Exploring novel paths towards protein signatures of chronic pain

David Gomez-Varela, PhD¹ and Manuela Schmidt, PhD¹

Abstract
Pain is a major symptom of many medical conditions and the worldwide number one reason for people to seek medical assistance. It affects the quality of life of patients and poses a heavy financial burden on society with high costs of treatment and lost productivity. Furthermore, the treatment of chronic pain presents a big challenge as pain therapeutics often lack efficacy and exhibit minimal safety profiles. The latter can be largely attributed to the fact that current therapies target molecules with key physiological functions throughout the body. In light of these difficulties, the identification of proteins specifically involved in chronic pain states is of paramount importance for designing selective interventions. Several profiling efforts have been employed with the aim to dissect the molecular underpinnings of chronic pain, both on the level of the transcriptome and proteome. However, generated results are often inconsistent and non-overlapping, which is largely due to inherent technical constraints. A potential solution may be offered by emerging strategies capable of performing standardized and reproducible proteome analysis, such as data-independent acquisition-mass spectrometry (DIA-MS). We have recently demonstrated the applicability of DIA-MS to interrogate chronic pain-related proteome alterations in mice. Based on our results, we aim to provide an overview on DIA-MS and its potential to contribute to the comprehensive characterization of molecular signatures underlying pain pathologies.

Keywords
Chronic pain, proteomics, data-independent acquisition-mass spectrometry, mass spectrometry, protein signature, mouse models of chronic pain

Date received: 21 June 2016; revised: 1 September 2016; accepted: 13 October 2016

Overview of current profiling approaches in pain research

While acute pain is an evolutionary adaptive response, chronic pain is considered a pathology of the nervous system. In vertebrates, a painful stimulus is detected by specialized peripheral sensory neurons like those of dorsal root ganglia (DRG). These DRG neurons serve as primary cellular detectors of noxious stimuli.¹ Their activity ultimately shapes the afferent message transmitted via the spinal cord to different regions of the central nervous system where the sensation of pain is generated. From a molecular point of view, the function of each relay station of this pain axis relies on dedicated protein machineries that are fine-tuned and highly regulated.¹–³

During the past decade, enormous efforts have been made towards the identification and characterization of key molecular pathways that orchestrate processes underlying chronic pain. Excellent previous studies documented pain-related transcriptome variations using microarrays and deep sequencing methods (RNA-Seq).⁴,⁵ Although these findings yielded important insights, the extent to which mRNA alterations account for protein variability is limited.⁶ It is thus desirable to directly assess chronic pain-induced proteome changes, as the proteome represents the composite readout of gene expression, translation, and posttranslational modulation.⁶ Many investigators have employed MS in order to reveal proteome changes in tissues from both, rodent models⁷–¹² and patients suffering from chronic pain.¹³,¹⁴ Despite the body of knowledge on potentially regulated proteins generated by these studies, the

¹Max-Planck Institute of Experimental Medicine, Göttingen, Germany

Corresponding authors:
Manuela Schmidt, Somatosensory Signaling and Systems Biology Group, Max-Planck Institute of Experimental Medicine, Göttingen D-37075, Germany.
Email: mschmidt@em.mpg.de
David Gomez-Varela, Somatosensory Signaling and Systems Biology Group, Max-Planck Institute of Experimental Medicine, Göttingen D-37075, Germany.
Email: gomezvarela@em.mpg.de
 Emerging DIA-MS-based proteomics

In the past years, MS has significantly advanced in the search of novel solutions to circumvent these commonly encountered problems of classical DDA experiments.

Highly optimized and extended DDA profiling has successfully achieved extensive proteome coverage. Highly optimized and extended DDA profiling has successfully achieved extensive proteome coverage. Still, limitations hinder its broad implementation in the pain research community: on one hand, the need of long instrument times and on the other hand, the lack of tools that can be shared to standardize the detection of these large sets of proteins.

