Infrared photoactivation enables nano-DESI MS of protein complexes in tissue on a linear ion trap mass spectrometer.

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Abstract

Native mass spectrometry analysis of proteins directly from tissues can be performed using nanospray-desorption electrospray ionization (nano-DESI). Typically, supplementary collisional activation is essential to decluster protein complex ions from solvent, salt, detergent and lipid clusters that comprise the ion beam. As an alternative, we have implemented declustering by infrared (IR) photoactivation on a linear ion trap mass spectrometer equipped with a CO_2 laser (λ = 10.6 μm). The prototype system demonstrates declustering of intact protein complex ions up to approx. 50 kDa in molecular weight that were sampled directly from brain and eye lens tissues by nano-DESI. For example, signals attributable to different metal binding states of $hSOD1^{G93A}$ homodimers (approx. 32 kDa) separated by only approx. 6 Th (10^+ ions) were resolved with IR declustering, but not with collisional activation. We found IR declustering to outperform collisional activation in its ability to reduce chemical background attributable to non-specific clusters in the nano-DESI ion beam. The prototype system also demonstrates *in situ* native MS on a low-cost mass spectrometer and the potential of linear ion trap mass spectrometers for this type of analysis.

Introduction

Native protein analysis directly from tissue by nanospray desorption electrospray ionization (nano-DESI) enables spatially resolved native top-down mass spectrometry (nTDMS), and mass spectrometry imaging (MSI) of protein complexes exceeding 100 kDa.[1,2] So far, this methodology has been limited to high-performance mass spectrometers. The cost of high-performance mass spectrometers is prohibitive to wider adoption of *in situ* native mass spectrometry in structural biology applications and more affordable instrumentation is a request of the broader native MS community. [3] One factor that limits native MS performance is the need for declustering, i.e. removal of solvent, salts and detergent from protein ions without disrupting non-covalent interactions in order to obtain accurate mass measurements.[4,5] The ubiquity of collisional activation means it is often used for declustering in native MS experiments.[6-9] The associated elevation of ion kinetic energy complicates transmission of the ions in the mass spectrometer and can be detrimental to sensitivity without further tuning, e.g., increasing trapping gas pressure[8,10,11] or potential surface optimization.[7,12,13] Each of these adds an additional level of complexity requiring extra pumping capacity or more sophisticated ion optic elements.

Infrared multiphoton dissociation (IRMPD) is a charge-independent alternative to collision-induced dissociation (CID), that has shown promise for declustering ions from the chemical background and adducts.[14] Brodbelt and co-workers demonstrated increased peptide and protein ion signal intensity after IRMPD of non-specific chemical signal on a custom linear ion trap (LIT) mass spectrometer.[15] Their study indicated that proteins incorporated in salt/solvent clusters could be recovered as intact, protonated molecular ions. Separately, several studies have combined native MS and IRMPD-equipped ion trap mass spectrometers to fragment protein complexes[16-19] and release membrane proteins from detergent micelles (with subsequent m/z analysis by TOF or orbitrap mass analyzers).[17-19]

Our recent work has focused on the use of nano-DESI under native-like conditions for the analysis of intact protein complexes directly from tissue sections. Native nano-DESI produces extremely heterogeneous ion beams from an electrospray containing intact proteins and protein complexes (i.e., the analytes of interest) mixed with non-specific clusters of solvent, salts, detergent micelles, and abundant endogenous biomolecules (e.g., lipids) leading to substantial non-specific chemical signal across a broad m/z range. Currently, collisional activation in the ion source and/or dedicated collision cell is used to release protonated protein complexes from the clusters. Although beneficial, native nano-DESI spectra still suffer from chemical background and low signal abundance with this approach, and to date it has not been possible to transfer native nano-DESI to simpler, lower cost mass spectrometers that feature less sophisticated ion optics.

Here, we demonstrate that the nano-DESI ion beam can be declustered by IRMPD allowing release of intact protein ions and enabling native protein analysis from tissue on a LIT mass spectrometer. We attached our home-built nano-DESI ion source[20] to a LIT mass spectrometer modified with a continuous wave infrared laser (CO_2, λ=10.6 μm). We observed a dramatic reduction in chemical background in nano-DESI spectra with IR activation of the ion beam compared with inactivated and collisionally-activated ion beams. Our results suggest that IR declustering is a promising alternative to collision-based declustering for native protein analysis from complex biological environments.

Materials and Methods

Materials

MS-grade water was purchased from Fisher Scientific. HPLC-grade ammonium acetate was bought from J.T. Baker (Deventer, The Netherlands). C_8E_4 detergent and polypropylene glycol were obtained from Merck (Gillingham, UK). Helium gas (99.996% purity) was obtained from BOC (Guildford, UK). Calibration solutions were purchased from Thermo Fisher Scientific (Waltham, MA).

Animal Tissues

Fresh frozen brains from SOD1G93A C57BL/6 transgenic mice were the gift of Dr Richard Mead (University of Sheffield, UK).[21] Each brain was bisected down the midline and the left hemisphere was mounted to a chuck with ice. Sagittal cryosections were prepared by cutting from the midline with a CM1810 Cryostat (Leica Microsystems, Wetzlar, Germany) and thaw mounted to glass microscope slides before storage at -80 °C until analysis.

