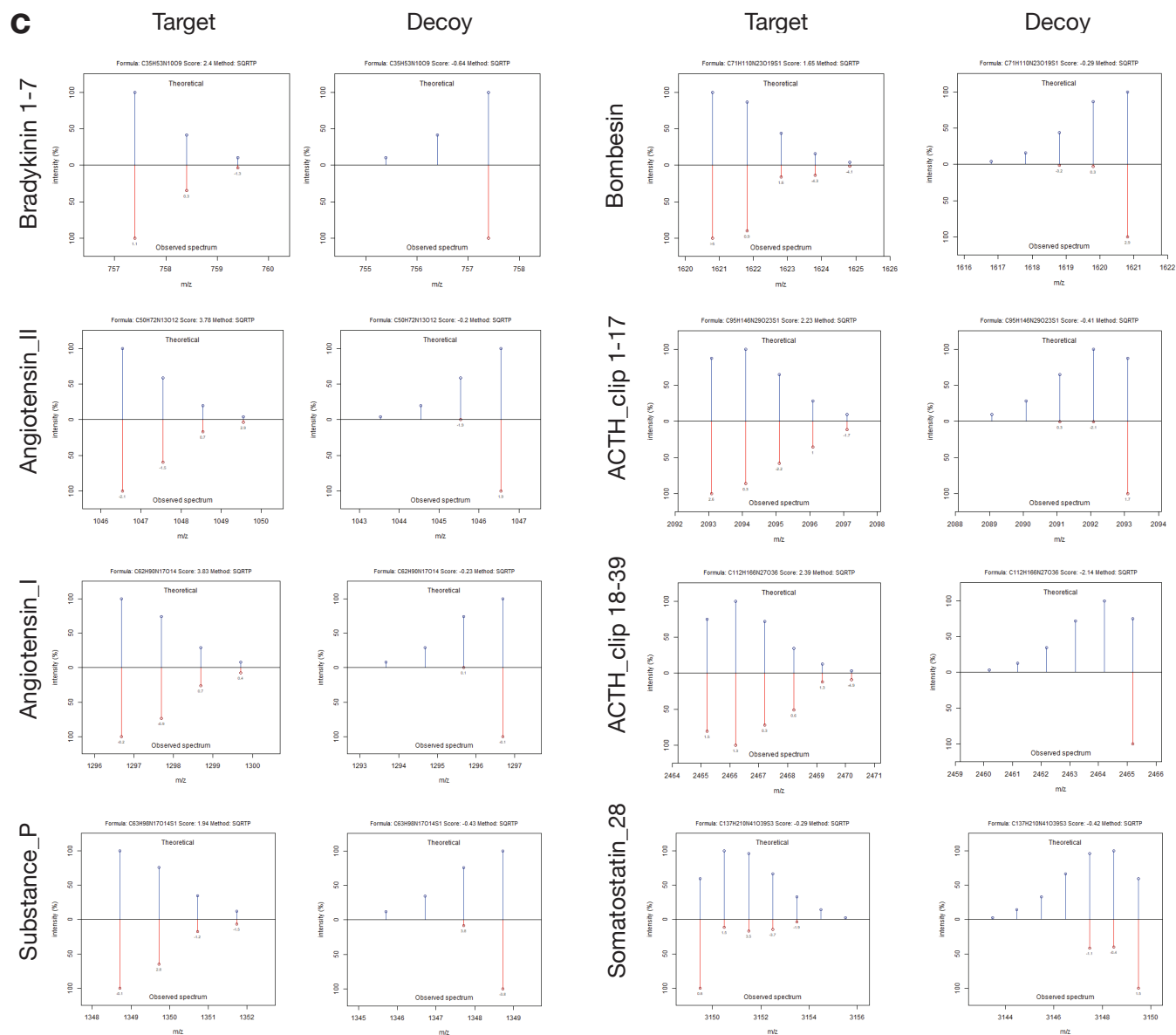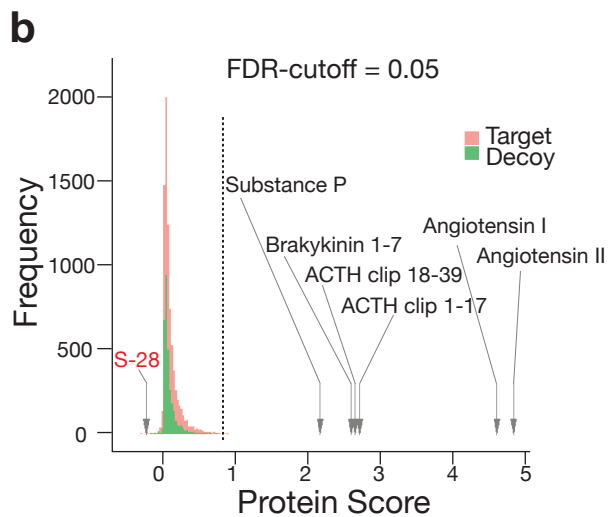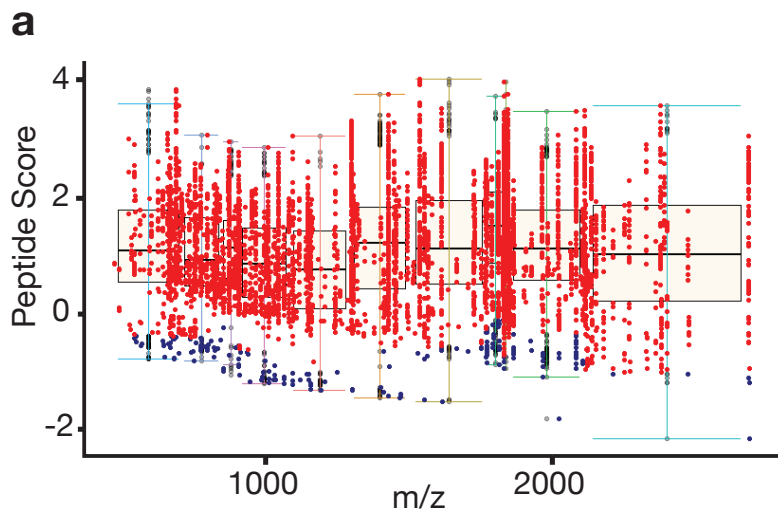**Supplementary Information**

