A large, abstract visualization of mass spectrometry data. It features a dark blue background with numerous vertical lines of varying heights and colors (yellow, green, red, white, cyan) representing ion signals. Overlaid on this are several colorful, interconnected lines and polygons in shades of yellow, green, red, white, and cyan, suggesting a complex network or pathway of data points.

- **PASEF™ on a timsTOF Pro defines new performance standards for shotgun proteomics with dramatic improvements in MS/MS data acquisition rates and sensitivity**

Trapped ion mobility spectrometry coupled with quadrupole time-of-flight mass spectrometry (timsTOF Pro) offers a unique dimension of characterization and separation in complex mixtures. The timsTOF Pro instrument also enables the previously introduced “Parallel Accumulation Serial Fragmentation” (PASEF) method.<sup>[1]</sup>

In the first publication on PASEF, a proof of principle experiment was performed using direct infusion, which led to the prediction that PASEF could achieve dramatic improvements in

MS/MS data acquisition rates in shotgun proteomics experiments with improved sensitivity, with the possibility to address >170,000 precursors in a single shotgun proteomics experiment.

This predicted performance far exceeded the capabilities of instruments available for shotgun proteomics, providing results with greater specificity and biological relevance. In this application note,

*Keywords:*  
Trapped ion mobility spectrometry (TIMS), Parallel Accumulation Serial Fragmentation (PASEF)

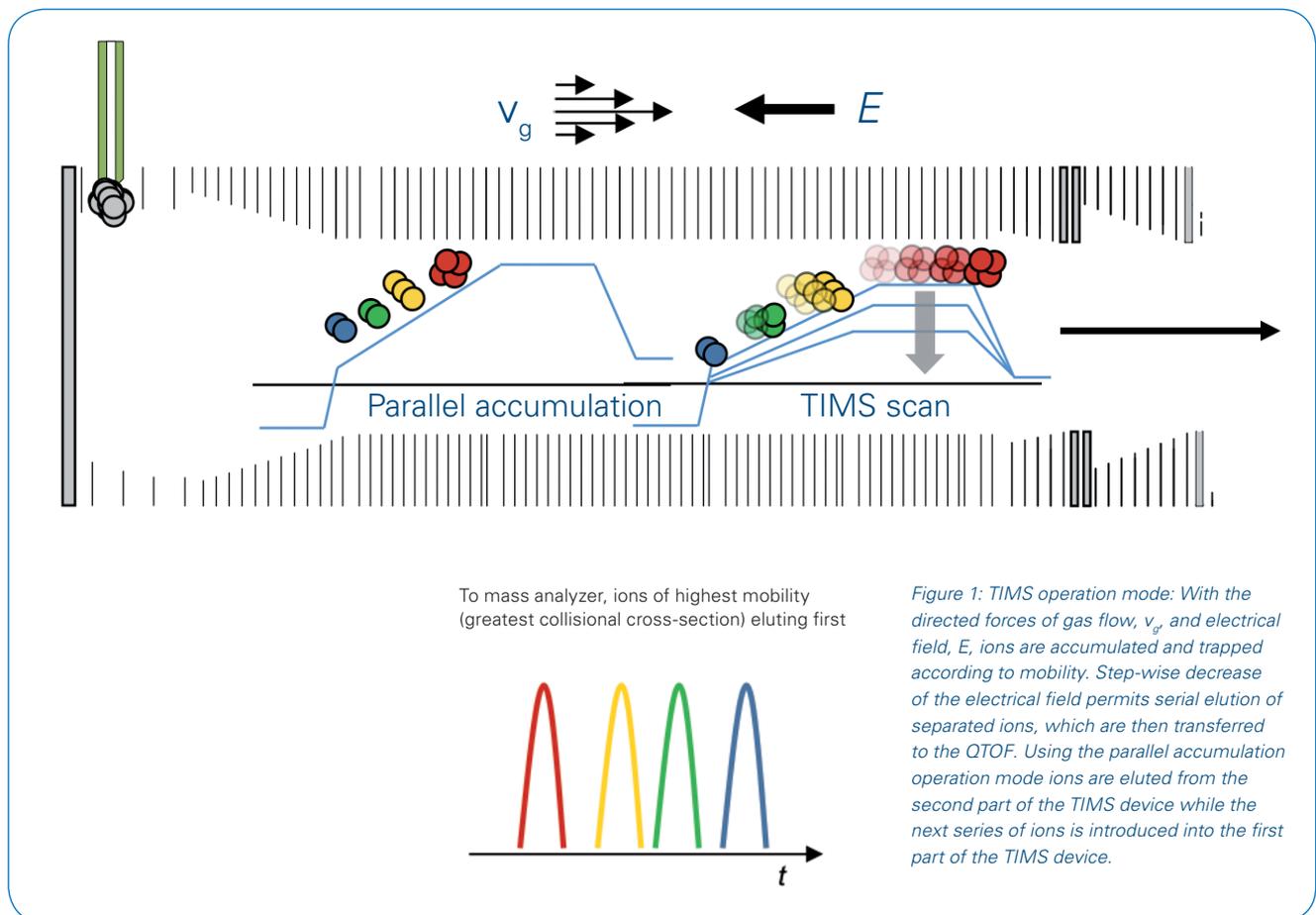
we demonstrate that with further hardware and software developments, the promise of PASEF has now been realized, and that data dependent shotgun proteomics experiments performed using PASEF can address 166,000 independent precursors from a HeLa digest using a 90 min gradient. The dual concentration effects from the separations by chromatography and trapped ion mobility spectrometry provide a gain of at least a factor of 10 in sensitivity and using just 200 ng of HeLa digest is sufficient to exceed what previously required 1-2  $\mu\text{g}$  of sample.<sup>[2]</sup> The 166,000 addressed precursors result in more than 35,000 unique peptide identifications and 5,500 protein identi-

