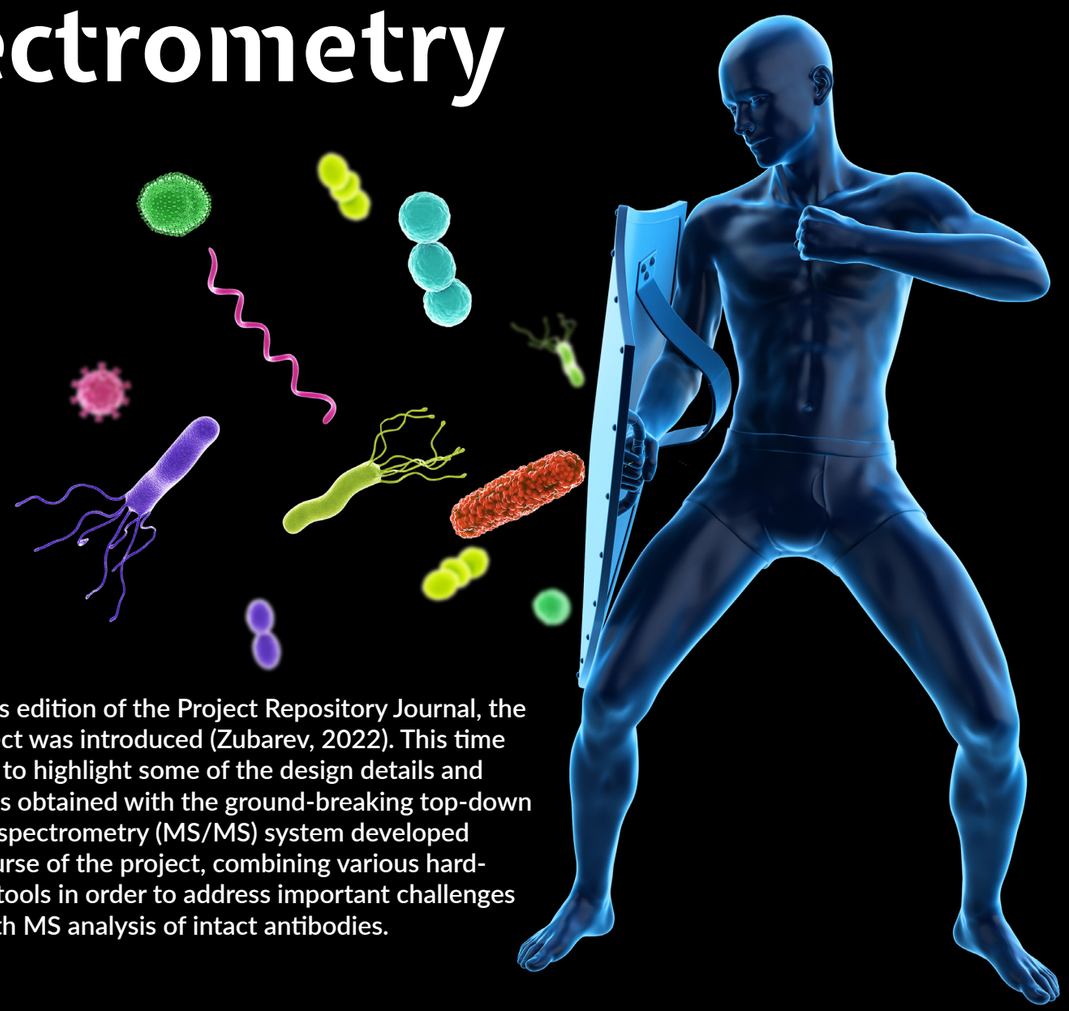
# The TopSpec system: new levels in antibody characterisation by top-down mass spectrometry



In the previous edition of the Project Repository Journal, the TopSpec project was introduced (Zubarev, 2022). This time we would like to highlight some of the design details and exciting results obtained with the ground-breaking top-down tandem mass spectrometry (MS/MS) system developed during the course of the project, combining various hard- and software tools in order to address important challenges associated with MS analysis of intact antibodies.