Automated annotation and visualisation of high-resolution spatial proteomic mass spectrometry imaging data using HIT-MAP
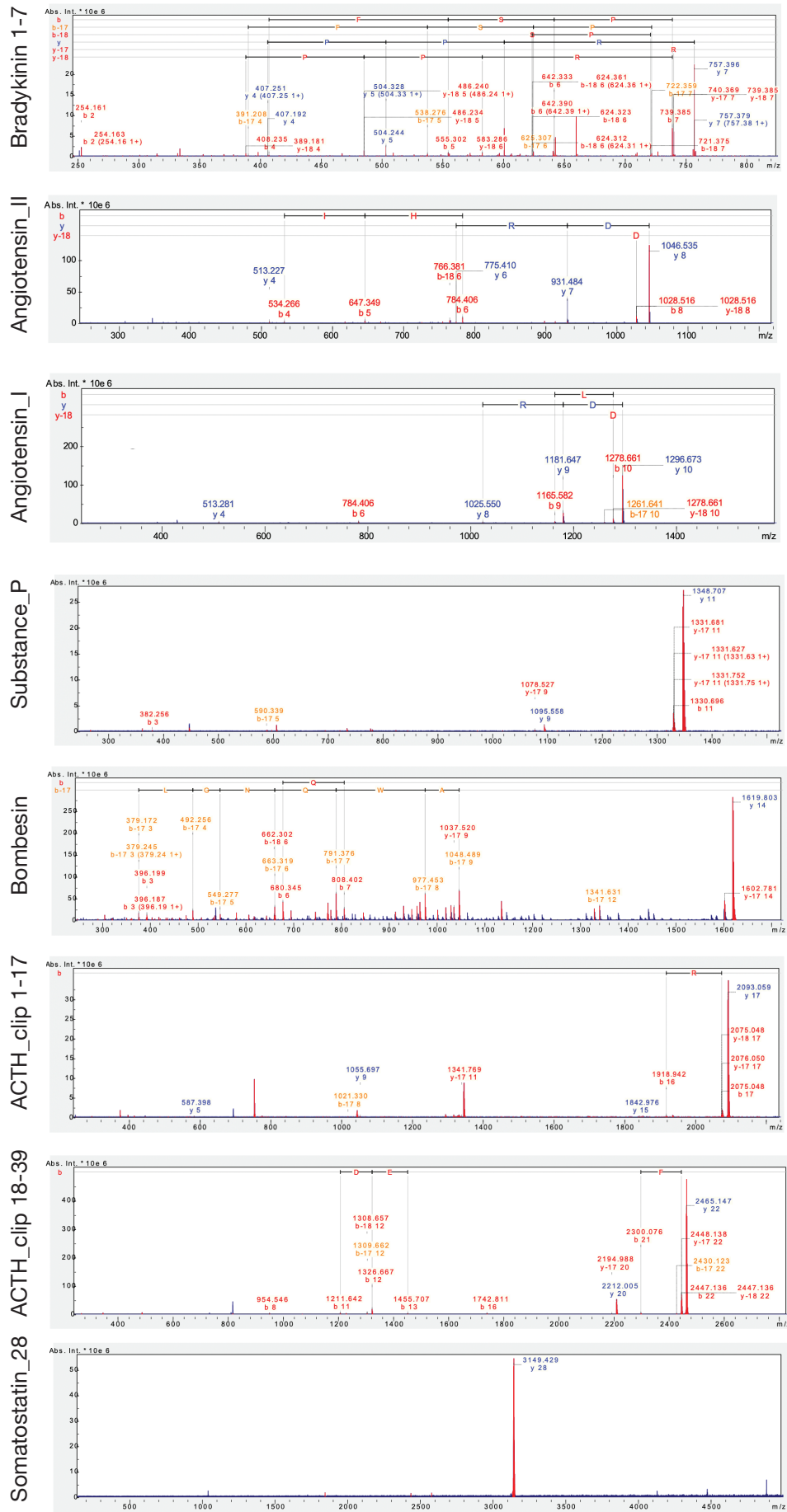
Guo *et al.*