fications from 200 ng of HeLa digest injected on column. The benefits of this approach for proteomics applications are demonstrated using a timsTOF Pro instrument (Bruker Daltonics) with the PASEF acquisition mode connected to a nanoElute UHPLC (Bruker Daltonics).

## Introduction

Multi-dimensional separations are necessary to obtain a more complete and accurate view of the content of complex proteomics samples in both discovery and targeted (quantitative) workflows, and ion mobility has been used as an additional separation device in commercial mass spectro-

meters since the 1960s. More recently trapped ion mobility spectrometry (TIMS) has been introduced and coupled to QTOF mass spectrometers. In TIMS, ions are propelled through a TIMS tunnel by a gas flow. An electric field traps each ion at the position where the push that it experiences from the gas flow matches the force of the electric field, resulting in separation by mobility, a function of their three-dimensional size and charge in the gas phase (Figure 1). Ramping down the electrical field allows a selective release of ions from the TIMS tunnel according to their ion mobility ( $\Omega/z$ ), where  $\Omega$  is the ion's collisional cross section (CCS). In contrast to traditional drift



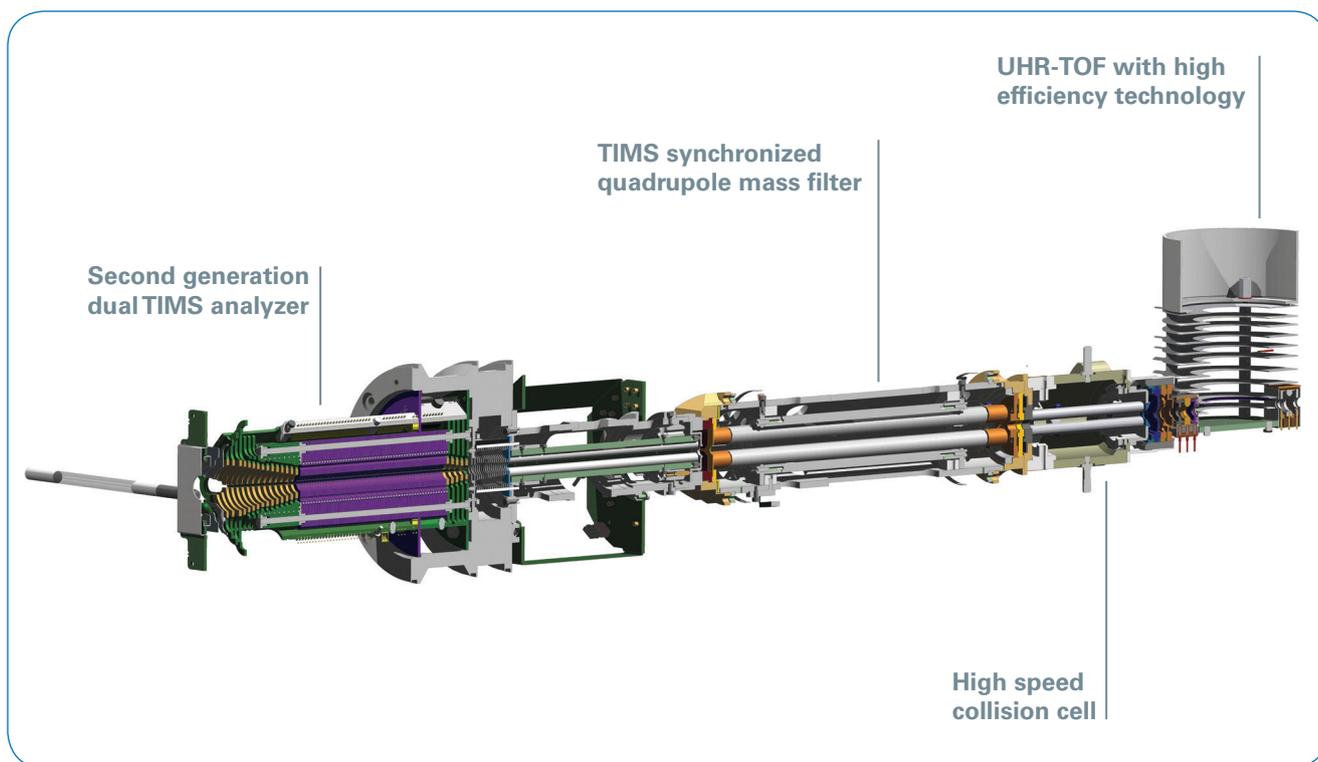


Figure 2. Ion optics of the timsTOF Pro instrument including a dual TIMS analyzer and QTOF mass spectrometer.

tube separation by size-to-charge, the larger ions elute first, followed by ions of decreasing cross section. In this study, a timsTOF Pro instrument (Bruker Daltonics) was used, with the eluted ions further analyzed via quadrupole time-of-flight mass spectrometry (Figure 2). The spectra generated during the TIMS elution cycle is termed a TIMS scan. The two dimensional separation of ions ( $1/K_0$  and  $m/z$ ) summed across a TIMS scan can be visualized as a TIMS MS heat map, with mobility and  $m/z$  as the y and x axes, respectively (Figure 3).