Adobe Stock © *SciePro*





## Omnitrap platform

At the core of the TopSpec project is the Omnitrap platform, developed and built by Fasmatech and available for Thermo Scientific™, Q-Exactive™ and Exploris™ 480 instruments (Papanastasiou *et al.*, 2022). The Omnitrap device adds a plethora of ion activation and fragmentation techniques to the system, vastly expanding its capabilities in probing intact antibody ions for enhanced amino acid sequence and modification information. The unique design of the Omnitrap platform allows for multi-stage ion activation with unprecedented flexibility.

The Omnitrap platform consists of nine consecutive quadrupole segments, labelled Q1–Q9, arranged in a linear configuration and grouped to form three principal trapping regions for processing ions. The quadrupole electrode geometry in terms of inscribed radius, segment length and machining tolerance was designed for optimal performance of the diverse set of functions available throughout the ion trap volume. The hyperbolic surface of the electrodes is wire-eroded to an accuracy of ~5 µm, while the overall construction is assembled with an accuracy of <20 µm.

Radial confinement of the ions is accomplished by a pair of opposite-phase rectangular waveforms, typically operated at 250 $V_{0p}$. The frequency range of the waveforms extends from 100 kHz to 2.5 MHz, and phase-coherent frequency jumps can be performed throughout this frequency range for optimal ion isolation or activation. Ions are confined axially by switching DC voltages applied to individual segments. The number of DC levels made available to each segment during the course of an experiment is limitless, offering an unrestricted number of DC profiles that can be applied sequentially for axial transfer and processing of the ions. Dipolar resonance excitation is available in segments Q2 and Q5 through the application of single frequency or notched AC waveforms to collisionally activate or select ions for tandem MSn (MS, MS/MS, MS/MS/MS etc.) workflows, respectively. Single precursor ion isolation is exercised by applying a resolving DC component to the pole electrodes of segments Q2 and Q5.

Elevated vacuum pressure levels are desirable, both during axial injection of ions into the ion trap from an external source during collisional activation (CA), and for efficient thermalisation of ions during their transfer between the principal trapping regions. For this purpose, a pair of pulse valves is employed for injecting gas pulses into the trapping volume with a maximum repetition rate of 25 Hz. A pressure level of >$10^{-2}$ mbar is maintained for ~20 ms during pulsed introduction of argon and nitrogen gases, while pressure relaxes to background levels ($10^{-5}$ mbar) within a period of ~75 ms.

## Activation techniques currently available on the Omnitrap platform

**Slow heating collision-induced dissociation (CID)** using multiple excitation frequencies in resonance with precursor ions distributed over different charge states is available in segments Q2 and Q5.

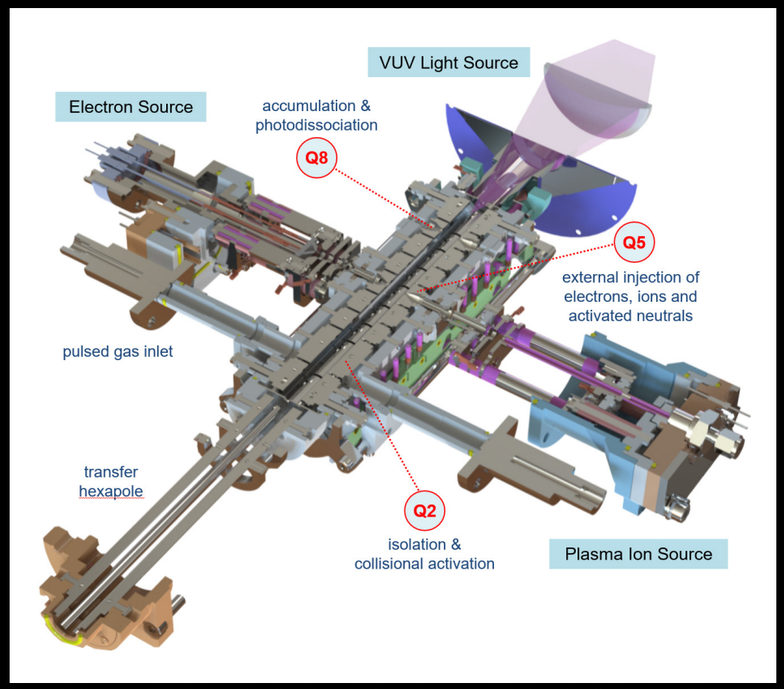



*Figure 1: Cross-sectional view of the Omnitrap platform. Ions are guided from the Orbitrap™ MS instrument to the Omnitrap platform by the transfer hexapole. Two pulsed gas inlets are connected to Q2, providing fast gas pulses for collisional cooling or collision-induced activation of ions. An electron source and a plasma ion source attached symmetrically on either side of segment Q5 are used for external injection of charged particles for ion-particle reactions. Optical access is provided through 2.0 mm holes in segment Q8, as well as along the axis of the ion trap through a viewport disposed at the back end of the device.*

