Published in final edited form as:

Proteomics. 2015 May ; 15(9): 1486–1502. doi:10.1002/pmic.201400448.

Emerging proteomic technologies for elucidating context-dependent cellular signaling events: A big challenge of tiny proportions

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Abstract

Aberrant cell signaling events either drive or compensate for nearly all pathologies. A thorough description and quantification of maladaptive signaling flux in disease is a critical step in drug development, and complex proteomic approaches can provide valuable mechanistic insights. Traditional proteomics-based signaling analyses rely heavily on *in vitro* cellular monoculture. The characterization of these simplified systems generates a rich understanding of the basic components and complex interactions of many signaling networks, but they cannot capture the full complexity of the microenvironments in which pathologies are ultimately made manifest. Unfortunately, techniques that can directly interrogate signaling *in situ* often yield mass-limited starting materials that are incompatible with traditional proteomics workflows. This review provides an overview of established and emerging techniques that are applicable to context-dependent proteomics. Analytical approaches are illustrated through recent proteomics-based studies in which selective sample acquisition strategies preserve context-dependent information, and where the challenge of minimal starting material is met by optimized sensitivity and coverage. This review is organized into three major technological themes: (1) LC methods inline with mass spectrometry; (2) Antibody-based approaches; (3) MS Imaging with a discussion of data integration and systems modeling. Finally, we conclude with future perspectives and implications of context-dependent proteomics.

Keywords

Proteomics; cell signaling; context; data independent analysis; CyTOF; capillary electrophoresis; mass spectrometry imaging

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1. Introduction

One measure of the fidelity with which experimental models recapitulate disease states is the proportion of discoveries that translate to new drugs and therapeutics. Even using the most generous statistical assumptions, fewer than one in every six drug candidates that enter the clinical trial process ultimately obtain FDA approval and reach the market to impact patient care (1). When each indication for a drug is considered independently, this statistic falls closer to 10%, with a steep drop out of drug candidates during Phase II and Phase III trials and over 50% of failures attributed to lack of efficacy (2). One explanation that partially accounts for these metrics is that pre-clinical research studies that identify candidate drugs inadequately capture the biological complexity and context in which a therapeutic is meant to operate (3, 4). In brief, pathologies are made manifest in tissues, not in flasks.

A web of cellular signaling events, centered in the proteome, work in concert with nuclear co-regulators to orchestrate acute and chronic cellular responses. Aberrations within this process are central to nearly all pathologies, which make signaling network perturbations prime targets for drug development (5). Signaling flux depends not only on the presence and relative abundance of signaling proteins, but on a diversity of protein variants and modifications collectively known as proteoforms (6). Thus, while genomic and transcriptomic data inform the convergence of signaling inputs and outputs, a quantitative context-dependent accounting of the signaling proteome speaks most directly to the process of dynamic information transfer via post-translational modifications.

The signaling proteome is epigenetically primed but contextually modified by external physical and chemical factors. A clear and commonly cited illustration of this concept is the interactions of cancerous tumors with their microenvironment. The observation that tumor origins influence the organs where their metastases preferentially settle is contextually linked to tissue heterogeneity: Some driver mutations result in invasive signaling signatures that are suppressed by contextual factors in one organ only to find a more fertile microenvironment elsewhere. Along similar lines, numerous potential therapeutics may suppress aberrant signaling events when applied to *in vitro* tumor monocultures or ectopic sub-cutaneous tumors derived in mice, but these same drugs encounter resistance when tumors are housed in orthotopic tissues (7).

From a proteomic perspective, extracellular factors that modulate signaling include matrix proteins and secreted ligands along with their complementary receptor domains. The dynamic complement of intracellular signaling networks, interacting with and operating within a tissue microenvironment, serves as a fundamental biological unit; it defines cell context and performs in concert determine physiology. Thus, a systems-level comparison of protein interaction networks and post-translational modification dynamics between normally functioning and diseased tissues represents the ideal methodology for identifying drivers of aberrant signaling along with potential targets amenable to therapeutic intervention.

The process of separating out the distinct signaling states of each cell type present in a tissue limits the amount of sample that can be acquired for downstream analyses. Traditional proteomic workflows and instrumentation tend to be constrained by inadequate dynamic

range and sensitivity to study systems-wide, context-dependent cell signaling (8). Enrichment and fractionation can help to mitigate these constraints provided sufficient starting materials are available, and considerable proteomics knowledge is consequently derived from *in vitro* monocultured cell lines. The strength with which these preparations overcome practical limitations is complemented by their proven and continuing ability to provide a wealth of fundamental biological information concerning signaling network assembly and responses. Their weakness stems from their inadequate recapitulation of the *in vivo* microenvironment (Figure 1), and this flaw in turn, limits the predictive validity of traditional proteomics workflows.

From the perspective of cell culture, ongoing developments in the bioengineering of platforms and extracellular matrix substrates that incorporate physiological context already represent an ongoing revolution that currently culminates in the emergence of organ-on-a-chip devices (9). These new bioengineering platforms, reviewed elsewhere (10), more accurately mimic physiological context *in vitro* relative to monocultures, but they cannot supplant the systems-level signaling networks inherent to intact tissues. Furthermore, regardless of whether substrate is derived from a model or from tissue samples, context-specific signaling studies present challenges and technical demands that push the limits of existing technologies.

Ongoing technological and methodological advancements within the field of proteomics are increasingly well suited to the task of generating the multiplexed readouts necessary for systems-level signaling network analysis. One key issue involves achieving adequate sensitivity for protein or PTM quantification with the ideal goal of reaching single cell and single molecule quantification. Technical developments that facilitate the excision of precisely defined tissue regions for analysis, nanoscale sample preparations, and high sensitivity detection of rare intracellular events are all needed. The focus of this review is on emerging proteomic technologies and workflows that lend themselves to the interrogation of context-specific cell signaling, the majority of which rely on bottom-up strategies.

Alongside these emerging techniques are additional challenges that are rooted in the preparation, separation, detection, acquisition, and quantitation aspects of MS-based approaches. Despite these challenges, the studies presented in this review represent new methods and technological platforms that are enabling the acquisition of high quality, context-dependent proteomics data. This review is organized into three major technological themes (Summarized in Table 1): (1) Methods employing peptide separation inline with MS, (2) Methods using antibody-based approaches, and (3) MS Imaging (MSI). Even with ideal detection and quantification of signaling targets, each of these tissue-based approaches is limited to capturing *in vivo* signaling within a static and temporal snapshot. We therefore conclude this review with a discussion of the importance of bioinformatics and computational modeling for deciphering the meaning of *in situ* signaling states.

2. Peptide separation inline with bottom-up MS

Proteomic analyses of cell signaling events in organs and tissues routinely begin with homogenization. This blunt approach can accommodate the largest possible amount of starting material, which is clearly advantageous for protein-intensive analyses such as

affinity-purification or other preparative strategies for low-abundant protein and peptide enrichment. Unfortunately, bulk sample homogenization results in the aggregation of all distinct cell populations into an indistinguishable soup of protein. Aberrant signaling remains detectable in cell homogenate if these events are derived from dominant cell populations, but important pathologies may be rooted in the signaling perturbations of a small subset of resident cells. Signals derived from these less abundant cells can easily be negated by reciprocal activity from neighboring cells, and can often be altogether muted behind more intense signals derived from more abundant but less relevant cell types.