Whole, fresh sheep eyes were bought from DissectUK (Birmingham, UK). Eyes were harvested and transported with cold packs for dissection. Lenses were extracted from each eye, placed on aluminium foil and snap frozen in liquid nitrogen. All tissue was stored at -80 °C, sectioned at -22 – -24 °C to a thickness of 20 μm with a CM1810 Cryostat, thaw mounted to glass microscope slides and stored at -80 °C until analysis.

Linear ion trap modification for IRMPD.

An LTQ Velos Pro linear ion trap (LIT) mass spectrometer with scan range up to m/z 4000 (Thermo Fisher Scientific, San Jose, CA) was recovered from a decommissioned Orbitrap Elite mass spectrometer (Thermo Fisher Scientific). A CF flange containing an IR-transparent ZnSe window (Thorlabs, Newton, NJ) was attached to the rear of the LIT. A continuous wave CO_2 IR laser (λ=10.6 μm; max power; 20 W, model; FireStar V20, Synrad Inc, Mukilteo, WA), an inline red diode pilot laser and beam optics were recovered from a decommissioned LTQ-FT mass spectrometer (Thermo Fisher Scientific) and positioned at the rear of the LIT, see *Figure S1, Supporting Information*. With the LIT chamber open to atmosphere, the pilot laser beam was transmitted through the LIT low pressure

(LP) cell and high pressure (HP) cell for initial beam alignment. The raw 10.6 μm output (2.0 mm x 2.4 mm elliptical cross section, 7 mrad divergence) was guided approx. 300 mm to the 2 mm diameter end orifice of the LIT using non-focusing mirrors and ZnSe window; alignment with the ion beam axis was ensured using 3 more LIT orifices as apertures and optimising the beam transmission through all of these; transmission of the beam was calculated at ~20%. Transmission of the 10.6 μm beam was confirmed by heat-sensitive cards (Thorlabs) placed in the beam path at atmospheric pressure and by observation of fragmentation of caffeine/MRFA/ubiquitin ions with the system under vacuum. The CO_2 laser was triggered by an RSDG 805 arbitrary waveform generator (RS Group, London, UK) enabling duty cycle control between 20% - 80%. The specific laser duty cycle used is detailed with relevant results in absence of direct power measurement. The laser was operated continuously during ion accumulation (see results for accumulation times).

Nanospray-desorption electrospray ionization (nano-DESI) MS.

A home-built nano-DESI ion source[20] was attached to the atmospheric pressure interface of the LTQ Velos Pro. The solvent system was aqueous ammonium acetate (200 mM) + 0.125% (by volume) of the detergent C_8E_4 and set to a flow rate of 1.7 μL/min by external syringe pump. Electrospray voltage was set to 1.15 kV. The ion inlet temperature was 275 °C and the S-lens was set to 70%. Source pressure was approx. 1.5 Torr, LIT pressure was approx. 1.7e-5 Torr (separate pressure readbacks for each cell were not available). Helium was provided to the HP cell as the damping gas. Tissue sections were continuously sampled by scanning under the nano-DESI probe at a rate of 5 μm/s. Ions were accumulated and continuously irradiated by 10.6 μm photons along the beam path through the high-pressure cell and transfer optics (See *Figure S1, Supporting Information*). The automatic gain control (AGC) target was set to 5e4 charges with a maximum ion accumulation ("injection") time of 750 ms. Long injection times were necessary because of the low protein ion flux from direct tissue sampling, and typically the AGC target was not reached before injection. This requirement is also typical of these experiments on high-end Orbitrap systems. Ions were injected into the low-pressure cell for m/z analysis. Unless otherwise noted, the ion trap mode was set to "High mass" and scan rate was set to "Turbo" (125,000 $m/z.s^{-1}$, peak FWHM; 3) with two microscans averaged per scan. Mass calibration was performed with CalMix and PPG 2700. Mass spectra were externally recalibrated to nano-DESI spectra recorded using an orbitrap mass spectrometer (Orbitrap Eclipse, see below).

The same nano-DESI source was attached to an Orbitrap Eclipse (Thermo Scientific) equipped with the HMR^n option, as previously described,[1] and set-up as for the LTQ unless otherwise noted here. Electrospray voltage was set between 0.9 and 1.4 kV and the ion inlet temperature was 275 °C. The S-lens was set to 120%, in-source CID potential was 80 V with a scaling factor of 3%. Acquisition

mode was set to “High Mass”, “Intact Protein”, “High Pressure” (ion routing multipole pressure = 20 mTorr, ion trap high pressure cell = 3.5e-5 Torr) and selected ion monitoring (SIM, ion trap isolation m/z 3200±800). AGC target was set to 10,000% (5e6 charges) with a maximum injection time of 750 ms, and the resolution setting was 7500 FWHM at m/z 200. The system was calibrated with FlexMix (Thermo Fisher Scientific). For nano-DESI top-down MS^n experiments, the orbitrap resolution was set up to 500,000 FWHM at m/z 200.

Results and Discussion

Nano-DESI-IRMPD MS of protein complexes in brain tissue.

Our previous work using nano-DESI for native MS of proteins from tissue has required high-end mass spectrometers for reasons including their high mass resolving power, ion/ion reactions and ion mobility separation.[1,22] An instrument suitable for native MS does not necessarily require a diverse set of functionality if its primary role is intact mass analysis for example, which piqued our interest in using the LTQ Velos Pro. We have found it necessary to use collisional activation on the high-performance instruments to generate declustered protein ions with usable signal quality. As a testbed for IR declustering on a lower cost instrument, we modified the LIT mass spectrometer to enable continuous irradiation of the ion beam during ion accumulation in the high-pressure cell, thereby declustering protein complexes from the solvent, detergent, and salts before m/z analysis. Overall, there was a stark improvement in mass spectra obtained with IR activation which promises to enable native MS from challenging sample environments on lower cost systems.