Likewise, particular ions may be isolated in three dimensions according to their liquid chromatography (LC) retention time, TIMS elution time and  $m/z$  (in this study, by a quadrupole filter) and subjected to collision induced dissociation. In the generated

PASEF MS/MS heat map, fragments may be easily aligned with their precursors in the TIMS MS heat map, given that they appear at the same ion mobility (Figure 3).

While this orthogonal separation can provide a deeper look into complex proteomics samples, its utility for peptide identification may be challenged in many instruments due to insufficient sensitivity resulting from low ion abundance (leading to weak spectra from subsequent collision induced fragmentation), as well as high scanning speeds required to be compatible with the typical 100 ms TIMS scan times. Through the implementation of the "Parallel Accumulation Serial Fragmentation" (PASEF) method on a fast scanning QTOF instrument, fragment ion spectra from isolated precursor ions may be acquired more

rapidly without sacrificing spectral quality. By using a dual TIMS analyzer, ions may be nearly continuously queued for accumulation, sorting and elution by mobility, allowing a duty cycle near 100% (Figure 1). During the timeframe of each TIMS scan, the quadrupole switches its (mass) isolation position several times, enabling the collection of MS/MS spectra from multiple precursors (Figure 4). Spectra for low abundant precursors may be summed to improve signal intensities to increase both the number and confidence of peptide identifications. The use of ion mobility separation prior to MS/MS results in improved signal-to-noise ratios, as the targeted ion species is time focused and subsequently transferred to the collision cell only during the periods of time they elute from the TIMS device. Additionally, by virtue of this additional

dimension of separation, precursors of the same  $m/z$  that co-elute from the LC may be distinguishable which is not possible with mass based selection alone.