**Beam-type CID** is implemented by transferring ions from one trapping segment to another along the axis of the ion trap with kinetic energies of >10 eV. The degree of acceleration is controlled by the gradient of the DC profile established across neighbouring segments. Slow heating and beam-type CID are performed during a gas pulse transient, permitting a high level of control and optimisation of these processes.

**Electron-based dissociation** can be employed in Q5 during trapping via external injection of electrons generated by a hot filament and accelerated up to 1 keV. In the low energy regime (~0 eV), electron capture dissociation (ECD) is efficiently accomplished, while in the higher energy regime, electron meta-ionisation (EmI) and electron-induced dissociation (EID) phenomena are observed. EmI in particular leads to the detachment of one or more electrons from (de)protonated ions producing stable radical species, which offers a

unique opportunity for investigating fundamental aspects of the rich ion chemistry manifested in radical-driven dissociation processes and also for enhancing sequence coverage.

**Ion activation dissociation (IAD)** has also been implemented successfully. An energetic beam of ions from a low-temperature plasma is injected through segment Q5. The plasma medium is hydrogen gas producing a pulsed, high-density beam of energetic $H^+$, $H_2^+$, and $H_3^+$ ions used for ion activation dissociation. Experiments with small- to medium-size proteins produce rich fragmentation mass spectra characterised by strong meta-ionisation and charge reduction effects of the precursor ions and with all types of primary fragment ions observed.

**Photodissociation** by simultaneous or sequential irradiation with two laser sources is readily available with viewports located on either side of segment Q8 and through the back end of the device. Axial injection of lasers also allows photoactivated ExD experiments to be executed directly in segment Q5.

Figure 1 shows a cross-sectional view of the Omnitrap platform connected in series with a transfer hexapole ion guide, connecting the Orbitrap™ mass spectrometer.

## Possibilities when combining activation methods

As an example of what can be achieved with combined activation methods, Figure 2 shows top-down mass spectra of Herceptin® (Genentech), a monoclonal antibody used to treat breast and stomach cancer. The name of the game here is the so-called sequence coverage, which represents the level of information on the sequential arrangement of the different amino acids along the protein backbone. The more information on protein fragments can be extracted from the spectrum, the higher the sequence coverage and the more likely it is that the entire structure of the protein can be reconstructed. The signal-to-noise ratio of isotopically resolved fragments produced by MS2 CID of the intact non-reduced charge state 49+ ions was enhanced