Techniques that facilitate the isolation of distinct cell sub-populations within a tissue can circumvent some of these limitations prior to downstream MS analysis. These isolation techniques include laser capture microdissection (LCMD) of frozen or fixed tissue sections, and flow cytometry-based sorting of freshly dissociated samples. LCMD uses microscopy to assist near-infrared laser excision of specific regions or cell types within a thin tissue section. These sections tend to have depths of 10 μ m or less, translating to 10³ – 10⁴ cells. While serial sections of the same tissue can be pooled to increase the total extracted sample volume, low cell yield remains a defining challenge for MS applications of this technique. The challenge of resolving intracellular signaling divergences between subpopulations of cells within tissues can also be facilitated by flow cytometry. This is because cells derived from freshly dissociated tissues can be sorted into purified populations for subsequent lysis and analysis by LC-MS. Like LCMD, a major limitation of flow cytometry workflows is that total cell numbers, and thus total protein yield, are low.

Advances in microfluidic device platforms can address the additional challenge of enriching low abundance signaling proteoforms derived from microscale amounts of material acquired by LCMD and flow sorting. Once distinct tissue regions have been isolated and, if applicable, their signaling components enriched, the protein fraction can be digested and subject to bottom-up workflows that involve an analytical separation coupled to MS. Increasing selectivity in this way further depletes cell numbers and starting material relative to traditional signaling studies in cultured cells, which necessitates the highest MS sensitivity.

Three options for MS data acquisition are available; data dependent acquisition (DDA), targeted acquisition, and data independent acquisition (DIA). Finally, chromatographic reverse-phase LC separations are most frequently used for proteomics workflows, but continuing advances CE can provide valuable complementary proteomic coverage with potentially enhanced analytical sensitivity (11–13). In principle, cell isolation, targeted enrichment of signaling components, analytical separation, and data acquisition techniques can be combined in a number of ways to suit experimental demands. Under the umbrella of LC-MS, we discuss examples of how sample acquisition by either LCMD or flow cytometry have been analyzed by data dependent and targeted MS workflows. We conclude this section with two categories of emerging technologies that will facilitate ongoing efforts to study contextually dependent cell signaling; data independent acquisition strategies, and enhanced enrichment and peptide separation techniques.

2.1. Data Dependent LC-MS Acquisition

Data dependent acquisition is the most established MS technique for discovery-based proteomics. Traditional DDA approaches have well known sensitivity and dynamic range limitations for detecting and quantifying proteoforms. A recent analysis by Michalski et al suggests that data dependent sampling is capable of detecting only a tiny fraction, less than 20%, of the greater than 100,000 peptide ion species that elute in a typical LC MS run (14). Unsurprisingly, low abundant peptides with relatively small MS¹ ion intensities were least likely to be selected for sequencing, with instrument time dedicated to the fragmentation and sequencing of MS¹ ions in the high and moderately high intensity range. It's likely that the majority of important peptides for deciphering cell signaling events, those derived from lower abundant signaling molecules and/or carrying critical and dynamic PTMs, cluster among the peptide species least likely to be selected for MS² sequencing in traditional DDA workflows.

Recent advances in intelligent DDA workflows promise to improve the depth of peptides selected for MS² by using online spectral matching and various decision tree processes that optimize exclusion lists to prevent oversampling of abundant peptides, focus MS² sampling onto a protein list of interest (e.g. signaling interactome), or to enhance detection and site-localization of post-translationally modified peptide forms (15–17). While these approaches should theoretically improve proteome coverage, some questions remain about whether this will hold true in practice (15). Improvements in the depth of proteome coverage aside, intelligent DDA approaches are promising tools to improve phosphopeptide profiling and site determination (16). Improvements to instrumentation speed, resolution, and sensitivity are also contributing to notable advancements in depth of proteome coverage by DDA-MS. For instance, parallelization of precursor isolation, fragmentation, and data acquisition at very high sampling speeds in the Orbitrap Fusion by ThermoFisher Scientific enable a greater than 30% improvement in the number of detected proteins compared with a traditional sequential set up, with the biggest improvements coming from increased peptide spectral matches among lower abundant peptides (18). The number of proteins detected in a single run by the paralleled acquisition approach reaches near complete coverage from just a few micrograms of starting material in smaller proteomes such as yeast, albeit excluding all post-translationally modified peptide species (19). Instrumentation advances are likewise improving lower limits of detection, and moving DDA towards reliable qualitative profiling of context-specific signaling events.

Sensitive and accurate quantitative analysis represents an additional challenge for DDA-based interrogation of context-specific signaling. The stochasticity of ion selection inherent to DDA-MS can both adversely affect reproducibility and result in missing data. This problem already becomes apparent in relatively simple affinity-purified interactome samples, quantified by label-free MS¹ analysis or iTRAQ chemical tagging (20). Furthermore, quantitation by MS¹ filtering ideally requires at least one peptide spectral match from MS² scans to accurately identify peptide sequence, which tethers this strategy to the typical DDA-MS constraints of instrument scan speed and sensitivity. Nevertheless, MS¹-based quantitation of extracted ion chromatograms has recently been shown to be a promising strategy for label free quantification, particularly after careful chromatogram

alignment and isotope envelope analysis (21). Similarly careful chromatogram alignment between experimental samples can allow for a confident spectral match generated in a single sample to ascribe identities to peptide MS¹ chromatograms in the remaining samples where the same MS¹ ion was not selected for fragmentation (22). As with any label-free quantitative MS strategy, higher coefficients of variation can be expected because experimental samples are injected and processed separately. This may be particularly true for peptides from proteins hovering near the lower limits of quantification.

One alternative strategy for quantitative DDA-MS involves labeling peptides with mass tags that identify their experimental source, followed by pooling peptides in a 1:1 ratio. This strategy allows relative protein abundances in the resulting mixture to be injected, processed, and interpreted simultaneously in MS² as relative tagged peptide abundance ratio (e.g. iTRAQ, TMT). MS²-based quantitation of chemical tags is confounded by the often chimeric nature of fragmentation spectra, especially among lower intensity peptides (14), and the selective sampling necessary to distinguish contextual influences on signaling states is likely to result in sample quantities that render accurate analysis by chemical labeling strategies challenging (23).

Another alternative strategy involves metabolic labeling of proteins with selected amino acids constructed from stable heavy isotopes of carbon (¹³C) and/or nitrogen (¹⁵N). Relative protein abundances can be analyzed and interpreted as the ratio between signals originating from “heavy” SILAC labeled and light “un-labeled” experimental groups. While SILAC experiments originated and are predominantly performed in cell culture, SILAC-based quantitation with whole organism metabolic labeling has been performed in a number of important species, including *C. elegans* (24), *Drosophila* (25, 26), and mice (27, 28).

In the first SILAC mouse study, Kruger and colleagues compared wild type mice with ¹³C₆-lysine SILAC-labeled counterparts bearing genetic constructs rendering them deficient in the expression of specific proteins. Not only did this SILAC-based MS approach confirm the absence of protein expression, this technique further revealed important biological pathways by which Kindlin-3 deficiency alters structural proteins of erythrocytes to result in anemia (27).

More recently, SILAC-labeled mouse models have been used to study phosphoproteomic changes during progressive stages of skin carcinogenesis, thus providing interesting context-specific signatures of cell signaling changes occurring from the initial application of a carcinogen, to the onset of a premalignant lesion, to the development of squamous cell carcinoma. In this study, Zanivan and colleagues were able to quantify 3,457 proteins and 5,249 phosphorylation sites, one-third of which were altered in tumor progression. Thus, in-vivo SILAC labeling could be used to analyze altered proteomic and phosphoproteomic signatures between benign and malignant tumor tissues (28). While whole animal in vivo SILAC labeling can be costly, especially for more complex organisms, its strength lies in its ability to provide quantitative proteomics data to signaling studies where the phenotype is fundamentally dependent on an intact biological system in its full complexity.