One emerging group of MS strategies that have the potential to overcome these bottlenecks is so-called data-independent acquisition (DIA)-MS. DIA-MS is not only capable of detecting and fragmenting the majority of peptides present in a complex sample, but it also does so with high reproducibility enabling standardization among laboratories. In contrast to DDA-MS, DIA-MS performs concurrent selection and fragmentation of nearly all peptides entering the mass spectrometer, on the basis of their mass/charge (m/z) ratio and irrespective of their intensity (as long as their intensity is above the detection limit of the mass spectrometer). The measured fragmentation data (composed of retention time, intensity, and m/z data from each fragmented ion) for each generated spectrum are stored in DIA digital maps, which are unique for a given sample. However, such digital maps are highly complex as each generated spectrum contains information from different peptides (referred to as a chimeric spectrum); this prevents the use of conventional database search tools to identify unique peptides. Hence, reference spectral libraries are employed to query these DIA digital maps in a targeted way. A reference spectral library is built from several highly optimized DDA-MS runs on the sample of interest in order to achieve the most extended number of peptide identifications possible. This compendium of peptides contains the necessary physicochemical information to uniquely identify them in the aforementioned chimeric spectra of DIA digital maps, which are produced by subsequent analysis of the same sample in DIA-MS mode. The use of these reference spectral libraries permits the quantification of thousands of proteins across different samples with unprecedented reproducibility.

Another interesting feature of DIA-MS is that DIA digital maps can be queried in silico any time using new/extended spectral libraries thereby opening the opportunity to test novel hypotheses without the need to perform laborious new experiments. These characteristics, together with the availability of both DIA digital maps and reference spectral libraries in growing public repositories (PeptideAtlas, positions DIA-MS as a promising tool towards highly reproducible and standardized protein profiling.

Indeed, several reports highlighted the potential of DIA-MS to interrogate pathology-related proteomes, such as signatures for tumor characteristics or drug-related protein–protein interactions.
We applied this spectral library for the targeted search of DIA digital maps obtained from DRG after induction of inflammatory and neuropathic pain as well as respective controls (CFA vs. Vehicle and SNI vs. Sham). As a result, we obtained a quantitative comparison of 2526 proteins across all biological replicates of the four conditions tested. Interestingly, these experiments revealed significant and largely pain model-specific alterations of 129 proteins, among which are targets that have either previously been reported to be relevant for nociception or represent yet uncharacterized proteins. In addition, the high statistical power of our DIA-MS results allowed us to uncover global alterations in diverse cellular protein networks, such as protein maturation and mitochondrial function. A comparison with known biology, combined with our functional validation experiments using independent animal cohorts, demonstrates that DRG protein networks relevant for pain and somatosensory signaling were indeed identified.

DRG constitute a major peripheral part of the pain axis. Yet, pain is perceived and strongly modulated by an intricate interplay of many regions of the central nervous system along the pain axis. Elegant studies have documented prominent pain-induced plasticity in the dorsal horn of the spinal cord as well as in diverse supraspinal sites. For example, several forms of synaptic plasticity in the anterior cingulate cortex are implicated in emotional-aversive aspects of pain including pain-related anxiety. Moreover, distinct pain-associated modifications in neuronal activity were found in the prefrontal cortex as well as in the amygdala.

It is this complexity underlying pain perception and modulation that renders the search for molecular signatures of pain a challenging endeavor. Nonetheless, the features offered by DIA-MS towards the standardization of protein profiling provide novel opportunities. These include the generation of tissue-specific spectral libraries and the applicability of existing spectral libraries to explore proteome changes in any region of the pain axis.

Conclusions and outlook

In spite of its likely impact on molecular pain research, several limitations, which are currently preventing DIA-MS from reaching its full potential have yet to be solved.