The system was tested by analysis of protein-ligand and protein-metal complexes from mouse brain. We sampled directly from the brainstem of a mouse brain tissue section from the G93A disease model of amyotrophic lateral sclerosis (ALS), in which a mutant form of the human protein SOD1 ($hSOD1^{G93A}$) is expressed. Without ion activation, and with in-source collisional activation, protein peaks were not detectable (**Figure 1a**). With IR activation (duty cycle 28%) the background chemical signal was reduced considerably, and protein signals were detectable (**Figure 1a**).

$hSOD1^{G93A}$ exhibits irregular metal ion binding (i.e., dimers binding 2, 3 metal ions) in addition to formation of the holo-form (binding 4 metal ions).[23] The characteristic pattern of three peaks separated by approx. 63 Da in mass is observable in the nano-DESI-IR-LIT mass spectrum (**Figure 1a & b**, dimers in charge states 11+, 10+ & 9+). Other protein complexes in this tissue were also detected, including Arf3 and Arf1 (both molecular weights correspond to the protein in complex with their endogenous ligand guanosine disphosphate, GDP) and carbonic anhydrase 2 bound to its endogenous Zn^{2+} cofactor. Mass spectra from the IR-LIT correlate with nano-DESI spectra obtained from the same mouse model on an Orbitrap Eclipse using in-source CID for declustering, and detection in the orbitrap mass analyser (**Figure 1c**, *Figure S2, Supporting Information*). The Orbitrap

Eclipse was developed with consideration for analysis of native MS of protein complexes.[12] The orbitrap analyzer demonstrated the highest performance with much higher signal intensity and low noise, in part owing to processing applied to the raw time-domain transient. The short time domain transient (here, transient duration = 16 ms) is beneficial for high signal-to-noise analysis of intact proteins at the expense of isotopic resolution.[24] The LIT also does not isotopically resolve the protein signals. Absence of isotopic resolution prevents charge assignment for a single peak, so spectrum post-processing, such as deconvolution of the charge state envelope, can be used to determine protein intact mass.[25-27] Deconvolution of orbitrap and LIT mass spectra was performed with UniDec[26] (settings in *Table S1, Supporting Information*) showing compatibility for mass measurement of proteins ~30 kDa, and with sufficient resolution to resolve the three metal-bound hSOD1^{G93A} complexes (**Figure 1d**, *Table S2, Supporting Information*).

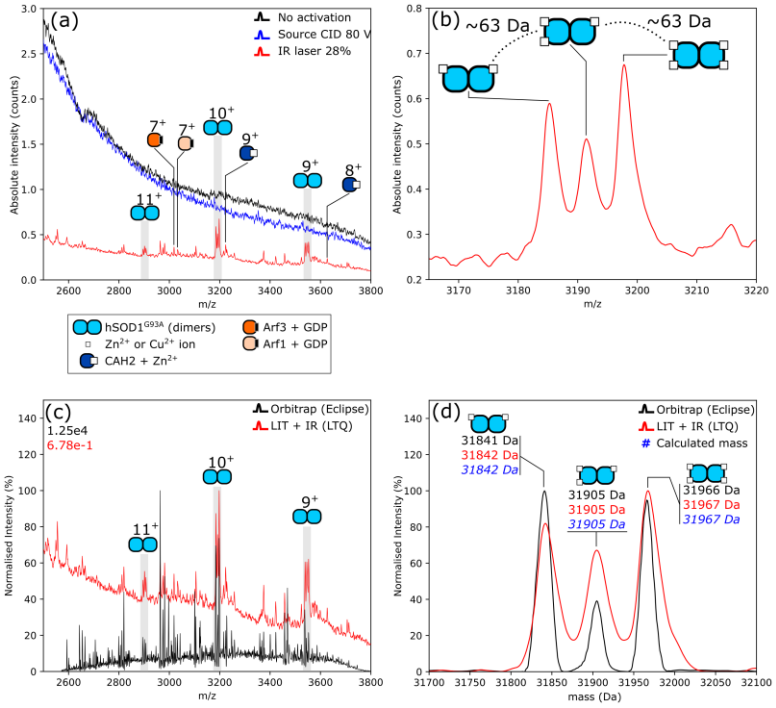

***Figure 1**: Nano-DESI mass spectra of transgenic mouse brainstem tissue (a) Analysis using the prototype IR-LIT platform: laser off (black trace), with source CID (80 V, blue trace) and with laser duty cycle set to 28% (red trace). (b) A distinctive triplet of signals, corresponding to endogenously occurring hSOD1^{G93A} homodimers in metal-deficient (2 and 3 metal ions) and holo (four metal ions) forms (charge states 11+, 10+ & 9+). (c) Nano-DESI mass spectra acquired on the Orbitrap Eclipse platform; orbitrap analyser (black trace, resolution setting = 7500 FWHM at m/z 200), and on the IR-LIT platform; linear ion trap (red trace). Spectra are intensity normalised, maximum signal intensity for each spectrum is shown in the top left. (d) Deconvoluted mass spectrum of hSOD1^{G93A} complexes using mass spectra from orbitrap (black) and LIT (IR-LIT, red). The calculated mass for each complex is shown in blue (additional information in Table S2, Supporting Information).*

Native nano-DESI-IR-LIT MS of proteins in eye lens tissue

The eye lens features abundant soluble proteins which form a variety of oligomeric complexes. The abundance of these complexes made them suitable to evaluate the effect of varied laser power. As with the brain tissue, protein ions were more effectively declustered by IR activation than with collisional activation, retain non-covalent interactions, and the chemical background is reduced.