Proteomic analyses of context dependent signaling are dependent on selective in situ sampling, including LCMD and flow sorting of freshly dissociated tissues. While they are equally compatible with other MS acquisition strategies, selective sampling techniques are predominantly described in conjunction with DDA-MS owing to its earlier and more widespread adoption relative to other acquisition strategies.

A recent review by Lagenkamp *et al* on context-specific biology in the endothelium contains a comprehensive and thoughtful summary pertaining to state-of-the-art LCMD for 'omics analyses using shotgun MS (29). Several studies are cited in this review that describe the identification and quantitation of biomolecules linked to various pathologies from as few as 2000 captured cells. More recently, Wisniewski *et al* described a workflow to process laser dissected formalin fixed and paraffin embedded (FFPE) colonic adenoma tissue using filter assisted sample preparation (FASP) to enhance overall protein recovery by enabling the inclusion and cleanup SDS-like detergents in tissue lysis buffer (30). Subsequent peptide fractionation was performed with microtip strong-anion exchange to enhance coverage, while LC-MS/MS shotgun analysis was performed on a Thermo Q Exactive instrument. Although the LCMD region only produced an estimated peptide input of 6 µg, this workflow identified of over 55,000 peptides in each sample representing a total of 9,910 proteins. Similarly, He *et al* performed FASP using approximately 5000 immunohistochemically identified CD90+ cells, excised by LCMD from FFPE glioblastoma cancer tissue biopsies (31). Despite the low cell count, 674 high confidence proteins were identified from peptide digests by LC-MS analysis using a Thermo Orbitrap Elite mass spectrometer.

By applying LCMD preparative techniques to excise a specific subset of cells predefined by immunohistochemistry, and combining this with state-of-the-art high-resolution MS instrumentation for LC-MS based shotgun proteomics, these studies were able to make progress towards overcoming the tissue size limitations inherent to LCMD. Additionally, FFPE tissues were used in both cases, and while new proteomics techniques for fixed samples are maximizing protein and peptide extraction from archived samples (32), more comprehensive coverage may have been achievable from LCMD segments harvested from freshly frozen unfixed tissues unencumbered by the chemical modifications induced by formalin and paraformaldehyde fixation (33).

Conversely, neither study specifically sought to examine one or more PTMs or protein-protein interactions on which microenvironmental signaling between distinct cellular subpopulations is built. Although this may be possible, the reliable analysis of these cell-signaling hallmarks, within the tissue size constraints of LCMD, requires enrichment and purification strategies for key peptide modifications that can be appropriately scaled and sufficiently sensitive. Emerging technologies to meet this challenge are discussed below (Section 2.4).

The challenge of resolving intracellular signaling divergences between subpopulations of cells within tissues can also be facilitated by flow cytometry. This is because cells derived from freshly dissociated tissues can be sorted into purified populations for subsequent lysis and analysis by LC-MS. Like LCMD, a major limitation of flow cytometry workflows is that total cell numbers, and thus total protein yield, are low. Di Palma *et al* used FACS and

2D LC fractionation to detect 15,775 peptides corresponding to 3775 proteins derived from the lysate of 5,000 GFP labeled mouse colon stem cells [18].

The Di Palma *et al* study illustrates the strong potential of traditional flow-cytometry platforms for studying micro-scale proteomes. In order to translate this technique to signaling, the common limitation of identifying stoichiometrically unfavored PTMs and low abundant signaling molecules will need to be addressed. This will require new enrichment techniques that minimize loss. In this spirit, Martin *et al* applied 5000 FACS sorted cells to a microfluidic sample preparation apparatus for online lysis, digestion, and desalting [19]. This efficient preparative step was directly upstream of a targeted approach that monitored 32 peptides corresponding to 17 proteins spanning a dynamic range of four orders of magnitude, with a quantitative percent coefficient of variance (% CV) of approximately 24%.

The challenges of enriching post-translationally modified sub-proteomes from limited quantities of starting materials persist. The identification of molecules that define a sub-population, in tissue sections for isolation by LCMD or in cells for isolation by flow sorting, presents additional challenges for this approach. As discussed below, until reliable and specific markers of sub-populations are characterized across multiple species, strict selection of cell types for contextual signaling analysis in sub-populations will remain problematic. Finally, DDA has well known sensitivity limitations for detecting and quantifying proteoforms at lower dynamic ranges of biological expression. These limitations are further exacerbated when the quantity of starting material is constrained.

2.2. Targeted LC-MS Data Acquisition

The analytical study of context dependent signaling requires adequate lower limits of detection and quantitation. An alternative or complementary strategy to *a priori* enrichment of low abundant targets lays in increased analytical sensitivity and improved quantitative accuracy at the instrumentation level. To this end, targeted data acquisition is sufficiently sensitive to monitor signaling pathway activation with input amounts well within the ranges obtained using LCMD or *ex vivo* cell sorting.

MS-based strategies for targeted quantitative proteomics are becoming more established. MRM and SRM of proteotypic and modified peptides is currently the most sensitive MS-based analysis tool that can be applied to contextual signaling within tissues. High sensitivity in MRM is achieved by focusing exclusively on those ions that correspond to a particular MS¹ precursor while scanning through a handful of defining MS/MS transition group fragment ions (36). Advances in chromatographic resolution and reproducibility, combined with *a priori* knowledge of peptides and their retention times, enables individual MRM transition group analyses to be chromatographically scheduled so that a single injection of a complex sample can yield a highly multiplexed assay. Recently, Wolf-Yadlin *et al* used this approach to quantify 222 tyrosine-phosphopeptides relevant to EGF signaling, and characterized phosphorylation across 7 time points subsequent to EGF ligand stimulation. The number of quantified phosphopeptides in this study was attained with lysate derived from the equivalent of 10 million cells, which represents two-orders of magnitude more starting material than typical LCMD tissue specimen harvests (37). Applying a similar

approach to signaling within small sample amounts that can be isolated from *in situ* signaling studies presents an even deeper technical challenge.

To address this challenge, the Koomen lab is developing MRM-assays for monitoring key members of signaling pathways derived from clinical tissue specimens without prior phosphopeptide enrichment. Chen *et al.* used MRM to quantitatively analyze 22 proteins involved in Wnt-Beta Catenin signaling from 50,000 laser-captured cells (corresponding to approximately 28 μg) excised from a colon cancer specimen (38). Similarly, Xiang *et al* monitored 10 NF κ B-related signaling proteins in 500,000 CD138⁺ multiple myeloma tumor cells freshly sorted from patient bone marrow. Interestingly, the lysate from these cells only generated 8 μg of protein, representing 1/12 of what the authors observed with a similar number of cells in culture (approximately 100 μg) -although distinguishing experimental error from a biological event as the true source of this discrepancy becomes difficult (39).

These studies embody the promise held by targeted MS strategies to detect and quantify signaling *in situ* from purified, LCMS or flow sorted tissue samples or, in principle, from systems like organ-on-a-chip, where cells are grown in an artificial approximation of *in vivo* biological context. The advantage of MRM is that assay design theoretically only requires a priori knowledge of a peptide's sequence and chromatographic behavior. In practice, it is often more practical to build assays from existing shotgun observations of peptides, obtained either from prior discovery-based experiments or from the analysis of LC-MS data of synthetic peptide analogues that can be labeled with heavy isotopes of nitrogen (¹⁵N) and carbon (¹³C).

