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Resolving cellular signaling in space and time: From organelle proteomics to spatial phosphoproteomics

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Cellular signaling is inherently organized in space and time, requiring coordinated control of protein localization, molecular interactions, and enzymatic activity across subcellular compartments. Recent advances in chemical biology, protein engineering, and quantitative proteomics have made it possible to interrogate these dimensions in an integrated manner. Here, we highlight emerging strategies to resolve signaling organization across three interconnected dimensions: organelle-resolved proteome mapping to define spatial context, proximity labeling to capture local protein interaction networks, and spatially resolved phosphoproteomics to quantify signaling outputs. Developments in proximity labeling, including split, conditionally activated and light-gated enzymes, enable temporally controlled, context-dependent profiling of transient protein assemblies in living cells. Advances in high-throughput and low-input phosphoproteomics, together with improved computational frameworks for kinase activity inference and subcellular enrichment strategies, are enabling spatially resolved measurement of signaling activity. Together, these approaches are shifting the field from static localization maps toward dynamic models of signaling networks.

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Introduction

Cells interpret extracellular and intracellular cues within spatially organized signaling networks: proteins are localized within organelles, concentrated in microdomains, assembled into protein complexes, and dynamically modified by posttranslational modifications in response to perturbations [1]. Understanding how signaling networks operate therefore requires not only identifying their molecular components but also where they are in the cell, with whom they interact, and when they are enzymatically active or modified—ideally measured in living cells and under physiological conditions.

These three dimensions—localization, molecular interaction, and posttranslational modification—have largely been studied in isolation, in part because the experimental approaches to measure them simultaneously and quantitatively at scale have until recently been lacking. Advances in chemical biology, protein engineering, and quantitative proteomics are now making it possible to interrogate these dimensions in parallel. Here we describe recent methodological developments and applications to resolve cellular signaling organization across space and time within a framework of three interconnected dimensions: organelle-resolved proteome mapping to define spatial context [1,2], proximity-based labeling strategies to capture local protein interaction networks with temporal and conditional precision [3], and spatially resolved posttranslational modification measurements with the focus on phosphorylation [4]. Throughout this article, we highlight practical considerations for experimental design which are summarized in Table 1.

Localization–defining spatial context through organelle-resolved proteomics

Cells are highly compartmentalized into organelles enabling distinct biochemical processes to occur in parallel. The subcellular localization of a protein influences its function by constraining its physicochemical environment and the available biomolecules for interactions [5]. Accordingly, multifunctional proteins can execute distinct and independent physiological roles depending on their spatial context [5]. This spatial encoding of function is particularly relevant for signaling proteins, whose activity is often gated by compartment-specific access to substrates, scaffolds, or regulatory cofactors [1,5,6]. Recent advances in quantitative spatial proteomics now allow systematic mapping of organellar proteomes and how these dynamically reorganize under perturbations, for example, through subcellular fractionation and affinity-based purification of individual organelles (Figure 1).