Nano-DESI analysis of eye lens without source CID generated few protein signals and the noise level was high (**Figure 2a**). With the source CID potential set to 100 V (system maximum for LTQ Velos Pro), some abundant proteins were detected, e.g., γ-crystallin S (20.86 kDa monomer, W5QH67, *Figure S3 & Table S3, Supporting Information*). Peak broadening due to neutral loss fragmentation with this level of source CID was not detected for these proteins which indicates poor declustering performance with collisional activation rather than signal being absent because of protein fragmentation. Conversely, a laser duty cycle set to 30% effectively declustered protein ions and reduced chemical noise. Intact non-covalent protein complexes of β-B2-crystallin (46.42 kDa homodimer. B2 subunit; 23.21 kDa, identification reported previously[2]), β-B2/B3-crystallin (47.43 kDa heterodimer. B3 subunit; 24.21 kDa, *Figure S4 & Table S4, Supporting Information*) and β-B2/A2-crystallin (45.37 kDa heterodimer. A2; 22.16 kDa, *Figure S5, Supporting Information*), and galectin-related inter-fiber protein (GRIFIN[28], 15.83 kDa homodimer, *Figure S6, Supporting Information*) were detected in multiple charge states across the m/z range. Protein ions detected with the IR-LIT system correspond to high-resolution nano-DESI MS spectra obtained using the Orbitrap Eclipse (*Figure S7*, *Supporting Information*). Laser duty cycle exceeding 30% resulted in noticeable broadening of the peak owing to neutral loss fragmentation, indicating a threshold at which IR declustering becomes detrimental and operates in the IRMPD MS^n modality capable of covalent bond dissociation (**Figure 2b**).[29] Certainly, at 50% duty cycle IRMPD fragmentation was the dominant process, whereas the optimum for declustering was between 20-30%.

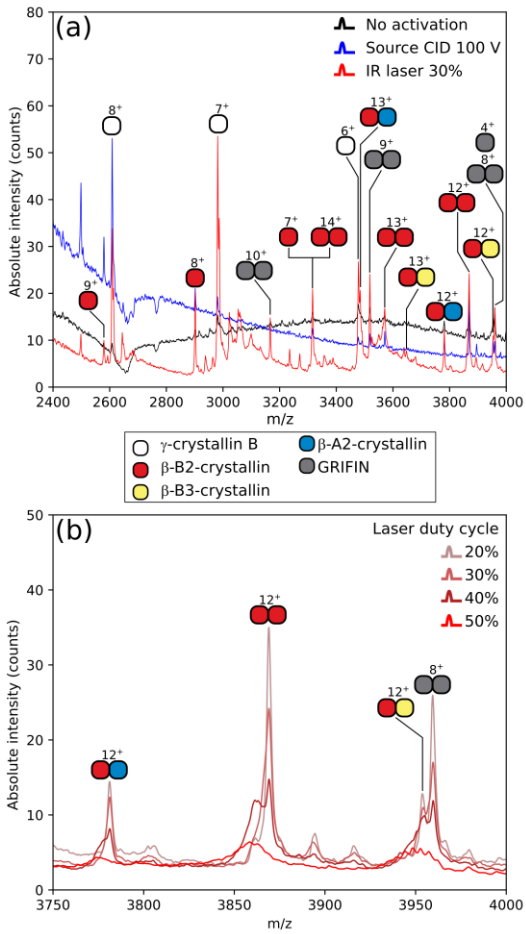

***Figure 2**: IR declustering of proteins sampled from sheep eye lens by nano-DESI. (a) laser off (black trace), with source CID (100 V, blue trace) and with laser on and duty cycle set to 30% (red trace). (b) The influence of laser duty cycle on intact protein signal. Higher duty cycle resulted in IRMPD fragmentation of the intact proteins, detectable as reduction of peak intensity and evidence of neutral loss.*

Perspective on linear ion traps for native MS.

The native MS community has called for lower cost instrumentation to enable wider adoption of native MS in structural biology.[3] Complexity contributes to the cost of high-performance mass spectrometers used for native MS: State-of-the-art QTOF and hybrid Orbitrap mass spectrometers require precision manufacturing and sophisticated ion optics. The vacuum requirements of these instruments are often extreme, e.g. Orbitrap analysers operating at approx. 1e-9 mBar, which is complicated by adjacent cells of higher pressure required for trapping and CID. Conversely, LITs are robust, sensitive, and versatile, yet are low-cost systems with moderate vacuum requirements (approx. 10e-5 mBar). Perhaps the biggest drawback is that LITs are not high-resolution accurate mass instruments which limits their use in top-down protein analysis; however, “high resolution” native MS is usually performed with low mass resolving powers on orbitrap systems, and desolvation/declustering limits the effective mass resolution on QTOF systems.[5] So for MS^1 experiments, high mass resolving power is not required for many native MS applications as long as declustering performance is sufficient. Furthermore, LITs featuring MS^n capability can still enable

protein complex stoichiometry to be investigated using resonant CID to eject monomers and the fragmentation could be performed with IRMPD at higher powers;[30] the use of IRMPD for this purpose would avoid the "1/3 cut-off rule" of resonant CID. Alternatively, beam-type CID could be implemented as in the recently released Stellar MS.[31]