While the upfront assay design and validation requires considerable time and expertise, established targeted proteotypic and PTM-specific MRM assays are relatively easy to implement. Furthermore, advanced C18-based chromatographic retention time (RT) normalization strategies (40) will allow MRM assays to be more easily and reproducibly shared between instruments and labs. Assay libraries of entire proteomes have been generated and released into the public domain (41–43), and these efforts are ongoing. Curated MRM assay libraries for protein PTMs represent a new challenge for MRM-based proteomics. Where traditional MRM assays typically use the highest intensity transitions derived from tryptic peptides, constrained MRM assays (44) are designed to quantify specific amino acid residues that are post-translationally modified or unique to a particular isoform. This MRM variant therefore necessitates the inclusion of specific transitions regardless of their ionization efficiency/signal quality and may require alternative enzymatic digestion strategies. Constrained MRM assay libraries that carefully identify and quantify peptide modification states and peptide isoforms may be time consuming and expensive to generate, but they can be key components of context dependent cell signaling studies. The generation of context-dependent constrained MRM data will require sufficient sensitivity to detect endogenous signaling in as little as a few nanograms of initially-injected protein digest. As discussed in section 2.4, emerging techniques to either enrich for low abundant signaling targets or to achieve more efficient separation help to profile the signaling proteome *in situ*. The scope of this challenge is great, but the clinical implications are enormous.

2.3 Emerging MS approaches: Data Independent Acquisition

With high sensitivity proteomics platforms (e.g. MRM assays) there is a practical limit to the total number of proteins and/or protein modifications that can be detected in a given technical sample run, which can reduce the comprehensiveness of the proteomic analysis. This can be mitigated by including additional multiplexes of a given sample to increase the total number of targets that can be detected and analyzed, however the low cell and protein yields inherent to LCMD workflows often preclude that strategy for target expansion. Furthermore, the targets of interest (and modified residue, if desired) must be identified *a priori* and only those targets are analyzed in the experiment. To obtain unbiased proteome analysis, shotgun DDA MS is traditionally used, however, as mentioned previously, this strategy is limited in terms of the comprehensiveness and dynamic range of proteome coverage obtainable. Emerging DIA strategies are combining the strengths of targeted and shotgun DDA MS while bringing unique approaches to mitigate the limitations of either of traditional approaches to proteomic quantitation.

In contrast to shotgun MS, where the instrument is programmed to make stochastic decisions regarding peptide selection for MS/MS fragmentation, the DIA approach uses defined precursor windows to fragment all co-eluting species within a given m/z range over the course of a chromatographic run. The resulting MS/MS spectra depict a complex chimeric fragmentation pattern that is derived from a multitude of precursor peptide ions, and the challenge of deciphering and assigning sequence identities requires sophisticated bioinformatics approaches that have only recently been developed.

Modern bioinformatics approaches that enable DIA analysis allow both the identification of the peptide species from complex fragmentation spectra, and also their reliable and accurate quantification. DIA using sequential narrow m/z windows was first applied in 2004 in a study by the Yate's group with downstream complementary use of MRM assays for selected protein quantitative follow up (45). Wang and colleagues applied MS spectral libraries for the interpretation of DIA data specifically allowing for the assignment of a protein based on the correct matching of a few preselected peptides present amongst the complex peptide composition of the MS library(46). Building on the strengths of MRM targeted acquisition strategies and enabled by the development faster MS scanning instrumentation, Gillet *et al* in the Aebersold lab described the extraction of MRM-like peptide transition group data from complex chimeric peptide spectra composed of all ions fragmented within a series of small defined m/z windows (e.g. in their initial example, 32 windows, each spanning 25 m/z) (47). This approach was termed SWATH for its use of the serial window acquisition strategy. DIA approaches such as SWATH combine the advantages of both the unbiased data acquisition with subsequent data mining being targeted to peptides of interest that represent large number of selected proteins of interest. This contrasts with the traditional MRM approach, in which data acquisition is limited to specific transition groups as they elute from the LC system, which limits the number of proteins that can be quantified at any one time.

One of the distinctive benefits of the untargeted acquisition inherent to DIA MS is that all observable ions are captured from a given sample injection, and represent a map of the

observable proteome for a sample at a given point in time. This map can be read against a library of peptide transition group ‘assays’ to identify and quantify proteins of interest, and can be reiteratively analyzed as new hypotheses develop out of initial observations. Theoretically, an ion library composed of all MS observable proteotypic peptides from across the genome could be queried against a DIA file for comprehensive analysis of the proteoforms present in a specific sample at a given time. In practice, sensitivity and dynamic range limitation still constrain the depth of proteome coverage obtainable with current DIA technologies, but ongoing technological advancements continue to push these limits. The use of as many as 200 variable width precursor isolation windows (as opposed to the initial 32 fixed-width m/z-wide windows) by Hunter et al. (48), or multiple overlapping precursor isolation windows by Egerton et al. (49) are two examples of how the strategy for data acquisition can enhance precursor ion selectivity and overall sensitivity of DIA-MS. Thus, the capabilities of DIA even in its burgeoning state promise to be a revolution in the field for proteome profiling and quantitation, as is already evident in the handful of publications representing the first demonstrations of the biological applications for the technology.

The SWATH acquisition and analytical approach can achieve a low femtomolar (0.0456 fmol) limit of quantitation of N-linked glycopeptides in human plasma. Although this is approximately 2–3 times less sensitive than an MRM assay targeted to the same peptides, quantitative coefficients of variation were equivalent (CV of 13.4% and 14.9% for SRM and SWATH, respectively) (50). Despite its penalty in sensitivity relative to MRM, the DIA approach is compatible with massive multiplexing and has the unique capability of allowing iterative re-interrogation of previously acquired data against evolving and expanding transition libraries. This approach is particularly advantageous for the analysis of large sample numbers, which lends itself to the analysis of experiments that monitor signaling events across hundreds of clinical patient samples. Furthermore, in contrast to MRM, DIA allows datasets to be re-interrogated for targets of interest beyond those identified in an initial study design.

In late 2013, Lambert *et al* from the Gingras group and Collins *et al* from the Aebersold group published descriptions of the application of SWATH-MS to quantify affinity-purified protein complexes (AP-SWATH) (20, 51). In the Lambert *et al* paper, 1300 peptides from a CDK4 affinity purification were monitored in a comparison of quantitation between SWATH and the more traditional DDA analysis. This comparison illustrated the stochastic nature of DDA-based ion selection, which resulted in a lack of data matrix completeness across biological replicates and treatment groups.

Furthermore, SWATH-based DIA resulted in improved quantitative reproducibility compared to both an MS¹ quantitation and an iTRAQ-based DDA approach (20). The study by Collins *et al* used a similar strategy to enrich the 14-3-3 interactome, and monitored 1,967 binding proteins across all replicates of a 6-treatment, 4-time point study. This data matrix completeness enables confident true-interacting proteins to be differentiated from non-specific binding partners, which in turn allows the initial data set to be pared down to 567 high-confident interacting pairs (51). Furthermore, Collins *et al* re-queried the same AP SWATH files against a phosphospecific transition group library, which allowed for the

quantitative monitoring of 2,337 unique phosphosites within the original 14-3-3 interactome datasets (51).