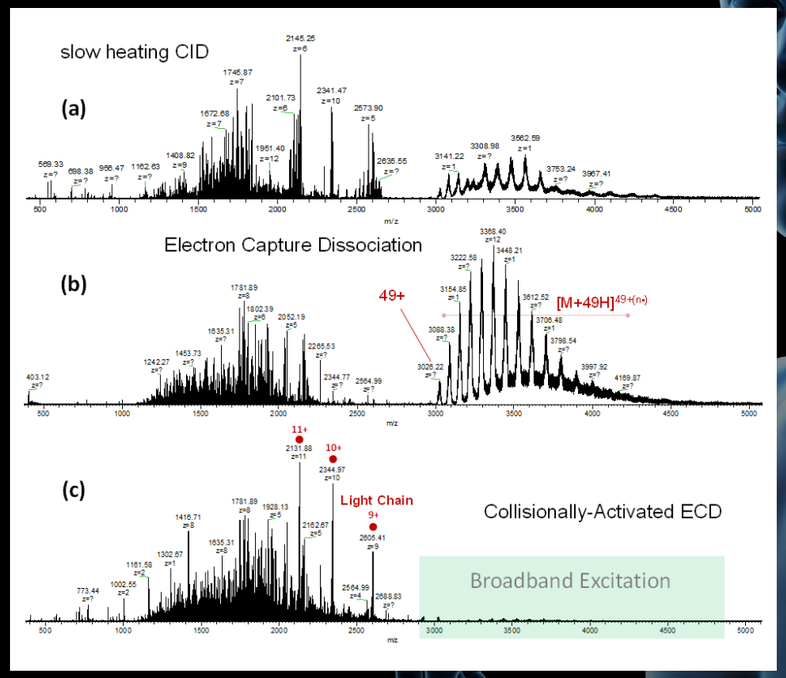



*Figure 2: Top-down mass spectra for Herceptin® obtained on a Thermo Scientific Q-Exactive Plus instrument equipped with an Omnitrap platform. (a) slow heating CID with broadband excitation of the intact non-reduced 49+ charge state precursor ion (b) ECD of the same precursor yields charged-reduced radical species (c) these dissociate into a wealth of information-rich fragments by collisional activation of the charge-reduced radical species.*

by broadband excitation of the higher-m/z first-generation fragments (Figure 2a), increasing the amount of identified fragments by 52 per cent. In another experiment, ECD of the same precursor yields charge-reduced radical species (Figure 2b). In a second step applied during the same scan, these radical species were collisionally activated using broadband excitation, increasing the information density of the fragments even further (Figure 2c). One of the most exciting aspects of this achievement is that these high-efficiency fragmentation methods are available on liquid chromatography (LC) time scales, offering great potential for integration of the system in current standard analytical workflows.

The MS/MS results presented in Figure 2 already provide a detailed look into the antibody structure. When deeper and more detailed insights are required, the performance and productivity of the system can be further enhanced by improved data acquisition processing approaches, another key objective of the TopSpec project. To this end, TopSpec partners led by Spectroswiss, an SME from Lausanne, Switzerland, developed and evaluated a high-performance data acquisition system, the FTMS Booster TD, including data processing software, Peak-by-Peak BioPharma.

The FTMS Booster TD is based on new-generation data acquisition electronics architecture. This architecture allows extensive real-time data processing, enabling the acquisition of an unreduced raw signal from the system. This signal is generated by ions as they describe oscillatory orbits inside the Orbitrap™ analyser during detection. The signal consists of many superimposed sinewaves, forming a transient which is deconvolved using a Fourier transform (FT) algorithm, where each discrete frequency component represents a mass-to-charge value of ions in the trap. FTMS Booster TD prototype systems were interfaced with several Omnitrap systems equipped with different versions of the Orbitrap™ analyser—a Q-Exactive





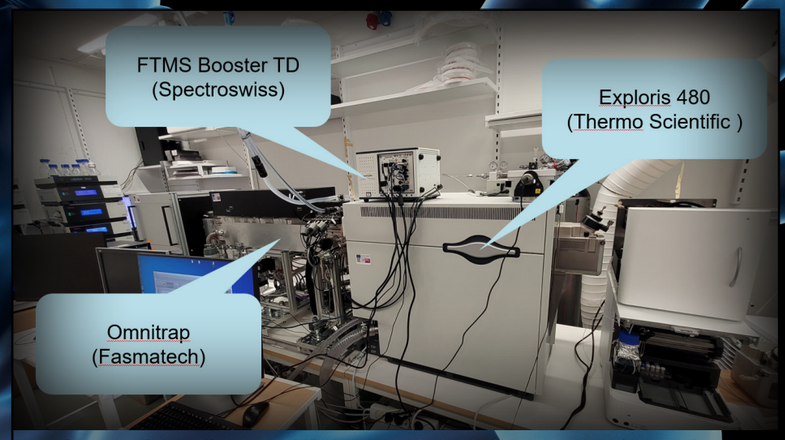



*Figure 3: A powerful triad for tandem MS of intact antibodies and beyond—state-of-the-art Exploris 480 Orbitrap™ mass spectrometer, Omnitrap platform and FTMS Booster TD prototype. This unique combination of technologies is installed at the Karolinska Institutet in Stockholm, Sweden.*