Supplementary Figure 1

a

b
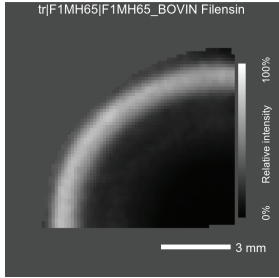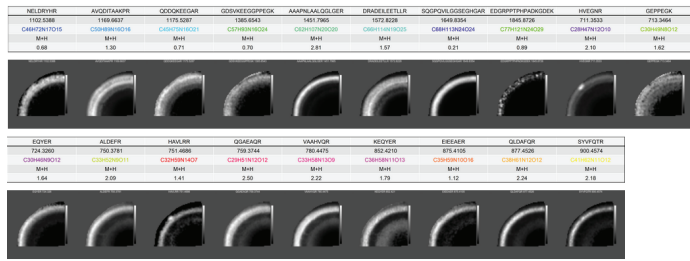

c

Supplementary Figure 1 cont.

d

Supplementary Figure 1 | **a.** Post-hoc IQR outlier analysis of peptide score across the mass ranges identified in the peptide calibrant identifies putative outlier peptides (blue dots) using an interquartile-range (IQR) function (box plots how minimum, maximum and interquartile range). **b.** The frequency distribution of protein candidate list scoring for the preliminary HIT-MAP analysis, annotating 7/8 proteins (excluding Somatostatin-28). **c.** The peptide mass fingerprint analysis demonstrating cross-referencing of the observed isotopic pattern to the simulated theoretical target or decoy isotopic pattern for the 8 peptide calibrants. **d.** MALDI-FT-ICR-MS/MS of the 8 peptides present with the calibrant.

Supplementary Figure 2

a Filensin

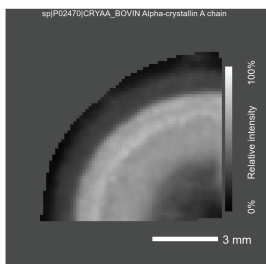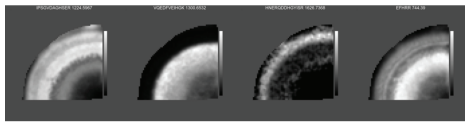| NELDRYHR	AVGDITAAKPR	QDDQKEEGAR	GDSVKEEGGPPEGK	AAAPNLAALQGLGER	DRADEILEETLLR	SQGPQVILGGSEGHGAR	EDGRPPTPHPADKGDEK	HVEGNR	GEPPEGK
1102.5386	1169.6637	1175.5287	1385.6543	1451.7965	1572.8228	1849.9354	1845.8726	711.3533	713.3464
C46H72N17O15	C53H89N18O16	C43H75N19O21	C57H89N18O24	C66H114N19O25	C68H113N24O24	C77H121N24O29	C78H124N31O36	C28H47N12O10	C30H49N9O12
M+H	M+H	M+H	M+H	M+H	M+H	M+H	M+H	M+H	M+H
0.68	1.30	0.71	0.70	2.81	1.57	0.21	0.89	2.10	1.62

EQYER	ALDEFR	HAVLRR	QQAEAQR	VAAHVQR	KEQYER	EIEEAER	QLDAFQR	SYVFQTR
724.3280	750.3781	751.4686	759.3744	780.4475	852.4210	875.4105	877.4526	900.4574
C33H46N9O12	C33H52N8O11	C32H59N14O7	C29H51N12O12	C33H58N13O9	C38H58N11O13	C33H59N10O16	C38H61N12O12	C41H62N10O12
M+H	M+H	M+H	M+H	M+H	M+H	M+H	M+H	M+H
1.64	2.09	1.41	2.50	2.22	1.79	1.12	2.24	2.18

tr|F1MH65|F1MH65_BOVIN Filensin

b Phakinin

LQAGALLQAK	YENEQPFR	VSQELEAQLR	YENEQPFRK	DVASYHALLDR	ATRSENWGALR	SSGLATAPAPSLER	AAQSPSSLDGLPASR	VYVGMAPSGPTGGLGAR
1012.6150	1082.4902	1172.6270	1210.5851	1259.6379	1290.6444	1356.7118	1456.7390	1589.8104
C45H82N13O13	C48H68N13O16	C49H86N15O16	C59H87N16O18	C55H91N16O17	C57H99N17O21	C60H102N19O23	C59H113N20O21S1	
M+H	M+H	M+H	M+H	M+H	M+H	M+H	M+H	M+H
0.28	0.93	-0.36	1.09	1.62	0.53	2.39	1.39	1.20