The observation by Lambert *et al* that SWATH-DIA could yield improved quantitative reproducibility stands as an important contrast to observations from a metabolomics-based comparison with DDA, which found that the more convoluted nature and quality of DIA-MS² spectra conferred a slight penalty onto SWATH data (52). A direct comparison using small molecules revealed superior reliability of the ion sampling inherent to SWATH relative to that of a DDA sampling approach (52, 53). The SWATH acquisition approach utilized in this comparative analysis was one of the first employed, with wide MS¹ selection windows of a fixed 27 Dalton width. Newer narrower and variable window protocols for SWATH-DIA allow fewer ions and decreased spectral complexity in each window, which may further improve SWATH-MS² quality and signal-to-noise ratios. Experiments dedicated to similar comparisons between DDA and DIA peptide quantitation, rather than small molecule and metabolite samples, are warranted and will further inform the field.

With respect to the limited sample quantities that are traditionally inherent to context-dependent cell-signaling studies, the typical injection mass of peptides on-column for a SWATH workflow corresponds to approximately 1 µg (or less) of enriched protein, which approaches sample amounts generated with LCMD. Ongoing developments in data acquisition parameters, instrument speed and sensitivity, and downstream analysis methods will help to increase the capabilities of quantitative DIA methods, and promise to expand its functional dynamic range. Such improvements will be required during the maturation of DIA-based MS, and for SWATH-like techniques to establish their place among techniques capable of studying context-dependent signaling from LCMD tissue samples.

2.4: Emerging techniques towards lower limits of detection and quantitation: Electrophoretic separation and microfluidic enrichment devices

The enrichment of low abundant protein and peptide targets is an important and powerful approach for an expanded interrogation of the signaling proteome. These strategies to study cell signaling include immunoaffinity purification of interacting proteins (co-IP) and the enrichment of peptides carrying a particular post-translational modification. The low protein quantities resulting from selective acquisition protocols such as LCMD and flow sorting represent a severe constraint that magnifies the challenge of quantitatively detecting the low abundant proteoforms that mediate crucial signaling events. To address this challenge, microfluidic lab-on-a-chip-type platforms can maintain low working volumes that both scale down the overall enrichment workflow and minimize sample loss in downstream processing steps. Additionally, the orthogonal separation principle behind CE, combined with its low working volumes, can be used in concert with chromatographic techniques to provide complementary data that expands the proteomic coverage of a workflow.

Lab-on-a-chip microfluidic devices represent an innovative strategy to address the challenges of micro-scale co-IP protein and PTM-specific peptide enrichment. In 2010, Sandison *et al* introduced a device that employed an antibody-functionalized polymethylsiloxane microcolumn and demonstrated efficient capture and detection of sub-nanogram amounts of target protein from approximately 4 µg of input material with a

system that was not directly coupled to MS (54). In another proof-of-concept work, chip-based histone immunoprecipitation, performed from as few as 50 cells, can be used to sequence bound chromatin regions (55). While this assay relies on PCR amplification, it clearly demonstrates that antibody-based immunoprecipitation is sufficiently sensitive for LCMD tissue segments. Indeed, Li *et al* (2002) fabricated a device with an immunoaffinity enrichment channel with an electrospray outlet, direct coupled to a TOF/TOF mass spectrometer (56). As proof of concept, the resulting enrichment 1 ng of a c-myc peptide spiked into 50 μ l of trypsin-digested serum resulted in a 10:1 signal-to-noise ratio of detected target peptide. Although these applications result from chips produced in-house, several commercial vendors now offer design and bulk production of custom microfluidic devices (e.g. Microunit, Thinxxs).

The Li group, using tryptic digests from purified alpha-casein, has also shown that off-chip (FeIII) IMAC micro-columns conceptually represent an emerging and efficient enrichment platform for phosphopeptide analysis (56). Other non-chip methods, including microtip enrichment and serial column set-ups have also been applied to metal-affinity enrichment of phosphopeptides from as little as 1 μ g of starting material (57–59). LCMD in-line with micro-scale enrichment coupled to highly-sensitive MS-based detection may provide a path forward for qualitative and quantitative investigations of contextual cell signaling; whether the quantity of purified target proteins from micro-scale sample acquisition and chip-processing workflows will be sufficiently sensitive and quantitative with current discovery LC-MS workflows and instrumentation remains to be seen.

Chromatographic and electrophoretic separations each have their strengths and limitations, but because the separative principle that underscores these techniques is different, their strengths and limitations are complementary. Indeed, orthogonal separation offered by CE-ESI-MS/MS can extend the coverage of the secreted *Mycobacterium marinum* proteome by 53% compared to UPLC-ESI-MS/MS(60). The low sample requirements of CE, combined with its high separation efficiency, ultra-low flow rate, and fast separation times make this technique attractive, but its routine application has historically been limited due to the technical complexities of interfacing CE with MS. Recent innovations continue to improve this compatibility, and commercially available sheath flow and direct ESI interfaces are available for CE-MS and have been applied to bottom-up proteomics (reviewed in (61)). From a proteomics perspective, a bottom-up experiment of pre-fractionated *E. coli* tryptic digests, using CE-ESI-MS/MS yielded 4902 peptides corresponding to 871 proteins, in analysis that consumed less than 1 μ g of sample (62). Zhu *et al* compared CE-ESI MS/MS with UPLC-ESI-MS/MS with an analysis of *E. coli* lysate using the same MS analysis time. They found that the UPLC-based method identified more (and complementary) peptides than its CE counterpart when 100 ng of sample was loaded. Interestingly, the reverse was true when the more mass limited 1 ng digest was loaded (63), suggesting a potential advantage of CE-MS to analyze the low sample volumes inherent in methods to capture context specific signaling.

CE-MS/MS has also been used as a platform to enhance sensitivity in targeted MRM assays. Leu-enkephalin has been quantified across nearly 4 orders of magnitude in a background of bovine albumin tryptic digest. This quantitation was accomplished in an assay time of less

than 6 min with a detection limit of 60 pM, which, given the small sample requirements of CE, corresponded to a total injection of 335 zmol of peptide (64). Similarly, Sun *et al* performed CE-MRM to 100 pg of cell lysate digest and were able to monitor 36 transitions, corresponding to nine abundant proteins, in under 10 minutes (65).

The integration of emerging microfluidic and CE technologies for sample enrichment and analysis can provide additional dimensions to *in situ* signaling studies while minimizing sample consumption. Coupled with the sensitivity of MRM or with the sensitivity and multiplexing of emerging DIA technologies, a workflow of optimized sample acquisition, microfluidic processing, high fidelity separation, and optimized detection by MS can maximize yield of quantitative data from a multitude of low abundant intermediates of context specific-specific signaling cascades.

3. Antibody-based Proteomics Tools

3.1. Profiling by Antibody Array

Antibody and other protein capture arrays and their reverse-phase protein array correlates are established techniques for the analysis of signaling in small-scale tissue sections. Classical capture-based arrays utilize immobilized high-affinity biomolecules, typically antibodies, that specifically bind protein antigens either directly or through intermediary peptide or small-molecules that in turn secondarily bind a protein of interest (66, 67). This technique can provide only relative quantitative information between samples by labeling test samples with a differentiating set of fluorescent dyes prior to capture, and subsequently reading sample-specific fluorescence intensities. Reverse-phase protein arrays, in which the sample lysate itself is immobilized and then blotted with labeled antibodies against various targets of interest, are inversions of this principle (68).