#### Two dimensional ( $m/z$ -mobility) data dependent precursor acquisition

Another challenge in fast data acquisition is the computational time required to determine the precursors that will be selected for MS/MS. Maximizing the number of quality MS/MS spectra, an essential step in successful shotgun proteomics experiments, requires consideration of relative ion intensities and resolutions, the latter of which is increased (thus increasing the number of viable precursors) with the second dimension of separation. With higher speed data acquisition capabilities, lower intensity targets may be fragmented multiple times. A rather simple and rapid algorithmic approach<sup>[3]</sup> was developed to efficiently schedule the subsequent fragmentation scans based on the initial detection of precursor ions during the MS scan in both the  $m/z$  and mobility dimensions. Even in very complex proteomics samples, computational time does not exceed 1 ms, easily supporting high throughput data collection.

## Experimental

A complex tryptic peptide mixture derived from HeLa cells was diluted with 0.1% formic acid (FA) in water to a concentration of 200 ng/ $\mu$ L. A nanoELute UHPLC (Bruker Daltonics) was coupled to the timsTOF Pro mass spectrometer. The peptide mixture (200 ng) was loaded onto a C18 column (25 cm X 75  $\mu$ m 1.6  $\mu$ m, IonOpticks, Australia). Chromatographic separation was carried out using a linear gradient of 2-37% of buffer B (0.1% FA in ACN) at a flow rate of 400 nL/min over 90 min. MS data was collected over a  $m/z$  range of 100 to 1700, and MS/MS range of 100 to 1700. During MS/MS data collection, each TIMS cycle was 1.1 s and included 1 MS + an average of 10 PASEF MS/MS scans. The acquired data were submitted to the MASCOT search engine for peptide identifications.

#### Rapid data collection...

As may be expected for this type of sample, the base peak chromatogram (BPC) (Figure 5, top) indicates an extremely complex collection of peptide species. Considering one narrow time slice of the LC gradient (61.7 min), the power of the PASEF method is readily seen. During this example

scan, 41 different precursors were fed into the scheduling algorithm and addressed by a total of 10 PASEF MS/MS scans. By examination of a single TIMS elution (time) heat map (Figure 5, bottom left), rapid quadrupole isolation window switching for the first 12 precursors is evident. Additional precursors are targeted in subsequent segments of the elution gradient. All precursors analyzed within this example PASEF cycle are indicated (Figure 5, bottom right), including the resequencing of targets of lower abundance. Within this single PASEF cycle (1.1 s), the equivalent of 119 separate MS/MS events (if collected without PASEF on a timsTOF mass spectrometer) were conducted.

#### ...while maintaining necessary sensitivity

Important proteomic features are often hidden within low intensity peaks of complex samples, peaks which might not be targeted by instruments with slower MS/MS acquisition rates. The sensitivity (through the TIMS time focusing and the ability to sum spectra of low abundance targets) and separation power (through differences in ion mobility) of the PASEF approach is