VVVDAPAGASSSMPLQR	DVASYHALLDREESS	RVVVDAPAGASSSMPLQR	IHIWER	MHLESK	QQAELAR	TVAVSGLVR	SENWGALR
1694.8687	1691.7871	1840.9698	740.3836	744.3709	815.4370	901.5465	932.4585
C71H124N21O24S1	C71H111N20O28	C79H140N25O27	C38H50N11O8	C31H56N9O10S1	C35H62N13O12	C39H73N12O12	C40H62N13O13
M+H	M+H	M+H	M+H	M+H	M+H	M+H	M+H
1.22	0.68	1.72	2.39	1.94	2.64	1.79	2.20

sp|Q28177|BFSP2_BOVIN Phakinin

c

Alpha Crystallin A Chain

TLGPFYPSR	QDDHGYISR	HFSPEDLTVK	TVLDSGISEVR	RTLGPFYPSR
1037.542	1090.491	1172.595	1175.627	1193.643
C49H73N12O13	C45H68N15O17	C53H82N13O17	C49H87N14O19	C55H85N16O14
M+H	M+H	M+H	M+H	M+H
1.97	2.20	0.78	2.19	1.06
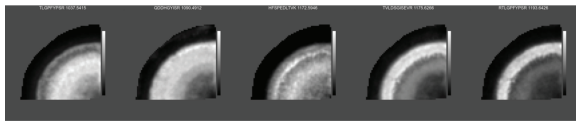
IPSGVDAGHSER	VQEDFVEIHGK	HNERQDDHGYISR	EFHRR
1224.597	1300.653	1626.737	744.390
C50H82N17O19	C58H90N15O19	C66H100N25O24	C32H50N13O8
M+H	M+H	M+H	M+H
2.52	2.41	2.05	2.73

sp|P02470|CRYAA_BOVIN Alpha-crystallin A chain

d

Alpha Crystallin B Chain

QDEHGFISR	EEKPAVTAAPK	VLGDVIEVHGK	DRFSVNLDVK	RPFFPFHSPSR	APSWIDTGLSEMR	VLGDVIEVHGKHEER
1088.5119	1140.6259	1165.6575	1192.6321	1374.7066	1462.6995	1716.9028
C46H70N15O16	C50H86N13O17	C52H89N14O16	C54H86N15O17	C66H93N19O14	C63H100N17O21S1	C74H122N23O24
M+H	M+H	M+H	M+H	M+H	M+H	M+H
1.95	2.25	2.15	2.82	2.33	1.96	0.74

IPADVDPLAITSSLSSDGVLTVNGPR	TIPITR	EFHRK	QASGPER	KQASGPER	FSVNLDVK	HFSPEELK
2594.3672	700.4352	716.3838	744.3635	872.4585	921.5040	986.4942
C112H189N30O40	C31H59N9O8	C32H50N11O8	C29H50N11O12	C35H62N13O13	C42H69N10O13	C43H68N11O14
M+H	M+H	M+H	M+H	M+H	M+H	M+H
2.37	2.55	2.77	2.60	2.69	1.67	1.78

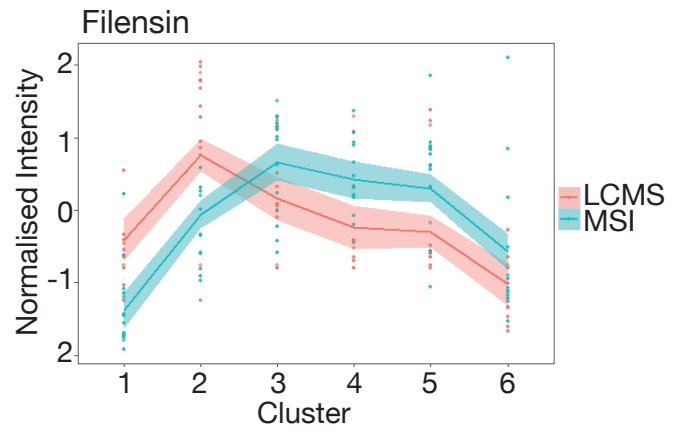sp|P02510|CRYAB_BOVIN Alpha-crystallin B chain

e
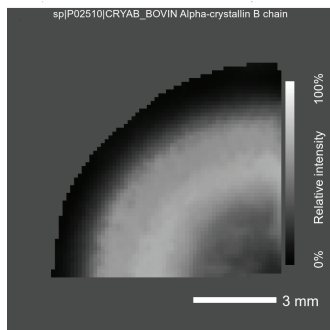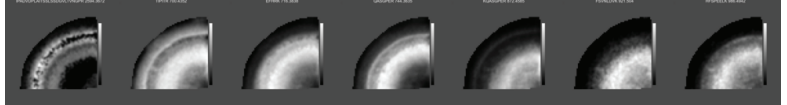
Beta Crystalin B2

DMQWHQR	TDSLSSLRPIK	IRDMQWHQR	RTDSLSSLRPIK	ITLYENPNFTGK	VDSQEHKITLYENPNFTGK	GAFHPSS	VDSQEHK
1000.4418	1216.6896	1269.6269	1372.7907	1396.7107	2220.0931	702.3206	842.4003
C42H62N15O12S1	C52H94N15O18	C54H85N20O14S1	C58H106N19O19	C64H98N15O20	C98H151N26O33	C31H44N9O10	C34H56N11O14
M+H	M+H	M+H	M+H	M+H	M+H	M+H	M+H
0.94	1.68	0.92	0.64	0.53	2.45	3.01	0.96

sp|P02522|CRBB2_BOVIN Beta-crystallin B2

Supplementary Figure 2 cont.