The specific use of these related technologies for proteomic profiling of clinical tissues is reviewed elsewhere (68). From the perspective of context dependent signaling, capture-based strategies rely both on high quality antibodies or other capture agents (with little or no cross reactivity and high specificity and sensitivity) and on *a priori* knowledge of their targets. Consequently, less studied proteins or post-translational modifications do not uniformly have reliable and consistent commercially available antibodies or other capture reagents. This limitation can skew experiments toward well-characterized “hub” kinases and signaling molecules at the expense of less common pathway discovery. Industrial-scale production and validation of high quality antibodies to a multitude of popular and more obscure targets would enhance the value of capture-based arrays for discovery level proteomic analysis of signaling from small amounts of clinical tissue specimens. One such initiative is exemplified by the systematic approach of the Structural Genomics Consortium led by Aled Edwards at the University of Toronto with collaborating institutions (69). A primary issue concerns the potential lack of specificity between targets and their capture agents, which is influenced by binding kinetics and protein concentrations. Therefore, while antibody arrays can provide valuable information about relatively well-characterized components of signaling pathways, the elucidation of novel interactions, less-known proteins, or unpredicted PTMs all present a much larger challenge.

3.2. Mass Flow Cytometry (CyTOF)

One major advantage of flow cytometry is its capacity for multiplexing using combinations of different detection fluorophores (70). While spectral overlap in the emission wavelengths of common fluorescent antibody conjugates limits multiplexing beyond 11–15 targets (70), flow mass cytometry, performed within an MS-workflow called CyTOF (cytometry by time of flight), creatively expands this number of targets (71–73). In brief, metal isotopes are conjugated to detection antibodies, which are then used to bind their intracellular targets. Labeled cells are sorted along narrow-bore capillaries into single cell streams, vaporized, and the resulting metal ions are fed into a TOF mass spectrometer (71). Each metal ion generates a discrete isotopic mass/charge (m/z) peak, and its net signal is directly proportional to the amount of labeled target within a given cell.

By circumventing the spectral overlap limitation of immunofluorescence, CyTOF is able to simultaneously detect a multitude of intracellular signaling events in suspended cells. The instrumentation is now capable of detecting up to 100 different signals, although the number of metal species for labeling antibodies is still constrained to approximately thirty two (74).

There are a number of CyTOF-based applications to study cell signaling, many of these have been directed towards understanding function and pharmacology in cell populations isolated from blood or freshly dissociated solid tissues (71, 75, 76). This work has been led by the Nolan group at Stanford, where the synthesis of both reagents and bioinformatics platforms continues to broaden the accessibility and scope of the CyTOF technique. A clear challenge precluding widespread CyTOF application stems from the potential lack of specific markers that can reliably differentiate between distinct cell populations present within the tissue microenvironments of various organs. In this respect, MS-based techniques that identify cell specific surface markers would greatly enhance context-specific cell signaling by facilitating the isolation of specific cell populations from tissues. Cell Surface Capture of N-linked membrane glycopeptides combined with MS analysis (77) has been used to identify several markers that differentiate viable pluripotent stem cell sub-populations (78) and heterogeneity among cardiomyocytes induced from embryonic stem cells *in vitro* (79). The key to the approach developed by Wollscheid's group is that labeling of the extracellular glyco-protein occurs on viable cells allowing one to infer protein orientation. These techniques can extend into the analysis of distinct cell-phenotypes, with examples that discriminate between malignant and near-metastatic tumor cells (80), and those factors that distinguish contractile smooth muscle cells from their secretory counterparts (81). These studies clearly indicate that MS-based workflows are well suited to the task of defining sub-populations and delineating the contextual-dependency of their signaling states.

3.3. Tissue based immuno-CyTOF

The CyTOF technique builds on existing flow cytometry approaches that themselves emerged as an extension of immunofluorescence platforms used to elucidate proteins, protein-protein interactions, and PTMs from thin slices of slide-mounted tissues.

Therefore, extending the use of CyTOF MS beyond cell suspensions onto tissue sections has clear advantages over fluorescence-based approaches. Unlike traditional

immunofluorescence, tissue sections beset with autofluorescence do not hinder CyTOF. Additionally, CyTOF in tissues does not require enzymatic and mechanical dissociation, which preserves biological signaling states and spatial context within a specimen. Finally, tissue analysis enables the detection and quantitation of cell-cell interactions, as well as the intracellular localization of signaling proteins and their PTM-states. For these reasons, the multiplexing capabilities of CyTOF represent a promising frontier for more comprehensive interrogation of cell-type specific signaling within the tissue context.

To this end, Giesen *et al* describe the use of metal-ion conjugated antibodies to probe the cellular heterogeneity and signaling events within tissues using CyTOF MS (82). Using a novel laser-ablation chamber that minimizes aerosol dispersion, they achieved a spatial resolution of 1 μm and lower MS sensitivity limit of 500 molecules, which was adequate for crude subcellular localization of a molecule between plasma membrane, cytosol, and nucleus. In their experiment, these authors analyzed 32 separate proteins and phosphorylation sites within a single paraffin-embedded formalin-fixed breast cancer tumor specimen. In addition to achieving a molecular-based replication of tumor classification performed by surgical pathologists, this study detected new levels of heterogeneity among cellular sub-types, and found evidence of cell-cell crosstalk at the tumor-stromal interface in the form of two phosphorylation sites on S6 protein.

One strength of the CyTOF technique is that it allows conjugated antibodies to specifically target less abundant proteins that may otherwise remain hidden by the dynamic range constraints of MS detection. As with antibody array based profiling, CyTOF still requires *a priori* knowledge of the targets to be analyzed and relies on the availability of high-quality specific antibodies in the experimental design. These limitations will continue to constrain the discovery capabilities of this technique, however CyTOF is positioned as a valuable tool for multiplexed profiling and validation of *in situ* signaling events.

4. MS Imaging

The most straightforward approach to study the proteome under maintained structural and contextual hierarchy is to analyze intact tissue sections. Immunohistochemistry best exemplifies the power of this approach, but a series of technological advances have for more than a decade facilitated MS imaging (MSI), providing a powerful complementary dimension of molecular information to intact tissue analysis. MSI is based in principle on ascribing spectral information to spatial coordinates. In effect, an abundance of biological molecules within the mass range of an instrument can be visualized as an m/z heat map distributed across a tissue section. This is technically accomplished by direct desorption and ionization of analytes from a 10–20 μm tissue section mounted on a slide and coupled to a compatible mass analyser.

Given the versatility of this technique, MSI workflows and capabilities are continuously evolving. Because direct analyte desorption and ionization from mounted tissues is conceptually related to MALDI ionization, MSI has traditionally been accomplished by mounting a tissue section onto a conductive slide, rinsing the section with an appropriate solvent to reduce contaminants and to enrich for a specific class of biomolecule, spraying

matrix onto the slide, and subjecting the defined area of interest to MALDI-TOF/TOF. A recent study used this simple approach to identify eight proteins that were differentially regulated in two subtypes of hepatic cholangiocarcinomas (83). After MSI acquisition, the authors eluted out the sinapinic acid matrix using ethanol and subjected the sections to Hematoxylin and Eosin staining. In doing so, this study highlights the capacity to co-register histological features with MSI data.