DIA digital maps are very complex and reliable extraction of protein information with spectral libraries is demanding. In our study, for example, the spectral library (and thus our DIA-MS results) did not contain information on many ion channels known to be involved in nociception and pain such as Nav1.7. The reasons can be manifold, ranging from relative low expression levels (and consequently difficulties in detection by DDA-MS used for spectral library generation) to insufficient solubilization — factors known to render membrane protein analysis challenging. Hence, optimization of biochemical sample preparation and spectral library generation will be key to obtain the most complete coverage of the

---

**Figure 1.** Schematic representation of an experimental workflow suitable for standardized and comparative protein profiling in any tissue of interest across mouse models of chronic pain.
proteome. In addition, recent advances in both targeted (using spectral library information\(^2\)) and untargeted approaches (using computational workflows\(^3\)) offer improved strategies to extract information from DIA digital maps. Moreover, novel MS acquisition strategies\(^4\) are designed to reduce the complexity of DIA maps and to simultaneously maximize the amount of protein information obtained. Further, while to date DDA-MS units are available in many research institutions, DIA-MS platforms are still in a nascent stage. We expect that the growing landscape of technological solutions together with the access to user-friendly DIA analysis software (e.g., Skyline, OpenSWATH) will facilitate the incorporation of DIA-MS into the researchers’ toolbox.

Despite the vast amount of research, pain management still relies on the “one drug fits all” model. However, cellular signaling is diverse and it has long been known that the function of a single protein may be modulated by its assembly into dynamic multi-protein complexes and networks—a fact represented by the concept of cellular “molecular machines.”\(^4\) Therefore, evolving strategies aimed at correcting dysfunctional networks may provide promising tools for achieving analgesia.\(^2\) Identifying the composition of such networks in a tissue-specific manner can greatly facilitate insights into functional misalignments of pathology-associated processes with the opportunity to modify them at distinct, critical hubs.\(^3\) This may provide major benefits: (i) increase of specificity, (ii) possibilities for discrete tuning of cellular functions, and consequently, (iii) less side effects on key physiological functions.

The results from our study serve as a proof-of-principle for the generation of molecular portraits of signaling networks underlying specific chronic pain states. The availability of large and tissue-specific spectral libraries will open the possibility to perform longitudinal studies investigating the molecular plasticity along the whole pain axis or even in any tissue of interest. On the clinical front, DIA-MS has the potential to expedite the long-awaited stratification of chronic pain conditions through sensitive and specific diagnostics tools using minimally invasive liquid biopsy matrices (e.g., plasma). Consequently, more efficacious and personalized pain therapies may be developed.

We are confident that forthcoming DIA-MS-based research efforts of the pain-community will help to initiate new turns on the path towards pain relief.

Acknowledgments
We are grateful to Allison Barry (IMPRS Neurosciences, Göttingen) for proof reading the manuscript.

Author Contributions
Both authors contributed equally.

Declaration of Conflicting Interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding
The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by the Emmy Noether-Program of the Deutsche Forschungsgemeinschaft (SCHM 2533/2-1 to MS), research grants of the Deutsche Forschungsgemeinschaft (GO 2481/3-1 to DGV; SCHM 2533/4-1 to MS) and the Max Planck Society. MS received a research award and travel support by the German Pain Society (DGSS), both of which were sponsored by Astellas Pharma GmbH (Germany). DGV received research support from Biognosys AG (Zurich, Switzerland). Neither funding from Astellas Pharma GmbH nor from Biognosys AG influenced the content of this article.