f Vimetin

g Actin Related Protein 8

h Visinin Like Protein 1

i Cortactin

j Arpin

k Tropomyosin 4

l Tropomyosin Alpha 1

m Myosin Light Chain 3

n Dynein Regulatory Complex Subunit 4

o Kinesin Family Member 14

p Ankyrin Repeat Domain 45
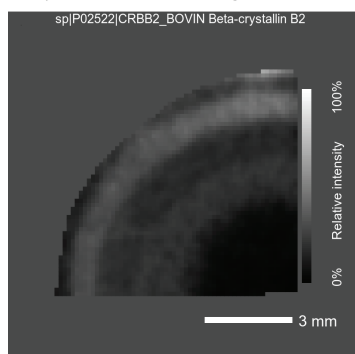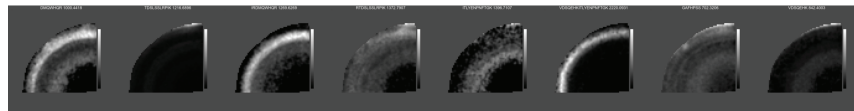
Supplementary Figure 2 | HIT-MAP analysis of published bovine lens(Wang et al. 2020). All peptide and protein cluster imaging outputs of annotated **a-e.** lens and **f-p.** cytoskeletal proteins, (scale bar = 3mm). Intensity scales represent relative intensity from 0% to 100%.

Supplementary Figure 3

a

Cluster Parameters
r = 1, k = 7
z = 1

Clusters
1 (red)
2 (blue)
3 (green)
4 (purple)
5 (orange)
6 (yellow)

b

LC-MS
MSI

6017
741 → 98
All

1893
624 → 96
Relative score > 0.25

245
90
560
Relative Intensity > -5

230
87
525
Relative score > 0.25
Relative Intensity > -5

c

Phakinin
Normalised Intensity
Cluster
LCMS
MSI

Filensin
Normalised Intensity
Cluster
LCMS
MSI

Beta Crystallin Beta 2
Normalised Intensity
Cluster
LCMS
MSI

Vimentin
Normalised Intensity
Cluster
LCMS
MSI
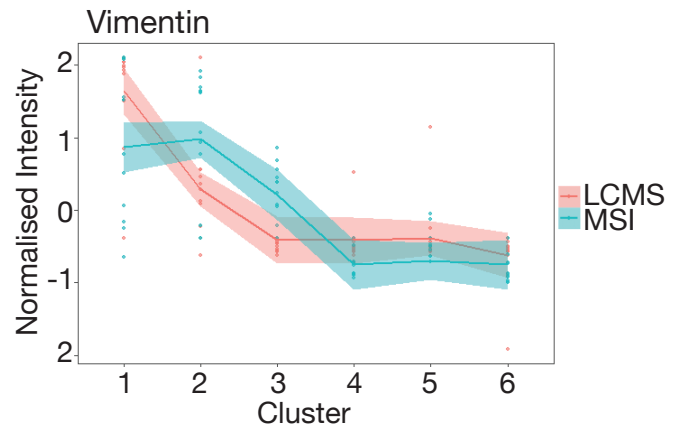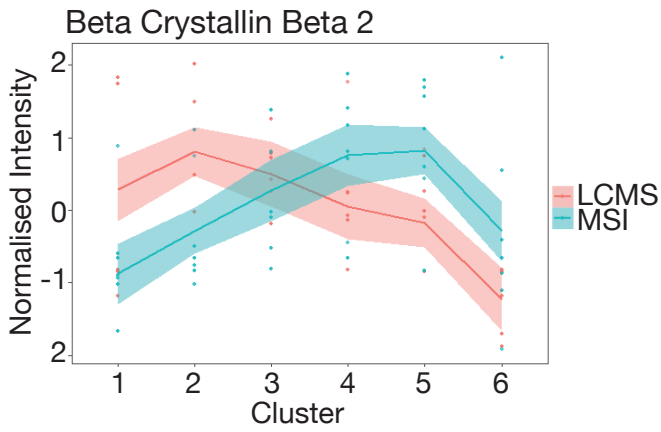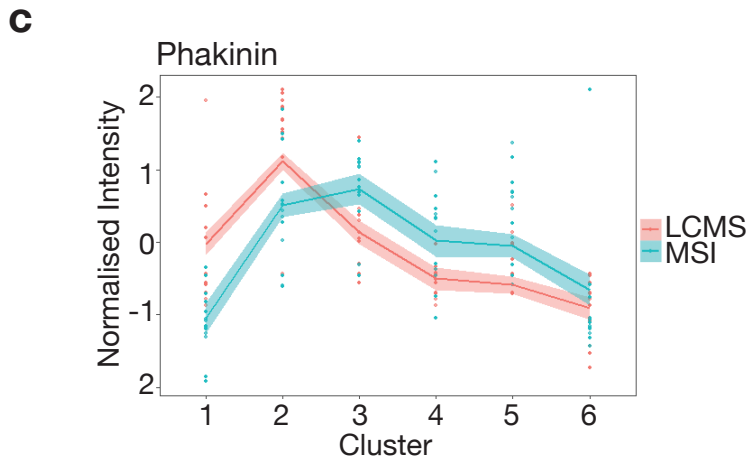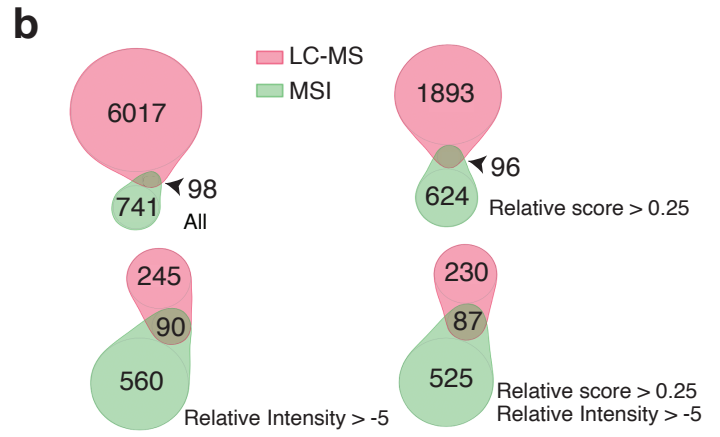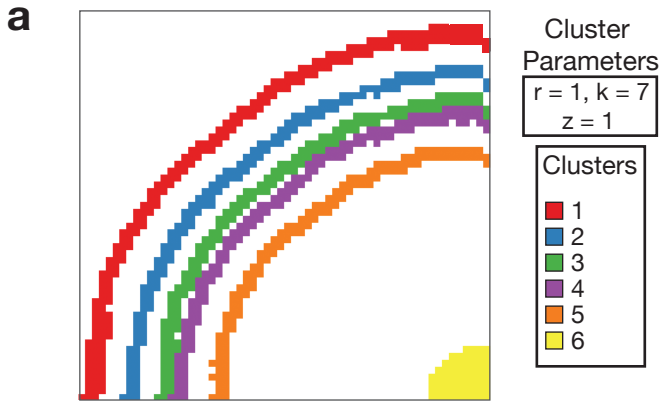
Supplementary Figure 3 | **a.** Segmentation of published bovine lens(Wang et al. 2020) for cross-referencing to spatial microLESA captured regions that underwent LC-MS/MS on a serially sectioned bovine lens MALDI dataset. **b.** Cross-referencing of the annotated peptides from the MALDI-MSI HIT-MAP pipeline with the LC-MS dataset. The relative overlap in co-annotated peptides increases when implementing either a relative score (ii), and relative intensity (iii) or both (iv), as compared to no normalisation (i). **c.** Spatial correlation of additional LC-MS/MS validated proteins annotated by the HIT-MAP pipeline. Intensity scales represent relative intensity from 0% to 100%.