The tendency of MALDI ionization to generate a plume of singly charged analytes places many proteins beyond the analytical reach of top-down tandem MS identification. In order to mitigate this potential limitation, the mounted tissue can be spotted and incubated with a protease prior to matrix deposition. This in-situ digestion strategy was pioneered in the Caprioli lab using an array printer to deposit discrete spots of trypsin (84), and remains the basis of more refined bottom-up proteomic iterations of MSI. A useful variant to this in situ digestion approach incorporates toluene or xylene washes to remove MS-incompatible paraffin, which facilitates bottom-up MALDI-MSI of archived patient specimens (85). Nakanishi *et al* recently illustrated this complementarity by performing MSI directly on Congo red stained and in situ tryptic digested formalin-fixed paraffin-embedded tissue sections (86). By relying on a combination of these MSI qualities Nakanishi *et al* could successfully identify and localize amyloidogenic proteins from tryptic peptides in amyloid plaques (86).

The ability to definitively and unambiguously identify proteins from singly charged peptides in a complex matrix remains a fundamental limitation of many MALDI-MSI workflows. Schober, *et al* (87) mitigated this problem by performing successive bottom-up proteomics on adjacent mouse brain sections using an LTQ orbitrap MS; the first section was analyzed with MALDI-MSI while the second section was homogenized, digested, fractionated, and analyzed with high mass accuracy using an LC/ESI-MS/MS strategy. Cross-referencing the protein lists generated with these complementary techniques ultimately produced definitive identities. In a similar sense, incorporating high-resolution MS instruments with improved desorption and ionization of small molecules and peptides represents the natural progression of MSI technology.

In an effort to ultimately map context-dependent, hypoxia-induced molecular changes in the tumor microenvironment, Chughatai *et al* demonstrated a similar parallel MS workflow based on tandem dimer Tomato (tdTomato) red fluorescent protein expression in a breast tumor xenograft model under a hypoxia response element-containing promoter (88). In order to definitively identify and to generate a list of readily detectable tdTomato tryptic peptides, fluorescent bands from tissue lysate were excised from gels, digested with trypsin, and subjected in parallel to nano-LC/MS with an LTQ Orbitrap and MALDI-MS using a qTOF. The spatial localization of one of these tdTomato peptides were subsequently determined by MALDI-MSI, and the image was compared with brightfield and fluorescence microscopy to reveal good correlation and exclusive expressions in hypoxic xenograft regions.

In addition, bottom-up MSI-based proteomics workflows that incorporate reporter constructs represent an important step towards providing co-localization information that more directly addresses contextual differences. Nevertheless, several important hurdles stand between

current MSI techniques and their routine use in context-specific measurements. Chief among these are the capacity to perform quantitative proteomic imaging experiments, and the power to sufficiently overcome the dynamic range of proteins in intact tissues and thereby achieve adequate sensitivity for lower abundance proteins and peptides.

In its simplest form, MSI directly measures analytes, but more complex mass spectrometric strategies that involve isotopic labeling and isobaric tags have the potential to endow MSI workflows with quantitative and comparative capacities. Rather than expressing marker proteins under a hypoxia-responsive promoter, Djidja *et al* overlaid bottom-up, relative MALDI-MSI intensities of several peptide targets MALDI-MSI with immunohistochemical staining of selected hypoxia protein markers. Djidja *et al* further complemented their in situ imaging data with in vitro SILAC-based LC-MS/MS quantitation of tumor cells grown in hypoxic or normoxic conditions; and correlated their results with LC-MS/MS results from LCMD excised and pimonidazole (hypoxia) stained regions using an LCQ-Orbitrap instrument (89).

The broad dynamic range of the proteome still remains a major challenge for direct MS-based bottom-up MSI analysis of tissues. The subset of peptides that are most susceptible to proteolysis and ionization, and derived from the most abundant proteins tend to dominate the MS signal at the expense of less abundant species. This challenge is most frequently addressed with a combination of sample fractionation and electrophoretic and/or chromatographic separation upstream of MS analysis, none of which are compatible with MSI techniques that rely on direct desorption of analytes from tissues. In searching for tdTomato-derived peptides, the Chughatai study illustrated this limitation: Ten separate tdTomato peptides were represented by twenty spectra –one single spectrum was derived from MALDI while nineteen were derived from ESI-LC-MS/MS (88).

Fortunately, several emerging variations of MSI have been making significant strides towards addressing this limitation. In one such example, Quanico *et al* combined MALDI-Orbitrap-MSI data with in situ tryptic peptide extraction using a liquid microjunction interface followed by off-tissue high-resolution nanoLC-MS/MS analysis (90). Although this technique has an estimated spatial resolution corresponding to an estimated 1900 cells, hierarchical molecular data clustering on MALDI-MSI results identified cell populations from molecular signatures within a tissue. In particular, this method was used for spatial back-correlation of nanoLC-MS/MS data. The strength of this approach is that it connects MSI with an additional separative component that consistently resulted in the identification of about 1500 proteins - albeit at the large expense of sample processing time and spatial resolution (90).

Park and Murray recently used an alternative approach to incorporate a pre-separation step between sample ion desorption and MS detection by using an IR laser-ablation technique, this group demonstrated that protein standards deposited onto a plate could be captured under ambient conditions, electrokinetically transferred to a capillary for electrophoretic separation coupled directly to ESI-MS analysis (91). The small sample requirements and high resolving efficiency of CE-based separations could lend themselves well to in situ MSI applications.

Other solutions to the dynamic range challenge that is inherent to MSI combine orthogonal ionization approaches with high-resolution MS instrumentation. This combination is critically important for top-down MSI approaches because these experiments require the generation and deconvolution of isotopic envelopes derived from multiple high-charge states of a particular protein. One orthogonal ionization approach makes use of IR-induced hydroxyl group excitation of intrinsic water molecules. This matrix-free ablation is accomplished under ambient conditions and coupled with an electrospray plume to provide a supply of multiply charged analytes (92). Sampson *et al* reported top-down analysis of protein samples using an IR-MALDESI ionization source at ambient temperature and pressure, coupled to an FT-ICR instrument (93). Although this initial top-down analysis was not performed on tissues, a subsequent manuscript from this group describes the IR-MALDESI/FT-ICR combination in the context of an imaging interface, which resulted in sufficient spatial resolution to resolve 45µm features of a cholesterol-derived ion on a calibration grid (94).

Interestingly, fundamental IR-MALDESI/FT-ICR research has revealed that a minute layer of ice can serve a useful matrix to facilitate ablation and improve sensitivity (95). From the perspective of context-dependent, top-down MSI, a recent study by Kiss *et al* could detect several proteins and identify haemoglobin from 50µm mouse lung sections using laser ablation ESI/FT-ICR (96). Although haemoglobin is highly abundant, this group was additionally able to identify and map an acetylation event. As such, this study represents the first report of top-down analysis of multiply charged proteins identified *in situ* by MSI, and demonstrates the feasibility of this approach for studying intact proteoforms and their distribution in tissues (96).

The ideal MSI experiment to studying context-dependent differential cell signaling events would achieve high-confidence proteoform identification across a wide dynamic range with single-cell spatial resolution. No single MS instrument or workflow can meet every need. Currently, the highest spatial resolution for MSI of a protein in the literature was 10 µm (97), but typical experiments result in a limited amount of confidently identified abundant proteins derived from spot sizes that are an order of magnitude larger, reviewed in (98). Nevertheless, the use of high-resolution instruments with matrix-free ionization and probe-based sampling (99) can already provide single cell analysis and even subcellular spatial resolution for phospholipids and smaller biomolecules (100), reviewed in (98). MSI workflows for proteomics are evolving towards these impressive capabilities.