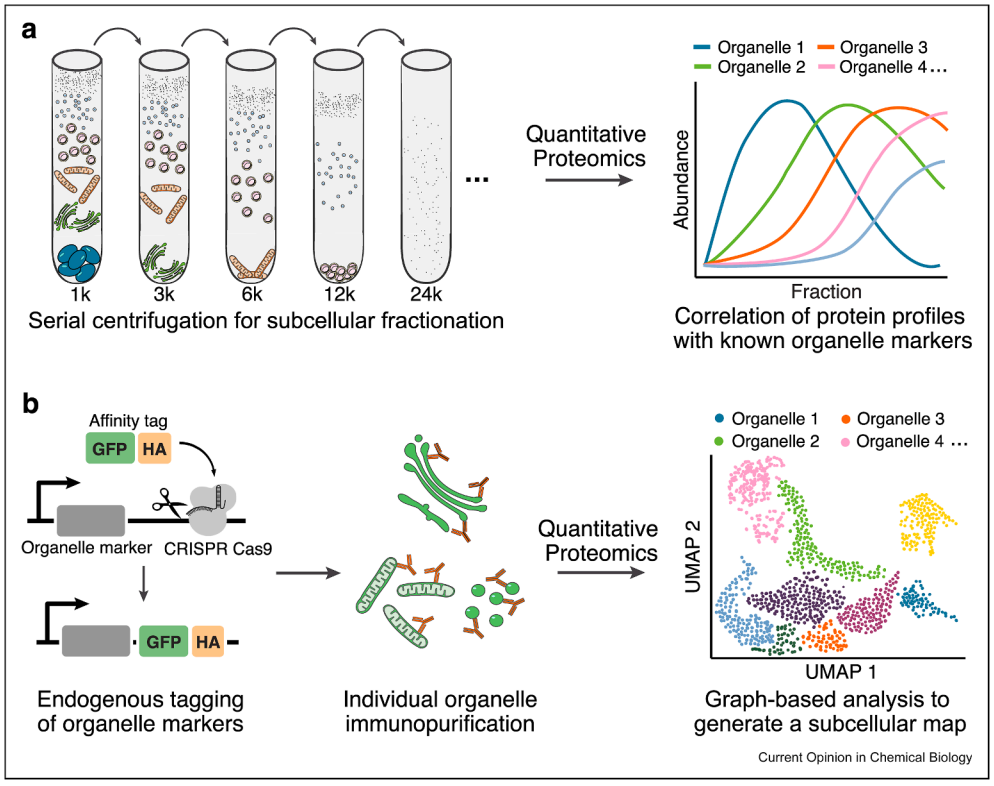
Table 1

Overview of spatial proteomics and phosphoproteomics approaches, summarizing key experimental requirements, and limitations. References to detailed protocols are provided for each method.

Approach	Methods	Experimental considerations
Subcellular Fractionation	LOPIT [62] hyperLOPIT [63] LOPIT-DC [7] DOM [10] SPLAT [64]	**Requirements** • Organelle markers for subcellular classification and validation. • Whole cell lysates as reference. • Normalization to total abundance. • Consistent cell lysis without organelle disruption. • Computational deconvolution for protein assignment. **Limitations** • Limited resolution as organelles of similar size/density. • Difficulty assigning multilocalizing proteins. • Variable organelle recovery efficiency.
Organelle IP	Lyso-IP [65] Endo-IP [20,21] Golgi-IP [19] Melano-IP [22] Organelle-IP with endogenous tagging [25]	**Requirements** • Surface organelle marker as handle for IP. • Validation of tagged organelle marker location. • Avoiding overexpression artifacts (e.g. through endogenous tagging). • Negative controls (different affinity tag, beads only). • Confirming organelle integrity following mild lysis and IP. **Limitations** • Independent engineered cell line and IP for each organelle. • One organelle per experiment limits multiplexing. • Limited to genetically tractable systems. • May miss transient organelle-associated proteins. • Cannot capture organelle contact site composition.
Proximity Labeling	TurboID/splitTurbo [66] APEX2 [67] iAPEX [35] LOV-Turbo [36]	**Requirements** • Genetic fusion of protein of interest with proximity labeling enzyme. • Confirmed fusion protein localization, function, and labeling efficiency. • Endogenous expression levels preferred to avoid mislocalization. • Optimization of labeling duration for signal-to-noise balance. • Negative controls (no biotin/H2O2, unfused/cytosolic localized enzyme) **Limitations** • Cannot distinguish direct vs. proximal interactions. • Background labeling using endogenous biotin (TurboID). • H_2O_2-induced oxidative stress and cytotoxicity (APEX2). • Potential perturbation of protein of interest localization and function by enzyme fusion. • Variability in labeling efficiency across subcellular environments.
Phosphoproteomics	Automated phosphoproteomics [39] SPARCE [42] μPhos [37] nanoPhos [43]	**Requirements** • Rapid cell lysis with phosphatase and protease inhibitors to preserve phosphorylation status. • Enrichment of phosphopeptides (IMAC/TiO_2). • Phosphosite localization scoring to resolve ambiguous sites (e.g., adjacent serines/threonines). • Normalization to total protein abundance to distinguish phosphorylation changes from expression changes. **Limitations** • Phosphopeptide changes might be confounded by protein abundance differences. • Occupancy/stoichiometry information is lost. • Bias toward phosphoserine/threonine; separate enrichment needed for high phosphotyrosine coverage. • Incomplete kinase-substrate annotations. • Unknown functional relevance of most detected phosphorylation sites.