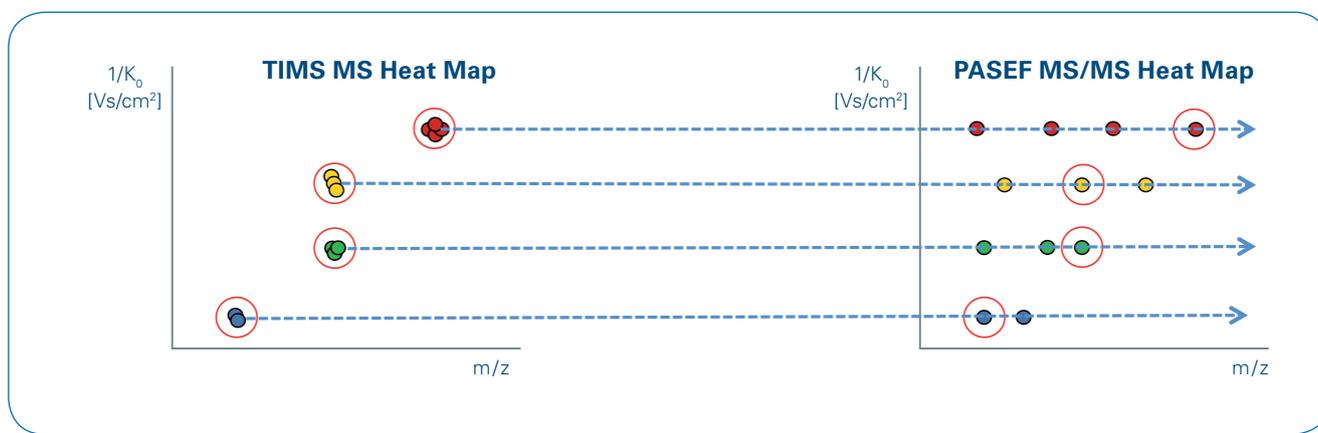


Figure 3. Illustration of TIMS MS (left) and PASEF MS/MS (right) heat maps resulting from TIMS separations. Although the data generated may be very complex, the characteristic ion mobility of targeted precursors and their fragments facilitates easy data alignment. Note that precursors of the same  $m/z$  (yellow and green) may be clearly separated by their mobility.

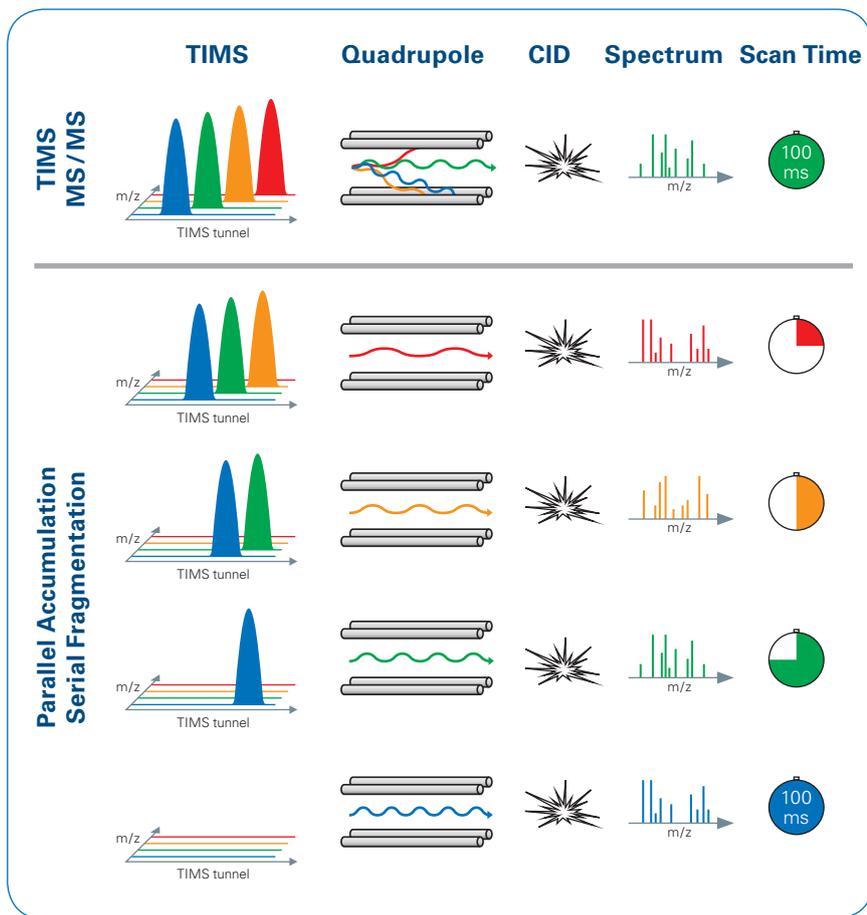


Figure 4. Illustration of the PASEF method in comparison with the standard TIMS MS/MS operation mode, with the same 100 ms timescale. Using PASEF (lower panel), the quadrupole switches its isolation position several times during each ion mobility scan, with multiple precursors isolated and subsequently transferred to the collision cell. In contrast, with the standard TIMS MS/MS approach (upper panel), only one precursor from each TIMS scan is selected.