References
1. Basbaum AI, Bautista DM, Scherrer G, et al. Cellular and molecular mechanisms of pain. Cell 2009; 139: 267–284.
2. Patapoutian A, Tate S and Woolf CJ. Transient receptor potential channels: targeting pain at the source. Nat Rev Drug Discov 2009; 8: 55–68.
3. Rouwette T, Avenali L, Sondermann J, et al. Modulation of nociceptive ion channels and receptors via protein–protein interaction: implications for pain relief. Channels (Austin) 2015; 9: 175–185.
4. Manteniotis S, Lehmann R, Flegel C, et al. Comprehensive RNA-Seq expression analysis of sensory ganglia with a focus on ion channels and GPCRs in Trigeminal ganglia. PloS One 2013; 8: e79523.
5. Usoskin D, Furlan A, Islam S, et al. Unbiased classification of sensory neuron types by large-scale single-cell RNA sequencing. Nat Neurosci 2015; 18: 145–153.
6. Schwанhauser B, Busse D, Li N, et al. Global quantification of mammalian gene expression control. Nature 2011; 473: 337–342.
7. Niederberger E and Geisslinger G. Proteomics in neuropathic pain research. Anesthesiology 2008; 108: 314–323.
8. Huang HL, Cendan CM, Roza C, et al. Proteomic profiling of neuromas reveals alterations in protein composition and local protein synthesis in hyper-excitabile nerves. Mol Pain 2008; 4: 33.
9. Melemedjian OK, Yassine HN, Shy A, et al. Proteomic and functional annotation analysis of injured peripheral nerves reveals ApoE as a protein upregulated by injury that is modulated by metformin treatment. Mol Pain 2013; 9: 14.
10. Michaeliievski I, Medzhiradzsky KF, Lynn A, et al. Axonal transport proteomics reveals mobilization of translation machinery to the lesion site in injured sciatic nerve. Mol Cell Proteomics 2010; 9: 976–987.
11. Vacca V, Marinelli S, Pieroni L, et al. Higher pain perception and lack of recovery from neuropathic pain in females: a behavioural, immunohistochemical, and proteomic
investigation on sex-related differences in mice. *Pain* 2014; 155: 388–402.
12. Zou W, Zhan X, Li M, et al. Identification of differentially expressed proteins in the spinal cord of neuropathic pain models with PKC gamma silence by proteomic analysis. *Brain Res* 2012; 1440: 34–46.
13. Olausson P, Gerdle B, Ghafori N, et al. Protein alterations in women with chronic widespread pain – an explorative proteome study of the trapezius muscle. *Sci Rep* 2015; 5: 11894.
14. Oki G, Wada T, Iba K, et al. Metallothionein deficiency in the injured peripheral nerves of complex regional pain syndrome as revealed by proteomics. *Pain* 2012; 153: 532–539.
15. Michalski A, Cox J and Mann M. More than 100,000 detectable peptide species elute in single shotgun proteomics runs but the majority is inaccessible to data-dependent LC-MS/MS. *J Proteome Res* 2011; 10: 1785–1793.
16. Domon B and Aebersold R. Options and considerations when selecting a quantitative proteomics strategy. *Nat Biotechnol* 2010; 28: 710–721.
17. Tabb DL, Vega-Montoto L, Rudnick PA, et al. Repeatability and reproducibility in proteomic identifications by liquid chromatography-tandem mass spectrometry. *J Proteome Res* 2010; 9: 761–776.
18. Sharma K, Schmitt S, Bergner CG, et al. Cell type- and brain region-resolved mouse brain proteome. *Nat Neurosci* 2015; 18: 1819–1831.
19. Bruderer R, Bernhardt OM, Gandhi T, et al. Extending the limits of quantitative proteome profiling with data-independent acquisition and application to acaminophen treated 3D liver microtissues. *Mol Cell Proteomics* 2015; 14: 1400–1410.
20. Gillet LC, Navarro P, Tate S, et al. Targeted data extraction of the MS/MS spectra generated by data-independent acquisition: a new concept for consistent and accurate proteome analysis. *Mol Cell Proteomics* 2012; 11: O111.016717.
21. Sajic T, Liu Y and Aebersold R. Using data-independent, high resolution mass spectrometry in protein biomarker research: perspectives and clinical applications. *Proteomics Clin Appl* 2015; 9: 307–321.