IP, immunoprecipitation; SPARCE, Streamlined Phosphoproteomic Analysis of Rare Cells.

Figure 1



Complementary approaches for mapping subcellular proteomes balancing proteome-wide coverage and organelle specificity. For subcellular fractionation, organelles are separated into fractions by serial centrifugation, and protein abundance profiles across fractions are quantified by mass spectrometry. Localization is inferred by correlation with known organelle markers **(a)**. For organelle immunoprecipitation, individual organelle markers are affinity-tagged (for example, endogenously tagged via CRISPR-Cas9), enabling selective isolation of individual organelles for quantitative proteomic analysis. Computational clustering resolves organelle-specific proteomes **(b)**.

Subcellular fractionation is a well-established method for globally assessing protein localization at a proteome scale (Figure 1a). Cells are lysed under mild conditions to preserve organelle integrity, followed by separation into 8–15 fractions using differential ultracentrifugation [7,8] or detergent-based fractionation [9]. Quantification of proteins across organellar fractions using mass spectrometry–based proteomics yields abundance profiles characteristic of the harboring organelles and protein localization is generally inferred based on correlation with profiles of known organelle markers. For protein quantification across fractions, tandem mass tag (TMT) labeling was initially used in combination with data-dependent acquisition (DDA) as introduced by localization of organelle proteins by isotope tagging (LOPIT) [7]. The multiplexed labeling enables quantification of all fractions within a single measurement, thereby minimizing missing values and ensuring reproducible protein abundance profiles across fractions. More recently, subcellular fractionation has been combined with data-independent acquisition (DIA)-based quantification [10–12]. Unlike DDA, which samples the peptide precursor ions semi-stochastically leading to missing values across runs, DIA systematically fragments all ions within defined mass windows, ensuring comprehensive and reproducible quantification with greater proteomic depth without requiring chemical labeling by TMT [13,14].

The analysis of subcellular fractionation datasets has advanced substantially through new computational tools with complementary functions. For data processing and quality control, DOM-ABC (dynamic organellar maps-analysis, benchmarking, and quality control) provides a user-friendly framework for standardized analysis and benchmarking of fractionation experiments [10]. For protein localization prediction, C-COMPASS (Cellular COMPartment clASSifier) uses a neural network classifier to assign proteins to compartments based on learned fractionation profile patterns [15]. As an alternative, semi-supervised Bayesian frameworks model localization as a probability distribution, enabling explicit quantification of uncertainty in protein assignments and integration of

fractionation data with orthogonal information such as Gene Ontology annotations [16]. For systematic evaluation, machine learning benchmarking efforts have assessed the relative performance of subcellular classification models, providing guidance on method selection [12]. Together, these tools enable robust extraction of localization information from large-scale fractionation datasets.

Since subcellular fractionation provides localization information for thousands of proteins in a single experiment, it is a powerful approach to determine perturbation-dependent changes on subcellular proteomes. This was exemplified by Currie et al. showing that unfolded protein response-mediated stress results in organelle-specific protein turnover rates [17]. However, disentangling closely related compartments is more challenging using subcellular fractionation by differential centrifugation and requires approaches that focus on individual compartments to provide higher specificity.