Supplementary Figure 4

a GABA receptor γ3 subunit

TTLGITTVLTMTTLSTIAR	SGSWRR
1994.1202	748.3849
C86H157N22O29S1	C31H50N13O9
M+H	M+H
2.72	1.87
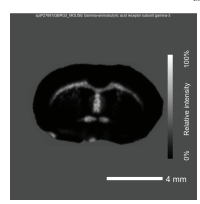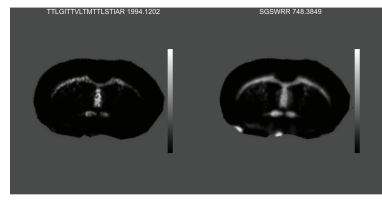
b GABA receptor δ subunit

EGGSRPGGPGGIR	VSLGITTVLTMTTLMVSAR
1196.613	1994.102
C48H82N19O17	C86H157N22O27S2
M+H	M+H
-0.10	1.89
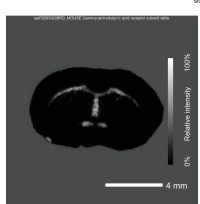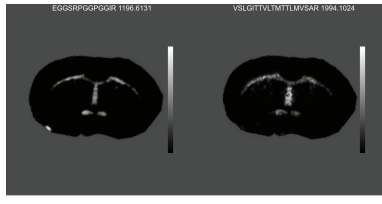
c Neuronal acetylcholine receptor β3 subunit

LPRWLCMK	SSSTYHPMAPWVKR	VYFGLK
1046.5638	1646.8108	726.4185
C48H80N13O9S2	C74H112N21O20S1	C37H56N7O8
M+H	M+H	M+H
1.12	-0.59	1.64
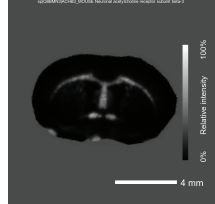
d Endophilin-A2

QGKIPDEELR	MPSKSMPPLDQPSCK	ITASSSFR
1184.6270	1645.7746	868.4523
C50H86N15O18	C69H117N18O22S3	C37H62N11O13
M+H	M+H	M+H
0.81	-0.02	1.96
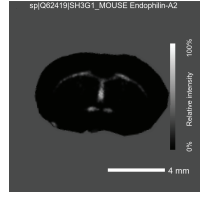
e Endophilin-B1