The lack of isotopic resolution means other approaches are needed to determine protein charge (and therefore, mass) in complex samples. In addition to spectral deconvolution used here, experimental methods harnessing gas-phase charge reduction through proton transfer charge reduction (PTCR)[32], electron transfer without dissociation (ETnoD),[33-36] electron capture without dissociation/electron capture charge reduction (ECnoD/ECCR),[37-39] and multiply-charged ion attachment[40] enable intact mass measurement without requiring high mass resolving power.[41] These technologies were developed, or are compatible, with ion traps; ETD was an available option for the LTQ Velos platform used here. We extensively use PTCR on the Orbitrap Eclipse platform to perform quick intact mass measurement for proteins in tissue samples where high-resolution experiments are impractical.[42] PTCR has also proven powerful for targeted native protein MS imaging experiments where it enables charge-based separation of overlapping ions[43,44] and in the characterisation of native MS samples with extreme heterogeneity.[45] The main obstacle for adoption of charge reduction methods on a LIT is the requirement of higher m/z range than used here (limited to m/z 4000) to enable detection of the charge-reduced product ions, especially when establishing the mass of larger (>30 kDa) proteins. In fact, extended m/z range would be a necessary feature of any LIT system developed for low cost native MS but the many examples of custom ion traps meeting this criterion show the technological capability exists.[16,32,46-48]

Perspective on IR declustering

Integration of a CO_2 laser for declustering is not cost prohibitive; a 30 W laser costs approx. £10,000/$12,000. System design to ensure laser safety is essential, but this should be a straightforward obstacle to overcome as IRMPD has been commercialised successfully before. Native MS using nano-DESI perhaps stands to benefit from the implementation of IR declustering more than "standard" native MS; Nano-DESI is usually performed with flow rates in the 0.3 – 2 μL/min regime, far higher than the low nL/min flow rates of nanoESI commonly used for native MS.[49] On our setup the nano-DESI emitter has an internal diameter of approx. 75 μm, necessary to ensure aspiration of the aqueous solvent by the mass spectrometer vacuum, but this produces large initial droplets from which production of desolvated protein ions is inefficient.[50] Even on high performance QTOF mass spectrometers, it was necessary to retrofit the ion inlet with a heated transfer tube to improve desclustering for experiments using this ion source.[22] In comparison, static nanoESI for native MS is typically performed with emitters in the 1 μm internal diameter regime. Thus, IR

declustering could be an essential approach for improving native nano-DESI performance across a broad range of mass spectrometers.

Conclusions

Native MS using nano-DESI on a linear ion trap mass spectrometer has been demonstrated by integration of a 10.6 μm laser to enhance protein ion declustering. The prototype IR-LIT enabled intact mass analysis of protein complexes up to 47 kDa in molecular weight directly from tissue. While simple and effective, the prototype system lacks synchronization of laser on/off, laser power and mass spectrometer scan events, which if implemented would allow finer control of *where* ions are irradiated along the beamline and more sophisticated experiments (e.g., MS^n). Currently ions are irradiated continuously. Nevertheless, our system indicates a substantial improvement to protein ion declustering from the complex ion beam generated by nano-DESI of tissues. The IR laser-based declustering enables analysis of protein complexes by nano-DESI on a LIT mass spectrometer for the first time.

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Ethics Statement

All studies using mice were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and all procedures were carried out under a Home Office project licence, reviewed and approved by the local ethics committee (University of Sheffield Animal Welfare and Ethical Review Body). All animal maintenance and day to day care was carried out in line with Home Office Code of Practice for Housing and Care of Animals Used in Scientific Procedures.