Organelle immunoprecipitation (IP), in which organelles are enriched by expressing an affinity-tagged organelle marker in cells followed by mild lysis and affinity purification of the intact organelles, provides higher specificity for mapping organelle proteomes. This strategy has extensively been used to map proteomes of individual organelles, such as lysosomes, mitochondria, endosomes, melanosomes and Golgi across cell types [18–22] and to monitor organelle proteome dynamics in disease contexts [23]. As an elegant example, Goochani et al. recently generated a lysosomal proteomic landscape using Lyso-IP in a cell-type-specific manner in intact mouse brains [24]. A limitation of organelle IP is that it requires expressing affinity-tagged organelle markers, which when exogenously expressed could lead to overexpression artifacts. To overcome this limitation, Hein et al. used CRISPR-Cas9 to endogenously tag organelle protein markers with the HA epitope in HEK293T cells (Figure 1b) [25]. Combining the library of engineered cell lines with rapid purification of individual organelles, quantitative mass spectrometry, and a graph-based analysis, the authors assigned the subcellular localization of over 7600 proteins and uncovered interconnections among cellular compartments [25]. Finally, Hein et al. used this strategy to study subcellular remodeling following human coronavirus OC43 (HCoV-OC43) viral infection, discovering that many proteins are regulated by changes in their spatial distribution rather than by changes in abundance [25]. While this study is a tour de force demonstrating the high resolution proteome-wide spatial dynamics that can be obtained using organelle IPs, it is worth noting that each organelle IP is a separate experiment, limiting the number of conditions that can be compared in such a study and illustrating the trade-off comparing subcellular fractionation and organelle IP.

While fractionation and organelle purification define the organelle-scale spatial context of signaling components, they do not resolve the dynamic molecular interactions through which signaling specificity is achieved within those compartments. Enzyme-based proximity labeling using enzymes such as APEX2 or TurboID enables nanometer-scale capture of local protein interaction networks without physical purification. In the next section, we discuss how these strategies extend from compartment mapping to interrogation of transient signaling complexes.

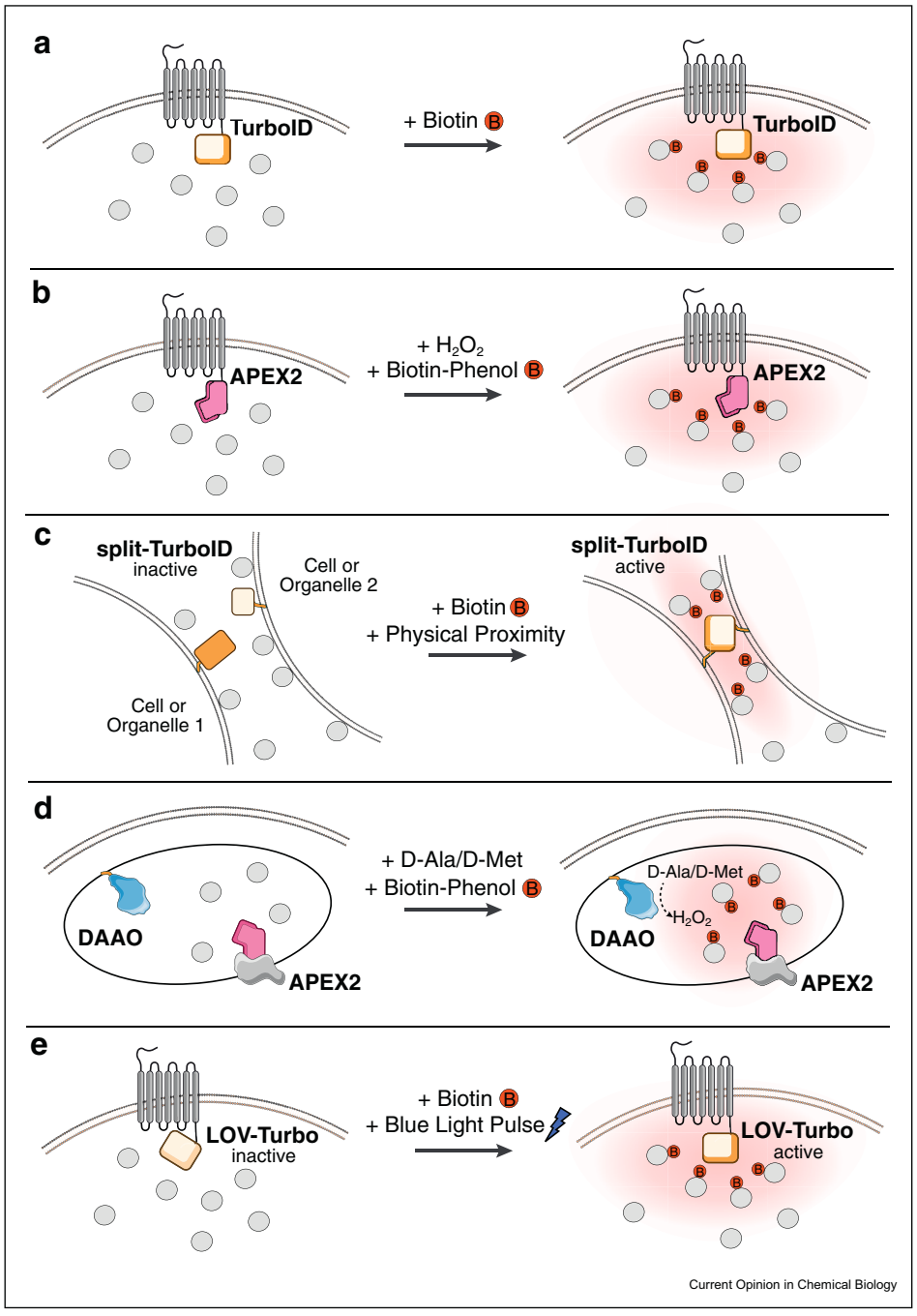
Interactions—capturing context-dependent protein interaction networks with proximity labeling