AAETKSSSEQELR	LGQAEKTELDAHLENLLSK
1435.702	2109.119
C57H99N18O25	C91H154N25O32
M+H	M+H
0.92	2.77
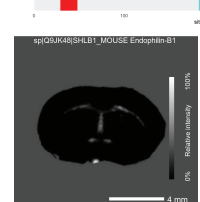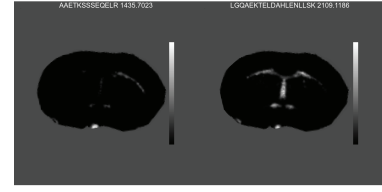
f Syntaxin-1A

EELEELMSDIK	HSEIIK
1335.6348	726.4145
C56H95N12O23S1	C32H56N9O10
M+H	M+H
-1.31	3.82
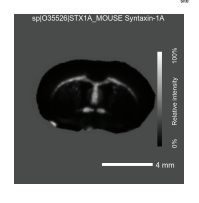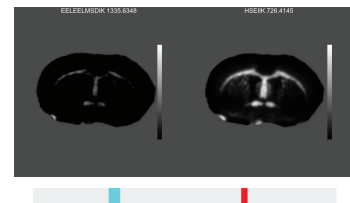
g Vesicle-associated membrane protein 7

LELLIDKTENLVDSSVTFK	AMCMK
2164.1747	583.2401
C97H163N22O33	C22H43N6O6S3
M+H	M+H
0.12	0.84
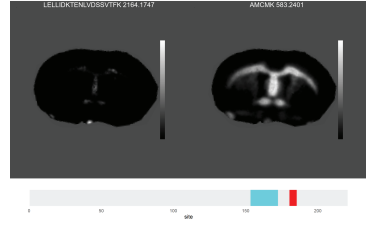
h Vesicle-associated membrane protein 8

GENLDHLRNK	KFWWK
1195.6178	794.4348
C49H83N18O17	C43H56N9O6
M+H	M+H
1.52	-0.32
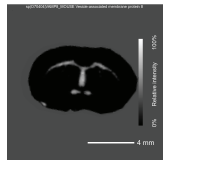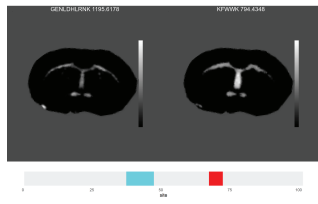
i Protein Kinase C eta type
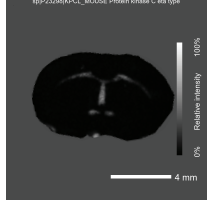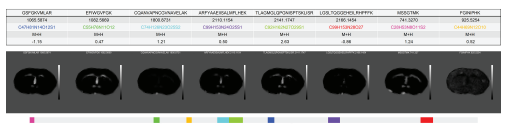
Supplementary Figure 4 cont.

j Smad nuclear interacting protein-1

SSDERPVSGQDR	VKPYIDLGSGNGTFLNNK	EVPVKEKPSFELSGALLEDTNTFR	HRGHSR	LVEYTR
1332.6138	2050.0988	2706.3985	749.3914	780.4250
C51H88N19O23	C93H149N24O29	C121H199N30O40	C29H49N19O6	C35H58N9O11
M+H	M+H	M+H	M+H	M+H
0.23	0.98	3.02	0.40	1.84
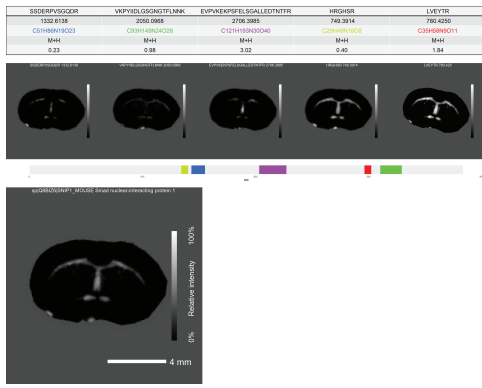
k Cytochrome c oxidase subunit 6A1