22. Ting YS, Egerton JD, Payne SH, et al. Peptide-centric proteome analysis: an alternative strategy for the analysis of tandem mass spectrometry data. *Mol Cell Proteomics* 2015; 14: 2301–2307.
23. Bruderer R, Bernhardt OM, Gandhi T, et al. Extending the limits of quantitative proteome profiling with data-independent acquisition and application to acaminophen-treated three-dimensional liver microtissues. *Mol Cell Proteomics* 2015; 14: 1400–1410.
24. Schubert OT, Gillet LC, Collins BC, et al. Building high-quality assay libraries for targeted analysis of SWATH MS data. *Nat Protoc* 2015; 10: 426–441.
25. Liu Y, Chen J, Sethi A, et al. Glycoproteomic analysis of prostate cancer tissues by SWATH mass spectrometry discovers N-acylethanolamine acid amidase and protein tyrosine kinase 7 as signatures for tumor aggressiveness. *Mol Cell Proteomics* 2014; 13: 1753–1768.
26. Surinova S, Radova L, Choi M, et al. Non-invasive prognostic protein biomarker signatures associated with colorectal cancer. *EMBO Mol Med* 2015; 7: 1153–1165.
27. Lambert JP, Ivoese G, Couzens AL, et al. Mapping differential interactomes by affinity purification coupled with data-independent mass spectrometry acquisition. *Nat Methods* 2013; 10: 1239–1245.
28. Rouwette T, Sondermann J, Avenali L, et al. Standardized profiling of the membrane-enriched proteome of mouse dorsal root ganglia (DRG) provides novel insights into chronic pain. *Mol Cell Proteomics* 2016; 15: 2152–2168.
29. Kuner R. Central mechanisms of pathological pain. *Nat Med* 2010; 16: 1258–1266.
30. Simonetti M, Hagenston AM, Vardeh D, et al. Nuclear calcium signaling in spinal neurons drives a genomic program required for persistent inflammatory pain. *Neuron* 2013; 77: 43–57.
31. Drdla R, Gassner M, Gingl E, et al. Induction of synaptic long-term potentiation after opioid withdrawal. *Science* 2009; 325: 207–210.
32. Drdla-Schutting R, Benrath J, Wunderbaldinger G, et al. Erasure of a spinal memory trace of pain by a brief, high-dose opioid administration. *Science* 2012; 335: 235–238.
33. Li XY, Ko HG, Chen T, et al. Alleviating neuropathic pain hypersensitivity by inhibiting PKMζeta in the anterior cingulate cortex. *Science* 2010; 330: 1400–1404.
34. Bliss TV, Collingridge GL, Kaang BK, et al. Synaptic plasticity in the anterior cingulate cortex in acute and chronic pain. *Nat Rev Neurosci* 2016; 17: 485–496.
35. Kiritoshi T, Ji G and Neugebauer V. Rescue of impaired mGLUR5-driven endocannabinoid signaling restores prefrontal cortical output to inhibit pain in arthritic rats. *J Neurosci* 2016; 36: 837–850.
36. Li Z, Ji G and Neugebauer V. Mitochondrial reactive oxygen species are activated by mGLUR5 through IP3 and activate ERK and PKA to increase excitability of amygdala neurons and pain behavior. *J Neurosci* 2011; 31: 1114–1127.
37. Tappe-Theodor A, Fu Y, Kuner R, et al. Homer1a signaling in the amygdala counteracts pain-related synaptic plasticity, mGLUR1 function and pain behaviors. *Mol Pain* 2011; 7: 38.
38. Tan S, Tan HT and Chung MC. Membrane proteins and membrane proteomics. *Proteomics* 2008; 8: 3924–3932.
39. Tsou CC, Avtonomov D, Larsen B, et al. DIA-Umpire: comprehensive computational framework for data-independent acquisition proteomics. *Nat Methods* 2015; 12: 258–264, 7 p following 64.
40. Williams BJ, Ciavarini SJ, Devlin C, et al. Multi-mode acquisition (MMA): an MS/MS acquisition strategy for maximizing selectivity, specificity and sensitivity of DIA product ion spectra. *Proteomics* 2016; 16: 2284–2301.
41. Alberts B. The cell as a collection of protein machines: preparing the next generation of molecular biologists. *Cell* 1998; 92: 291–294.
42. Borsook D, Hargreaves R, Bontra C, et al. Lost but making progress – where will new analgesic drugs come from? *Sci Transl Med* 2014; 6: 249sr3.
43. Barabasi AL, Gulbahce N and Loscalzo J. Network medicine: a network-based approach to human disease. *Nat Rev Genet* 2011; 12: 56–68.