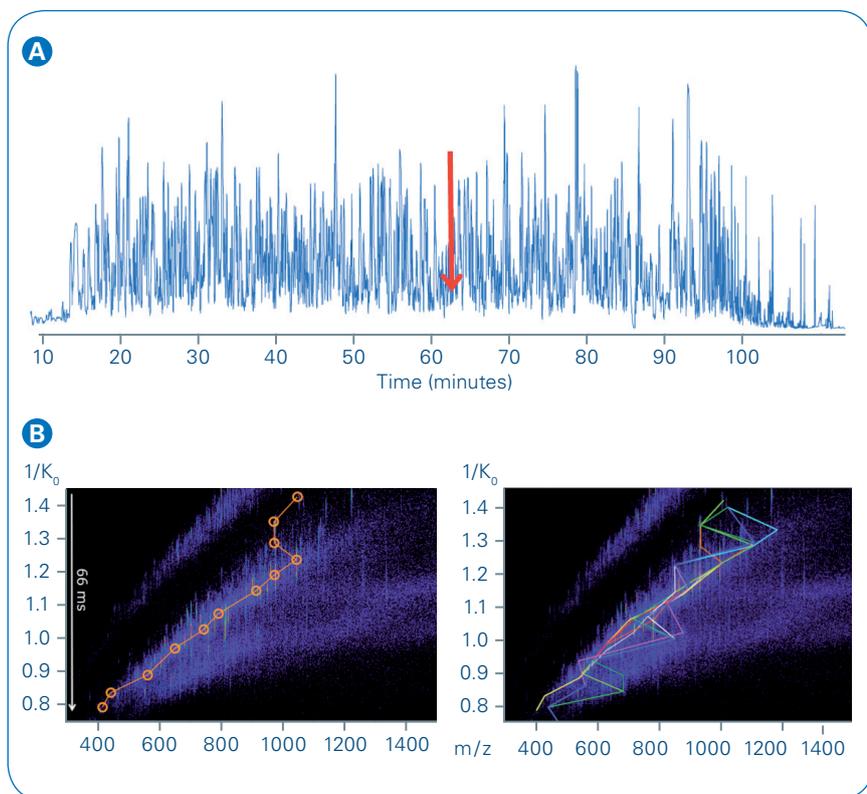
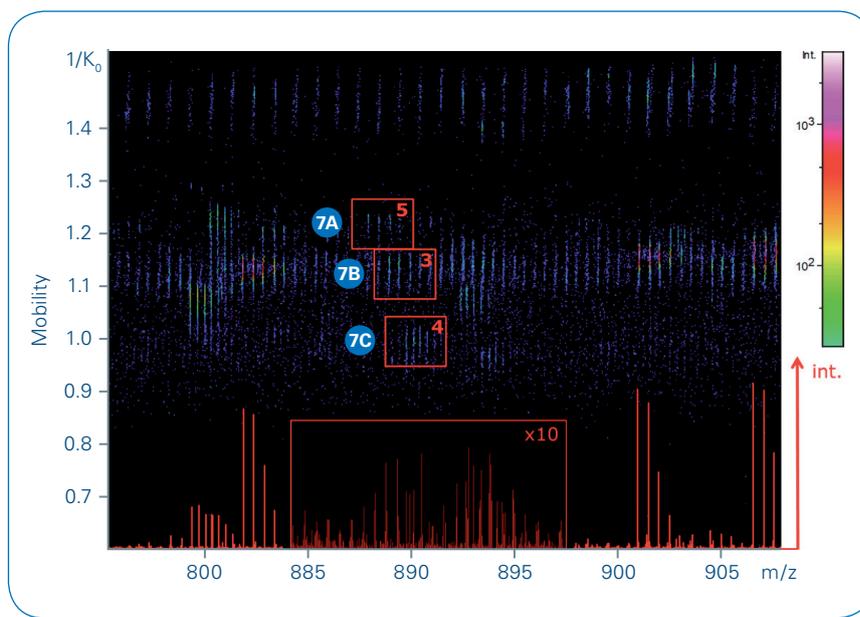