Plus (at Fasmatech, Athens, Greece), a Q-Exactive HF (at Institut Pasteur, Paris, France) and an Exploris 480 (at Karolinska Institutet, Stockholm, Sweden). The latter configuration is depicted in Figure 3 and represents an MSn system produced during the course of the TopSpec project that is truly beyond the state of the art.
The added ability to acquire the unreduced raw data allows to further enhance the analytical characteristics of the system, resulting in higher achievable resolution, sensitivity and mass accuracy. These analytical performance benefits translate into applied (biological) benefits, such as more complete—and more confident—primary structure information on antibodies. In addition to antibody sequencing, processing the unreduced data benefits other antibody analysis needs. For example, as demonstrated in the TopSpec project, rational processing of raw ion signals can help to resolve the extreme complexity of antibody-drug conjugate (ADC) mass spectra, as shown in Figure 4 (Nagornov, 2021).

The proteoform integration approach, described in Figure 4, facilitates analysis of the drug-to-antibody ratios (DARs)—a key critical quality attribute for antibody characterisation in drug discovery and of particular importance to biopharma applications. This approach serves as an example of the power of unreduced data processing for the structural analysis of antibodies. The proteoform integration and other advanced MS and MS/MS approaches are now offered by the TopSpec systems to tackle the most challenging tasks in the antibody structure analysis.

The main outcome of the TopSpec project, the TopSpec system itself, represents a highly versatile and high-performance tool for the structural analysis of intact

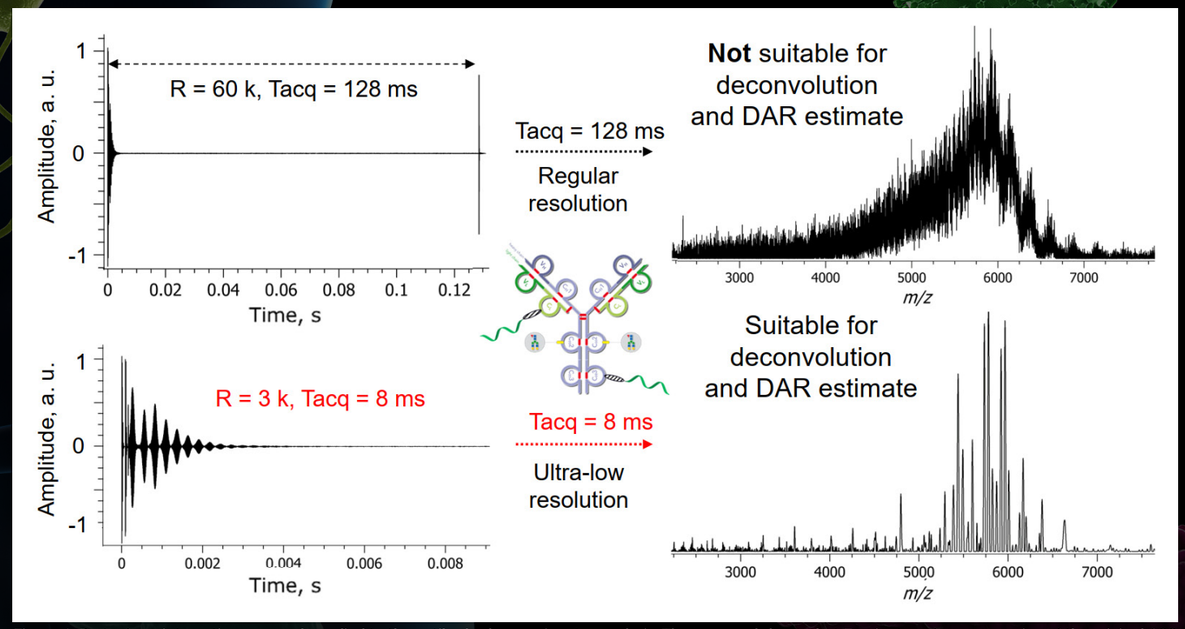



*Figure 4: Proteoform integration approach applied to the antibody-drug conjugates analysis. The top panel shows that Fourier transform processing of the original 128 ms time-domain signal acquired using the FTMS Booster TD on a Q-Exactive HF Orbitrap™ instrument leads to a very complex mass spectrum that shows interference of many individual proteoforms. This complexity presents a challenge for further analysis, such as deconvolution and extraction of the drug-to-antibody ratios (DARs). Instead, having access to the time-domain raw signal creates an opportunity to process only the first 8 ms part of the signal and generate a much more simple mass spectrum with proteoforms integrated. The lower spectral complexity achieved in this approach is suitable for further data processing and a direct DAR estimation.*