HEEHERPPFVAYPHLR	MASAVLSASR
2014.0042	992.5193
C92H133N28O24	C40H74N13O14S1
M+H	M+H
0.70	0.15

l Pyruvate Kinase

TVWVDYHNTQVVAVGGR	IGPEGLVTEVEHGGFLGRRK	GDAKTVWVDYHNTQVVAVGGR	DALGPEGR	QLFEELR	GVFPLLYR
2014.0505	2109.1087	2385.2310	814.4056	934.4993	964.5615
C91H141N26O26	C93H150N27O29	C106H169N31O32	C33H56N11O13	C42H68N11O13	C49H74N11O11
M+H	M+H	M+H	M+H	M+H	M+H
0.75	2.78	-0.46	-0.57	1.03	1.27
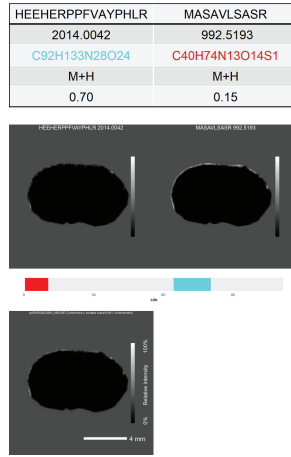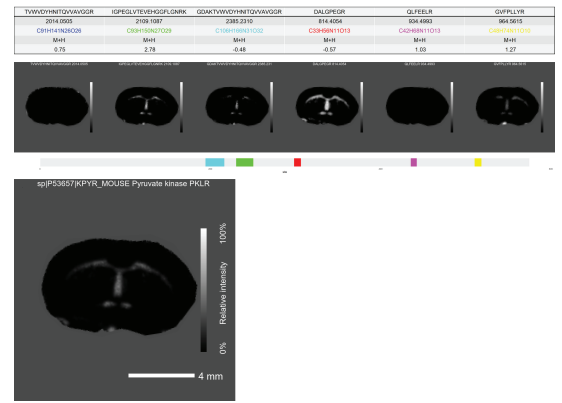
m Neutrophilic Granule Protein

AIEAYNQGRQGRPLFR	NIYEDAKYDIIGNILK
1875.994	1881.996
C82H131N28O23	C86H137N20O27
M+H	M+H
1.19	1.27
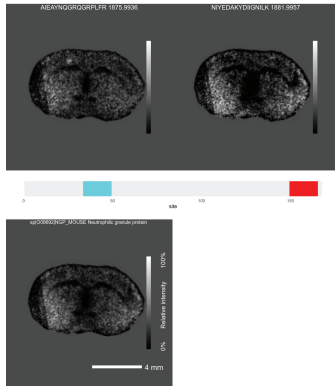
n Small EDRK rich factor 2

MTRGNQR	GNQRELAR
862.4312	943.5068
C32H60N15O11S1	C37H67N16O13
M+H	M+H
1.45	2.78
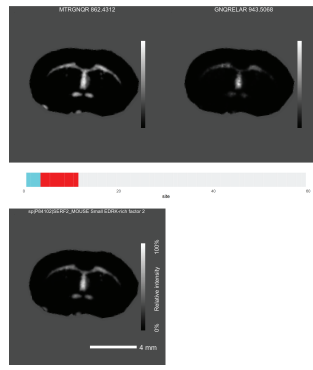
o Disintegrin and metalloproteinase domain-containing protein 22

QCKYIWGQK	EQRQLPQGDYVK	YIELMIVNDHLMFKK	LWETSI
1153.5823	1460.7492	1893.9965	748.3876
C53H81N14O13S1	C63H102N19O21	C88H141N20O22S2	C35H54N7O11
M+H	M+H	M+H	M+H
1.39	2.27	0.30	1.76
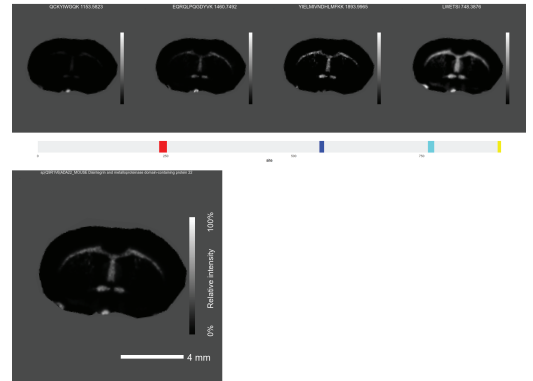
p Hyaluronan and proteoglycan link protein 3

YDVFCFAAALKGR	SFGDYQGR
1460.7355	929.4112
C68H102N17O17S1	C40H57N12O14
M+H	M+H
2.34	1.22
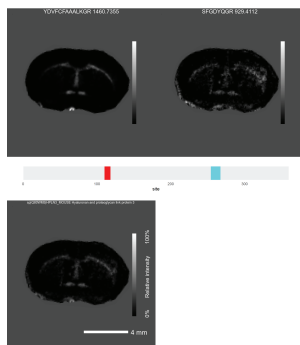
q Wnt-2b

ALMNLHNNR	QGYRHATR
1082.5524	988.5071
C44H76N17O13S1	C41H66N17O12
M+H	M+H
0.27	2.07
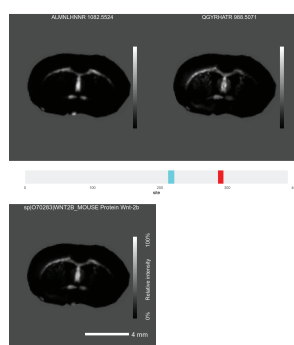
Supplementary Figure 4 | HIT-MAP analysis of murine brain. All peptide and protein cluster imaging outputs of annotated **a-c** neural receptors, **d-h** vesicle related proteins, **I** and **j** signalling proteins, **k** and **l** metabolic proteins, **m** and **n** Alzheimer's disease related proteins, and **o-q** extracellular proteins, scale bars = 4 mm. Intensity scales represent relative intensity from 0% to 100%.

Supplementary References

1. Wang, Z., Ryan, D. J. & Schey, K. L. Localization of the lens intermediate filament switch by imaging mass spectrometry. *Exp. Eye Res.* 108134 (2020). doi:10.1016/j.exer.2020.108134