Figure 5. LC MS/MS run, 200 ng HeLa digest, 90 min gradient. **A**: Base peak chromatogram. **B**: TIMS MS heat map at 61.7 min, with targeted precursors indicated. Bottom left, first PASEF MS/MS analysis event, precursors circled in orange. Bottom right, full PASEF MS/MS cycle, with each of the ten colored lines indicating targets from one TIMS elution segment. The most abundant precursors were sequenced in previous scan cycles and were dynamically excluded from re-sequencing.

Figure 6. Collection of precursors at  $m/z$  887.96, 888.94, and 889.78 indicated on a TIMS MS heat map, with the number of summed MS/MS spectra for each indicated. The base peak chromatogram for this narrow  $m/z$  range is shown in the bottom of the heat map.

demonstrated in Figure 6. Summed MS/MS spectra from three low intensity precursors of similar  $m/z$  in a dense region of the spectrum were readily identified with good sequence coverage, high MASCOT scores and low expectation values (probabilities to be a random match). Clearly, high performance of the (subsequent) QTOF is also critical, and the timsTOF Pro mass spectrometer provides high resolution, ppm accurate mass and high isotopic fidelity (True Isotopic Pattern, or TIP™). The benefit of this combined approach for shotgun proteomics applications is clearly indicated by the increase in confident peptide identifications (Figure 8). In the same sample (200 ng HeLa digest, eluted from the LC with a 90 min gradient), nearly twice as many peptides were identified using the PASEF approach as compared to analysis without trapped ion mobility spectrometry separation (using the same instrumentation, however, with the TIMS device switched off).



7A

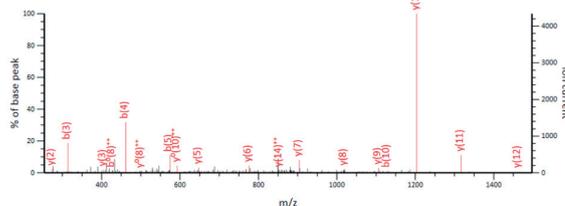
**Precursor**

Mass:  $m/z$  888.94 2+  
 CCS: 464.71 Å<sup>2</sup>  
 Intensity: 21219

**Fragment**

Repetitions: 3  
 Mascot Ions Score: 62  
 Expectation Value: 6.7e<sup>-7</sup>

A L E F I P S D Q G N E M V R



7B

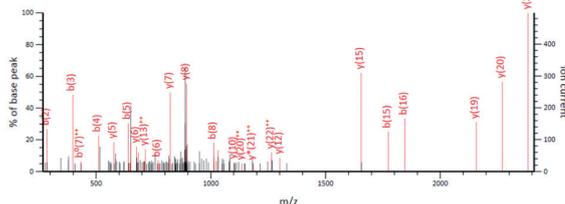
**Precursor**

Mass:  $m/z$  889.78 3+  
 CCS: 614.83 Å<sup>2</sup>  
 Intensity: 11844

**Fragment**

Repetitions: 4  
 Mascot Ions Score: 72  
 Expectation Value: 6.9e<sup>-8</sup>

H F L L E E D K P E E P T A H A F V S T L R



7C

**Precursor**

Mass:  $m/z$  887.96 2+  
 CCS: 498.32 Å<sup>2</sup>  
 Intensity: 10355

**Fragment**

Repetitions: 5  
 Mascot Ions Score: 35  
 Expectation Value: 3.0e<sup>-5</sup>

A L E Q L N G F E L A G R P M K

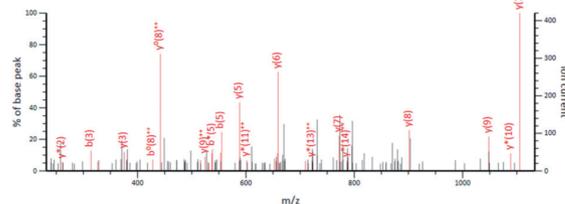


Figure 7. Example of low intensity peptide matches from HeLa cell analysis via PASEF differ by only 2 Da. While their isotopic patterns overlap in  $m/z$ , they are separated in the mobility dimension and the PASEF MS/MS results yield confident identifications.