5. Data Integration and Systems Modeling

MS-based investigation of *in situ* signaling continues to rely on biological specimens frozen at a single point in time, while signaling is a rapid, coordinated, and dynamic process. This is especially true in tissues, where various cell types are integrating a multitude of different stimuli at any given time. The interpretation of highly complex data, derived from comprehensive profiling of context-specific signaling states within discrete tissue cell populations, remains a significant challenge.

The first step in the analysis of complex signaling dynamics often involves the assembly or integration of data into informative visual maps of signaling networks. Gehlenberg et al reviewed many available tools that can facilitate this visualization, which are loosely organized into one of two terms: Protein-protein interaction network assemblies annotated according to experimental evidence of direct protein-protein binding, or functional pathway assembly annotated according to functional ontology (101). The visualization of signaling data by these means is an important component of systems analysis, but networks tend to be oversimplified and lacking in information that pertains to the activation state of a given molecule. These states, which are defined by one or more PTM events along multiple sites on the same protein, thereby define the binding capacity of that protein and its interactions with neighboring molecules. Thus, models and visualization tools that encompass the full complexity of signaling information transfer are critical components of network analysis. The use of more sophisticated models will enable a greater understanding and ultimately a prediction of these dynamics within normal and disease contexts.

Ideally, models that enable a comprehensive systems analysis of cell signaling in its full complexity will enable biologists to link static signaling snapshots with a dynamic biological phenotype. These models are mathematically sophisticated, but to be adopted and utilized by the broader scientific community they must be rendered accessible and intuitive to the biologists who will ultimately implement them. Already, software tools like Simmune (102), Morpheus (103), and VCell (104) are designed to simplify the mathematical and computational challenges of complex system modeling, and to render these tools to basic biologists. These software tools complement the large repository of proteomics databases and the literature that specifically describes protein-protein interactions and dynamic changes in PTMs. These combined resources are currently available for immediate implementation, and provide a foundation for the initial modeling of a system or pathway of interest.

From an experimental perspective, existing technologies like affinity-purification MS that analyze proteoforms and integrate co-IP data into protein interaction networks can fill in gaps in models of cellular communication. A description by Angermann *et al* of subcellular localization and MAPK-signaling during pheromone sensing in yeast represents a practical example of integrating signaling data with modeling software (105). A traditional scaffold-based kinase-target interaction was unable to account for the 40% phosphorylation of the yeast pheromone response protein, Fus3, within an observed time frame. However, a more refined model was able to predict a direct kinase-target binding interaction, and in doing so provided a biologically reasonable interpretation.

Thus, concept-based sophisticated modeling, in combination with highly controlled *in vitro* systems with defined perturbation conditions, can integrate and account for a large degree of experimentally derived information. This will become increasingly more important as higher throughput MS-based methods facilitate the routine generation of quantitative data for specific and multiple proteoforms. These computational strategies will become increasingly important for the interpretation of context-dependent *in situ* MS-based cell signaling data. Their applications are far reaching and hold great potential to help set a framework of understanding. Targeted therapeutic strategies could be experimentally tested and

interpreted in a conceptually similar manner, where discordant signaling events are interpreted in concert by the extent of their reversion to a native, disease-free state.

The implementation of sophisticated computational models, refined and informed by experimental data designed to integrate context-dependency in cell signaling events, represents an improved way to assess therapeutic potential *in silico* (106). Still, interpretive validity remains rooted in experimental design; Technologies and workflows are increasingly capable of generating quantitative, spatially resolved, proteomics data, and biological studies must be equally capable of accounting for context dependent signaling events.

6. Conclusion

Technical capabilities for studying and integrating the signaling proteome have vastly improved over the course of the past two-decades. These advancements have produced new insights into the basic assembly of interactomes and the alteration of protein-protein interaction networks by various chemical and biological perturbations. Despite experimental elegance and precise technical design, the majority of signaling studies have been performed using *in vitro* models, most often monocultures of immortalized cell lines, which limit their predictive validity beyond key signaling nodes. As the technologies outlined in this review mature, it will become imperative to combine data derived from basic signaling building blocks with insights into the communicative behavior of a cell in its more complete contextual milieu. The merger of existing *in vitro* techniques with novel context specific methodologies will provide the best clues to improving the efficiency with which pre-clinical discoveries translate into effective therapeutics.

Acknowledgments

SJP salary support is provided by a McKusick Postdoctoral Fellowship award through the National Marfan Foundation

KR holds a Postdoctoral Fellowship award from the Canadian Institutes of Health Research (FRN: MFE123700) and has received support from the NHLBI Program of Excellence in Glycosciences (PO1HL107153)

Work in support of this manuscript has been conducted through the NHLBI Johns Hopkins Proteomic Innovation Center in Heart Failure- HHSN268201000032C (JVE), the Glycoconjugates and Cardiovascular Disease PO1HL107153, and the Molecular Biology of Marfan Syndrome grant- R01AR041135 (JVE)

List of abbreviations

MSI	Mass Spectrometry Imaging
LCMD	Laser Capture Microdissection
DDA	Data Dependent Acquisition
DIA	Data Independent Acquisition
FFPE	Formalin fixed and paraffin embedded
FASP	Filter assisted sample preparation

RT	Retention time
SWATH	Serial window acquisition strategy
CyTOF	Cytometry by time of flight

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Figure 1.

Studies examining the signaling proteome are often performed in reduced preparations of monocultured cell lines (left schematic). Signaling within these cells occurs absent the typical complexity of microenvironmental cross talk that is present in the tissue context (right schematic). As a consequence, the signaling response to a given ligand of *in vitro* preparations can be quite different from the response that would occur in the complex *in situ* environment. The consequence can be extreme as a complete change in the functional response of a given cell type to a given stimulus between the *in vitro* and *in vivo* conditions. Thus, the different parameters that mediate and regulate signaling in a given cell between the *in vitro* and *in vivo* signaling state can result in different interpretations of how a particular genetic mutation or pathological event drive disease and also differences in the effectiveness of particular therapeutic agent. In the figure, red is used to imply activation of a particular signaling molecule, green is used to imply inhibition or a decrease in activity. Grey molecules are in a neutral or basal state. Each node with altered activity represents a potential target for therapeutic intervention.

Table 1

Summary of technologies covered in this review that can facilitate context-dependent proteomics analyses along with their major advantages and challenges. LC and CE provide peptide separation inline with MS; Antibody arrays and CyTOF are antibody-based approaches; MS Imaging (MSI) directly analyzes tissues.

	Selective Sample Acquisition	Advantages	Challenges
LC or CE separation* & Mass Spectrometry	Laser capture microdissection or tissue dissociation & cell sorting	Can be independent of <i>a priori</i> knowledge of targets (DDA, DIA)	<ul style="list-style-type: none"> • Small sample starting material • Sensitivity to low-abundant signaling events
Antibody Array	Laser capture microdissection or tissue dissociation & cell sorting	High Sensitivity	Requires <i>a priori</i> knowledge of targets and high-quality antibodies to recognize them
CyTOF	Tissue preparations on slides	Single-cell analysis of up to 32 (potentially 100) targets per cell	Requires <i>a priori</i> knowledge of targets and high-quality antibodies to recognize them
MALDI Imaging	Tissue preparations on slides	<i>In situ</i> discovery	Spatial resolution, sensitivity, and dynamic range

* LC = Liquid Chromatography; CE = Capillary Electrophoresis