References

(1) Hale, O. J.; Cooper, H. J. Native Mass Spectrometry Imaging of Proteins and Protein Complexes by Nano-DESI *Anal Chem* **2021**, *93*, 4619-4627, 10.1021/acs.analchem.0c05277
(2) Hale, O. J.; Cooper, H. J. Native Ambient Mass Spectrometry of an Intact Membrane Protein Assembly and Soluble Protein Assemblies Directly from Lens Tissue *Angew Chem Int Ed Engl* **2022**, *61*, e202201458, 10.1002/anie.202201458
(3) Harvey, S. R.; Gadkari, V. V.; Ruotolo, B. T.; Russell, D. H.; Wysocki, V. H.; Zhou, M. Expanding Native Mass Spectrometry to the Masses *J Am Soc Mass Spectrom* **2024**, *35*, 646-652, 10.1021/jasms.3c00352
(4) Lossl, P.; Snijder, J.; Heck, A. J. Boundaries of mass resolution in native mass spectrometry *J Am Soc Mass Spectrom* **2014**, *25*, 906-917, 10.1007/s13361-014-0874-3
(5) Tamara, S.; den Boer, M. A.; Heck, A. J. R. High-Resolution Native Mass Spectrometry *Chem Rev* **2022**, *122*, 7269-7326, 10.1021/acs.chemrev.1c00212
(6) Tolic, L. P.; Bruce, J. E.; Lei, Q. P.; Anderson, G. A.; Smith, R. D. In-trap cleanup of proteins from electrospray ionization using soft sustained off-resonance irradiation with fourier transform ion cyclotron resonance mass spectrometry *Anal Chem* **1998**, *70*, 405-408, 10.1021/ac970828c
(7) Gault, J.; Donlan, J. A.; Liko, I.; Hopper, J. T.; Gupta, K.; Housden, N. G.; Struwe, W. B.; Marty, M. T.; Mize, T.; Bechara, C.; Zhu, Y.; Wu, B.; Kleanthous, C.; Belov, M.; Damoc, E.; Makarov, A.; Robinson, C. V. High-resolution mass spectrometry of small molecules bound to membrane proteins *Nat Methods* **2016**, *13*, 333-336, 10.1038/nmeth.3771
(8) Sobott, F.; McCammon, M. G.; Hernandez, H.; Robinson, C. V. The flight of macromolecular complexes in a mass spectrometer *Philos Trans A Math Phys Eng Sci* **2005**, *363*, 379-389; discussion 389-391, 10.1098/rsta.2004.1498
(9) Benesch, J. L. Collisional activation of protein complexes: picking up the pieces *J Am Soc Mass Spectrom* **2009**, *20*, 341-348, 10.1016/j.jasms.2008.11.014
(10) Benesch, J. L.; Ruotolo, B. T.; Simmons, D. A.; Robinson, C. V. Protein complexes in the gas phase: technology for structural genomics and proteomics *Chem Rev* **2007**, *107*, 3544-3567, 10.1021/cr068289b
(11) Sobott, F.; Hernandez, H.; McCammon, M. G.; Tito, M. A.; Robinson, C. V. A tandem mass spectrometer for improved transmission and analysis of large macromolecular assemblies *Anal Chem* **2002**, *74*, 1402-1407, 10.1021/ac0110552
(12) Gault, J.; Liko, I.; Landreh, M.; Shutin, D.; Bolla, J. R.; Jefferies, D.; Agasid, M.; Yen, H. Y.; Ladds, M.; Lane, D. P.; Khalid, S.; Mullen, C.; Remes, P. M.; Huguet, R.; McAlister, G.; Goodwin, M.; Viner, R.; Syka, J. E. P.; Robinson, C. V. Combining native and 'omics' mass spectrometry to identify endogenous ligands bound to membrane proteins *Nat Methods* **2020**, *17*, 505-508, 10.1038/s41592-020-0821-0
(13) McGee, J. P.; Melani, R. D.; Goodwin, M.; McAlister, G.; Huguet, R.; Senko, M. W.; Compton, P. D.; Kelleher, N. L. Voltage Rollercoaster Filtering of Low-Mass Contaminants During Native Protein Analysis *J Am Soc Mass Spectrom* **2020**, *31*, 763-767, 10.1021/jasms.9b00037
(14) Little, D. P.; McLafferty, F. W. Infrared photodissociation of non-covalent adducts of electrosprayed nucleotide ions *J Am Soc Mass Spectrom* **1996**, *7*, 209-210, 10.1016/1044-0305(95)00680-X
(15) Gardner, M. W.; Brodbelt, J. S. Reduction of chemical noise in electrospray ionization mass spectrometry by supplemental IR activation *J Am Soc Mass Spectrom* **2009**, *20*, 2206-2210, 10.1016/j.jasms.2009.08.008
(16) Lee, K. W.; Harrilal, C. P.; Fu, L.; Eakins, G. S.; McLuckey, S. A. Digital ion trap mass analysis of high mass protein complexes using IR activation coupled with ion/ion reactions *Int J Mass Spectrom* **2020**, *458*, 116437, 10.1016/j.ijms.2020.116437

(17) Mikhailov, V. A.; Liko, I.; Mize, T. H.; Bush, M. F.; Benesch, J. L.; Robinson, C. V. Infrared Laser Activation of Soluble and Membrane Protein Assemblies in the Gas Phase *Anal Chem* **2016**, *88*, 7060-7067, 10.1021/acs.analchem.6b00645
(18) Lutomski, C. A.; El-Baba, T. J.; Hinkle, J. D.; Liko, I.; Bennett, J. L.; Kalmankar, N. V.; Dolan, A.; Kirschbaum, C.; Greis, K.; Urner, L. H.; Kapoor, P.; Yen, H. Y.; Pagel, K.; Mullen, C.; Syka, J. E. P.; Robinson, C. V. Infrared Multiphoton Dissociation Enables Top-Down Characterization of Membrane Protein Complexes and G Protein-Coupled Receptors *Angew Chem Int Ed Engl* **2023**, *62*, e202305694, 10.1002/anie.202305694
(19) Juliano, B. R.; Keating, J. W.; Ruotolo, B. T. Infrared Photoactivation Enables Improved Native Top-Down Mass Spectrometry of Transmembrane Proteins *Anal Chem* **2023**, *95*, 13361-13367, 10.1021/acs.analchem.3c02788
(20) Sisley, E. K.; Hale, O. J.; Styles, I. B.; Cooper, H. J. Native Ambient Mass Spectrometry Imaging of Ligand-Bound and Metal-Bound Proteins in Rat Brain *J Am Chem Soc* **2022**, *144*, 2120-2128, 10.1021/jacs.1c10032
(21) Mead, R. J.; Bennett, E. J.; Kennerley, A. J.; Sharp, P.; Sunyach, C.; Kasher, P.; Berwick, J.; Pettmann, B.; Battaglia, G.; Azzouz, M.; Grierson, A.; Shaw, P. J. Optimised and rapid pre-clinical screening in the SOD1(G93A) transgenic mouse model of amyotrophic lateral sclerosis (ALS) *PLoS One* **2011**, *6*, e23244, 10.1371/journal.pone.0023244
(22) Hale, O. J.; Hughes, J. W.; Cooper, H. J. Simultaneous spatial, conformational, and mass analysis of intact proteins and protein assemblies by nano-DESI travelling wave ion mobility mass spectrometry imaging *Int J Mass Spectrom* **2021**, *468*, 116656, ARTN 116656