## Discussion

The recent introduction of trapped ion mobility spectrometry (TIMS) has added an innovative dimension to quadrupole time-of-flight (QTOF). The incorporation of the previously described “Parallel Accumulation Serial Fragmentation” (PASEF)<sup>[1]</sup> method within Bruker’s proprietary timsTOF Pro instrument significantly magnifies these benefits. In this experiment, more than 160,000 independent precursors from a HeLa digest, separated using a 90 min LC gradient, were addressed, with very fast MS/MS data acquisition rates.

The timsTOF Pro used for this study provides critical advances in separation and sensitivity. Trapped ion mobility spectrometry separation alone leads to improved spectral quality by virtue of the reduction of background noise. The use of two regions of separation within the TIMS device enables 100% duty cycle so that no ions are lost, ions are accumulated in the first region of the TIMS device, while they are scanned out into the QTOF from the second region. The quadrupole of the timsTOF Pro can switch its isolation window on the millisecond time scale, enabling the QTOF to address >10 precursors in a typical 100 msec TIMS scan. The sensitivity is improved through the additional time focusing achieved by the TIMS, and additional sensitivity for low abundance precursors is achieved by addressing the same precursor in multiple PASEF scans.

Table 1 and 2: Instrumental Details

LC-Settings	
<b>LC-System</b>	nanoElute UHPLC system (Bruker Daltonics)
<b>Column</b>	25 cm X 75 µm 1.6 µm C18 column (IonOpticks, Australia)
<b>LC flow rate</b>	400 nL/min
<b>LC elution conditions</b>	2% to 37% (100% ACN, 0.1% FA)
<b>Column oven (Sonation) temperature</b>	50°C
<b>Source</b>	CaptiveSpray Ion Source (Bruker Daltonics)
<b>Ionization</b>	ESI (+)

MS-Settings	
<b>MS-System</b>	timsTOF Pro mass spectrometer (Bruker Daltonics)
<b>TIMS elution</b>	100 ms accumulation and ramp time (100% duty cycle)
<b>Cycle time</b>	1.1 s cycle with 1 MS + 10 PASEF MS/MS
<b>Scan range</b>	100 – 1700 m/z

Figure 8. Protein ID results. Approximately six times as many MS/MS spectra can be collected in the same time frame, with nearly double the number of unique peptide sequences and 1.5 times as many protein groups represented.

	Total number of MS/MS spectra	Precursors targeted	Peptide spectrum matches	Unique peptide sequences, FDR < 1%, (Mascot with Percolator)	Protein groups (Mascot with Percolator)
<b>TIMS-PASEF</b>	610,000	166,000	73,000	39,000	5,200
<b>TIMS off</b>	105,000	105,000	29,000	20,000	3,200

## Conclusions

“Parallel Accumulation Serial Fragmentation” (PASEF) on a timsTOF Pro instrument (Bruker Daltonics) has been shown to successfully deliver significantly higher speed and sensitivity in a data-dependent shotgun proteomics workflow, as demonstrated by the analysis of 166,000 independent precursors from a HeLa digest using a 90 min gradient. By applying PASEF, the number of identifications could be increased to more than 40,000 unique peptide identifications and 5,500 protein identifications from only 200 ng of HeLa digest injected on column. The number of precursors able to be targeted with this approach is dramatically increased by virtue of the rapid sample separation by both chromatography and trapped ion mobility spectrometry. These exceptional acquisition rates also support improved sensitivity, as low abundant precursors may be targeted several times. This unique combination of features provides a new standard for shotgun proteomics performance, enabling researchers to find and identify more biologically relevant proteins.



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- [2] Beck, et al (2015) The Impact II, a Very High-Resolution Quadrupole Time-of-Flight Instrument (OTOF) for Deep Shotgun Proteomics. *Mol Cell Proteomics.* 14(7):2014-29.
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