While spatial proteomics defines the cellular context in which signaling components reside, signaling specificity emerges from dynamic molecular interactions formed within that context. Affinity purification—mass spectrometry (AP-MS) is the method of choice for capturing stable protein complexes and high-affinity interactions at scale, but it provides limited information about the spatial context of the interactions and is poorly suited for transient interactions that can be lost during cell lysis and purification.

Proximity labeling through engineered enzymes such as the biotin ligase TurboID [26] (Figure 2a) and the peroxidase APEX2 [27] (Figure 2b) has overcome some of the limitations of AP-MS by covalently labeling proximal proteins with biotin in nanometer-scale radius in intact cells. Recent reviews have summarized advances in proximity labeling strategies and their differences in labeling kinetics [28]. Beyond mapping static interaction, we recently demonstrated that APEX2 can capture the interaction networks of G protein-coupled receptors (GPCRs), including transient interactions following receptor activation, with temporal resolution [29,30]. By comparing proteins labeled in the proximal environment of the activated GPCR to compartment-targeted spatial reference constructs, we were able to simultaneously model time-dependent subcellular location changes of the activated receptor [30].

Over recent years, developments in proximity labeling have focused on engineering new enzyme variants or labeling strategies that enable context-dependent labeling, enhance labeling specificity and activity, and improve temporal control (Figure 2c–e). To increase the peroxidase activity of APEX2, Tran et al. recently used MULTI-evolve, a rapid evolution framework that systematically engineers multimutants, to engineer a new enzyme variant, which is yet to be tested for proteomics applications [31]. For context-dependent proximity labeling, approaches such as split-TurboID [32] and Contact-ID [33] have been developed by splitting proximity labeling enzymes into two inactive fragments,

Figure 2



Developments in proximity labeling strategies for context-dependent labeling and enhanced temporal control. Proximity labeling using TurboID is initiated by the addition of biotin (**a**), while proximity labeling using the engineered peroxidase APEX2 is initiated by the addition of exogenous hydrogen peroxide H_2O_2 and biotin-phenol, covalently biotinylating proximal proteins **(b)**. In split-TurboID the enzyme is split into two inactive fragments, which reconstitute TurboID enzymatic activity when brought in proximity, for example, by organelle contact **(c)**. iAPEX combines the local production of H_2O_2 by the D-amino acid oxidase (DAAO) with the APEX2 peroxidase for compartment-specific labeling, also reducing oxidative stress **(d)**. LOV-Turbo utilizes a light-sensitive LOV domain to cage the TurboID enzyme and reversibly control its labeling activity with low-power blue light. Upon a blue light pulse, the enzyme undergoes a conformational change that enables biotinylation **(e)**.

which, upon coexpression in cells, are brought together by protein–protein interactions or organelle contacts leading to reconstitution of labeling activity (Figure 2c). As an example, split-TurboID has been elegantly applied to define the proteome at the astrocyte–neuron interfaces *in vivo* in the mouse brain [34].