10.1016/j.ijms.2021.116656
(23) Hale, O. J.; Wells, T. R.; Mead, R. J.; Cooper, H. J. Mass spectrometry imaging of SOD1 protein-metal complexes in SOD1G93A transgenic mice implicates demetalation with pathology *Nat Commun* **2024**, *15*, 6518, 10.1038/s41467-024-50514-7
(24) Makarov, A.; Denisov, E. Dynamics of ions of intact proteins in the Orbitrap mass analyzer *J Am Soc Mass Spectrom* **2009**, *20*, 1486-1495, 10.1016/j.jasms.2009.03.024
(25) Ferrige, A. G.; Seddon, M. J.; Green, B. N.; Jarvis, S. A.; Skilling, J. Disentangling Electrospray Spectra with Maximum-Entropy *Rapid Communications in Mass Spectrometry* **1992**, *6*, 707-711, DOI 10.1002/rcm.1290061115
(26) Marty, M. T.; Baldwin, A. J.; Marklund, E. G.; Hochberg, G. K.; Benesch, J. L.; Robinson, C. V. Bayesian deconvolution of mass and ion mobility spectra: from binary interactions to polydisperse ensembles *Anal Chem* **2015**, *87*, 4370-4376, 10.1021/acs.analchem.5b00140
(27) Cleary, S. P.; Li, H.; Bagal, D.; Loo, J. A.; Campuzano, I. D. G.; Prell, J. S. Extracting Charge and Mass Information from Highly Congested Mass Spectra Using Fourier-Domain Harmonics *J Am Soc Mass Spectrom* **2018**, *29*, 2067-2080, 10.1007/s13361-018-2018-7
(28) Ogden, A. T.; Nunes, I.; Ko, K.; Wu, S.; Hines, C. S.; Wang, A. F.; Hegde, R. S.; Lang, R. A. GRIFIN, a novel lens-specific protein related to the galectin family *J Biol Chem* **1998**, *273*, 28889-28896, 10.1074/jbc.273.44.28889
(29) Little, D. P.; Speir, J. P.; Senko, M. W.; O'Connor, P. B.; McLafferty, F. W. Infrared multiphoton dissociation of large multiply charged ions for biomolecule sequencing *Anal Chem* **1994**, *66*, 2809-2815, 10.1021/ac00090a004
(30) Madsen, J. A.; Gardner, M. W.; Smith, S. I.; Ledvina, A. R.; Coon, J. J.; Schwartz, J. C.; Stafford, G. C., Jr.; Brodbelt, J. S. Top-down protein fragmentation by infrared multiphoton dissociation in a dual pressure linear ion trap *Anal Chem* **2009**, *81*, 8677-8686, 10.1021/ac901554z
(31) Remes, P. M.; Jacob, C. C.; Heil, L. R.; Shulman, N.; MacLean, B. X.; MacCoss, M. J. Hybrid Quadrupole Mass Filter - Radial Ejection Linear Ion Trap and Intelligent Data Acquisition Enable Highly Multiplex Targeted Proteomics *bioRxiv* **2024**, 2024.2005.2031.596848, 10.1101/2024.05.31.596848
(32) Stephenson, J. L., Jr.; McLuckey, S. A. Ion/ion proton transfer reactions for protein mixture analysis *Anal Chem* **1996**, *68*, 4026-4032, 10.1021/ac9605657