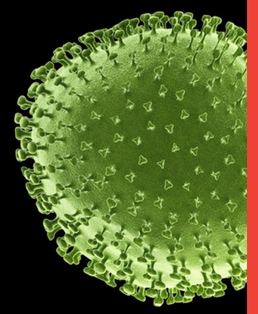
antibodies and other biomolecules. Combined with the applications developed at Karolinska Institute and Institut Pasteur, and the LC-compatible timescale, the system represents the new state-of-the-art analytical platform for the comprehensive characterisation of intact antibodies from complex samples. The current interest in the system from the structural biochemistry community, which has resulted in a number of sales already, is a testament to that.

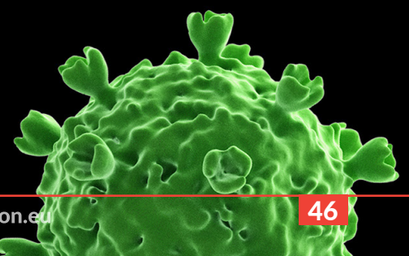
## References


Papanastasiou, D., Kounadis, D., Lekkas, A., Orfanopoulos, I., Mpozatzidis, A., Smyrnakis, A., Panagiotopoulos, E., Kosmopoulou, M., Reinhardt-Szyba, M., Fort, K., Makarov, A. and Zubarev, R.A. (2022) 'The Omnitrap Platform: A Versatile Segmented Linear Ion Trap for Multidimensional Multiple-Stage Tandem Mass Spectrometry', *Journal of the American Society for Mass Spectrometry*, 2022, 33(10), pp. 1990–2007. doi: 10.1021/jasms.2c00214.

Nagornov, K.O., Gasilova, N., Kozhinov, A.N., Virta, P., Holm, P., Menin, L., Nesatyy, V.J. and Tsybin, Y.O. (2021) 'Drug-to-Antibody Ratio Estimation via Proteoform Peak Integration in the Analysis of Antibody–Oligonucleotide Conjugates with Orbitrap™ Fourier Transform Mass Spectrometry', *Analytical Chemistry*, 2021, 93, pp. 12930–12937. doi: 10.1021/acs.analchem.1c02247.

Zubarev, R. (2022) 'Antibody profiling; from science fiction to reality', *Project Repository Journal*, 15, pp. 38–41. doi: 10.54050/PRj1519532.



## PROJECT NAME

TopSpec

## PROJECT SUMMARY


In the TopSpec project, tools to establish the sequence repertoire of human antibodies and their respective antigens have been developed to better understand how the human organism defends itself against diseases. Results obtained confirm that this is a breakthrough technology that will revolutionise academic, clinical and industrial proteomics and dramatically advance the development of antibody- and protein-based therapeutics.


## PROJECT PARTNERS

Karolinska Institute, Sweden; Fasmatech, Greece; Thermo Fisher Scientific, Germany; Spectroswiss, Switzerland; BioMotif, Sweden; Nottingham Trent University, United Kingdom; Institut Pasteur, France; and Spectrometry Vision, The Netherlands.

## PROJECT LEAD PROFILE

Roman Zubarev is a professor of medicinal proteomics in the Department of Medical Biochemistry and Biophysics at the Karolinska Institutet. His research focuses on the use of mass spectrometry in biology and medicine. In 1997 Zubarev discovered the phenomenon of electron-capture dissociation (ECD) of polypeptides. He later developed ECD and other ion-electron reactions as analytical techniques in Odense and Uppsala.

## PROJECT CONTACTS


**Prof. Dr Roman Zubarev**
Biomedicum, 9A, Floor 9, Solnavägen 9,
171 65 Solna, Sweden

☎ +46 8 524 875 94
✉ roman.zubarev@ki.se
https://ki.se/en/mbb/roman-zubarev-group
https://topspec.ki.se/



## FUNDING

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No. 829157.