(33) Lermyte, F.; Lacki, M. K.; Valkenborg, D.; Gambin, A.; Sobott, F. Conformational Space and Stability of ETD Charge Reduction Products of Ubiquitin *J Am Soc Mass Spectrom* **2017**, *28*, 69-76, 10.1007/s13361-016-1444-7
(34) Lermyte, F.; Williams, J. P.; Brown, J. M.; Martin, E. M.; Sobott, F. Extensive Charge Reduction and Dissociation of Intact Protein Complexes Following Electron Transfer on a Quadrupole-Ion Mobility-Time-of-Flight MS *J Am Soc Mass Spectrom* **2015**, *26*, 1068-1076, 10.1007/s13361-015-1124-z
(35) Jhingree, J. R.; Beveridge, R.; Dickinson, E. R.; Williams, J. P.; Brown, J. M.; Bellina, B.; Banan, P. E. Electron transfer with no dissociation ion mobility-mass spectrometry (ETnoD IM-MS). The effect of charge reduction on protein conformation *Int J Mass Spectrom* **2017**, *413*, 43-51, 10.1016/j.ijms.2016.08.006
(36) Kuo, C. Y.; Zheng, Y. F.; Wang, W. C.; Toh, J. T.; Hsu, Y. M.; Chien, H. J.; Chang, C. J.; Lai, C. C. Direct Identification of Intact Proteins Using a Low-Resolution Mass Spectrometer with CID(n)/ETnoD *J Am Soc Mass Spectrom* **2024**, *35*, 1507-1515, 10.1021/jasms.4c00108
(37) Zubarev, R. A.; Kelleher, N. L.; McLafferty, F. W. Electron capture dissociation of multiply charged protein cations. A nonergodic process *J Am Chem Soc* **1998**, *120*, 3265-3266, DOI 10.1021/ja973478k
(38) Le Huray, K. I. P.; Woerner, T. P.; Moreira, T.; Reinhardt-Szyba, M.; Devine, P. W. A.; Bond, N. J.; Fort, K. L.; Makarov, A. A.; Sobott, F. To 200,000 m/z and beyond: native electron capture charge reduction mass spectrometry deconvolves heterogeneous signals in large biopharmaceutical analytes *bioRxiv* **2024**, 2024.2002.2019.581059, 10.1101/2024.02.19.581059
(39) Shaw, J. B.; Harvey, S. R.; Du, C.; Xu, Z.; Edgington, R. M.; Olmedillas, E.; Saphire, E. O.; Wysocki, V. H. Protein complex heterogeneity and topology revealed by electron capture charge reduction and surface induced dissociation *bioRxiv* **2024**, 2024.2003.2007.583498, 10.1101/2024.03.07.583498
(40) Abdillahi, A. M.; Lee, K. W.; McLuckey, S. A. Mass Analysis of Macro-molecular Analytes via Multiply-Charged Ion Attachment *Anal Chem* **2020**, *92*, 16301-16306, 10.1021/acs.analchem.0c04335
(41) Karch, K. R.; Snyder, D. T.; Harvey, S. R.; Wysocki, V. H. Native Mass Spectrometry: Recent Progress and Remaining Challenges *Annu Rev Biophys* **2022**, *51*, 157-179, 10.1146/annurev-biophys-092721-085421
(42) Hale, O. J.; Hughes, J. W.; Sisley, E. K.; Cooper, H. J. Native Ambient Mass Spectrometry Enables Analysis of Intact Endogenous Protein Assemblies up to 145 kDa Directly from Tissue *Anal Chem* **2022**, *94*, 5608-5614, 10.1021/acs.analchem.1c05353
(43) Hale, O. J.; Cooper, H. J.; Marty, M. T. High-Throughput Deconvolution of Native Protein Mass Spectrometry Imaging Data Sets for Mass Domain Analysis *Anal Chem* **2023**, *95*, 14009-14015, 10.1021/acs.analchem.3c02616
(44) Illes-Toth, E.; Hale, O. J.; Hughes, J. W.; Strittmatter, N.; Rose, J.; Clayton, B.; Sargeant, R.; Jones, S.; Dannhorn, A.; Goodwin, R. J. A.; Cooper, H. J. Mass Spectrometry Detection and Imaging of a Non-Covalent Protein-Drug Complex in Tissue from Orally Dosed Rats *Angew Chem Int Ed Engl* **2022**, *61*, e202202075, 10.1002/anie.202202075
(45) Schachner, L. F.; Mullen, C.; Phung, W.; Hinkle, J. D.; Beardsley, M. I.; Bentley, T.; Day, P.; Tsai, C.; Sukumaran, S.; Baginski, T.; DiCara, D.; Agard, N. J.; Masureel, M.; Gober, J.; ElSohly, A. M.; Melani, R.; Syka, J. E. P.; Huguet, R.; Marty, M. T.; Sandoval, W. Exposing the molecular heterogeneity of glycosylated biotherapeutics *Nat Commun* **2024**, *15*, 3259, 10.1038/s41467-024-47693-8
(46) Patil, A. A.; Liu, Z. X.; Chiu, Y. P.; Lai, T. K. L.; Chou, S. W.; Cheng, C. Y.; Su, W. M.; Liao, H. T.; Agcaoili, J. B. A.; Peng, W. P. Development of a linear ion trap mass spectrometer capable of analyzing megadalton MALDI ions *Talanta* **2023**, *259*, 124555, 10.1016/j.talanta.2023.124555
(47) Prentice, B. M.; McLuckey, S. A. Analysis of high mass-to-charge ions in a quadrupole ion trap mass spectrometer via an end-cap quadrupolar direct current downscan *Anal Chem* **2012**, *84*, 7562-7569, 10.1021/ac301741a

(48) Zhang, H.; Jia, H.; Hong, J.; Zhang, M.; Jiang, T.; Xu, W. Development of a High-Field "Brick" Mass Spectrometer with Extended Mass Range and Capable of Characterizing Native Proteins *Anal Chem* **2023**, *95*, 13503-13508, 10.1021/acs.analchem.3c01769
(49) Wilm, M.; Mann, M. Analytical properties of the nanoelectrospray ion source *Anal Chem* **1996**, *68*, 1-8, 10.1021/ac9509519
(50) Hsu, C. Y.; Prabhu, G. R. D.; Chang, C. H.; Hsu, P. C.; Buchowiecki, K.; Urban, P. L. Are Most Micrometer Droplets (>10 mum) Wasted in Electrospray Ionization? An Insight from Real-Time High-Speed Imaging *Anal Chem* **2023**, *95*, 14702-14709, 10.1021/acs.analchem.3c02799