To increase labeling specificity within defined subcellular compartments and reduce cellular toxicity associated with H_2O_2 treatment, Sroka et al. developed an *in situ* labeling strategy termed as iAPEX. This approach combines APEX2 with a D-amino acid oxidase (DAAO) from *Rhodotorula gracilis* to locally generate hydrogen peroxide within cells (Figure 2d) [35]. Application of iAPEX to primary cilia, a prototypical signaling microdomain, resulted in improved sensitivity and spatial specificity for identifying ciliary proteins compared with conventional APEX2 labeling. Furthermore, the iAPEX system enables peroxidase-based proximity labeling *in vivo*, as demonstrated in Xenopus embryos, given its limited toxicity by generating H_2O_2 *in situ*.

For temporal control of proximity labeling, Lee et al. engineered a light-gated proximity labeling enzyme, by installing the light-sensitive LOV domain into TurboID to rapidly and reversibly control its labeling activity with low-power blue light (Figure 2e) [36]. This reversibility is particularly advantageous in biotin-rich environments, including neuronal systems, where constitutive TurboID can accumulate background labeling over time. Interestingly, the authors further showed that LOV-Turbo can be activated by the luciferase NanoLuc via BRET, enabling selective biotinylation of protein subcomplexes defined by proximity between a LOV-Turbo-fusion protein and a NanoLuc-fused protein [36].

Together, these advances shift proximity labeling from constitutive proximity mapping toward conditionally controlled, temporally resolved interrogation of signaling complexes in their cellular context.

Signaling outputs–quantifying signaling using high-throughput phosphoproteomics and kinase activity sensors

While the subcellular localization and protein interactions define where signaling occurs and which proteins participate, post-translational modifications provide a readout of signaling activity. Protein phosphorylation is the most common post-translational modification in cellular signaling, dynamically modulating protein activity, interactions, and localization, amongst other processes. MS-based phosphoproteomics has therefore become the method of choice for interrogating signaling networks at the systems level as it directly measures the signaling state of the cell, by providing the signature of phosphorylated proteins and sites in a given moment.

Recent advances have increased the scalability, throughput, and sensitivity of phosphoproteomics through low-input workflows, automated enrichment strategies, and improved instrumentation combined with DIA-based quantification [37–42]. For example, Oliinyk et al. developed microPhos (μPhos) for rapid (<8 h) phosphopeptide enrichment from cells cultured in a 96-well format, which maintains quantitative reproducibility and sensitivity with low-microgram input amounts [37]. To further increase scalability, nanoPhos has pushed phosphoproteomics toward nanogram-scale inputs and its integration with Deep Visual Proteomics has enabled probing cell type-specific phosphoproteomes in heterogeneous tissues [43]. Combining low-input workflows with automated enrichment of phosphopeptides will, in the future, facilitate systems-scale studies to probe signaling dynamics across many conditions simultaneously [39], for example, for decrypting drug actions by measuring signaling network changes in a dose- and time-dependent manner [44,45].

With increasing depth and throughput of phosphoproteomic data, computational inference of kinase activity has become an integral layer to interpret phosphorylation dynamics across multiple experimental conditions. One challenge for kinase activity predictions has been the low coverage of known kinase-substrate relationships, which is generally biased toward well-studied kinases and excludes the vast majority of the 10s of 1000s of phosphorylation sites routinely quantified in phosphoproteomic experiments and leave the majority of detected phosphorylation sites ('dark phosphosites' [46]) without assigned upstream kinases. To overcome this, two comprehensive kinase libraries have been established for human serine/threonine [47] and tyrosine kinases [48] defining intrinsic sequence preferences for 100s of kinases. These resources provide a more complete and less biased foundation for interpreting phosphoproteomic data and linking phosphorylation dynamics to upstream kinase activity. It is worth noting, however, that these atlases are based on *in vitro* kinase reactions using peptide libraries, which may not fully recapitulate phosphorylation selectivity in cells and tissues.

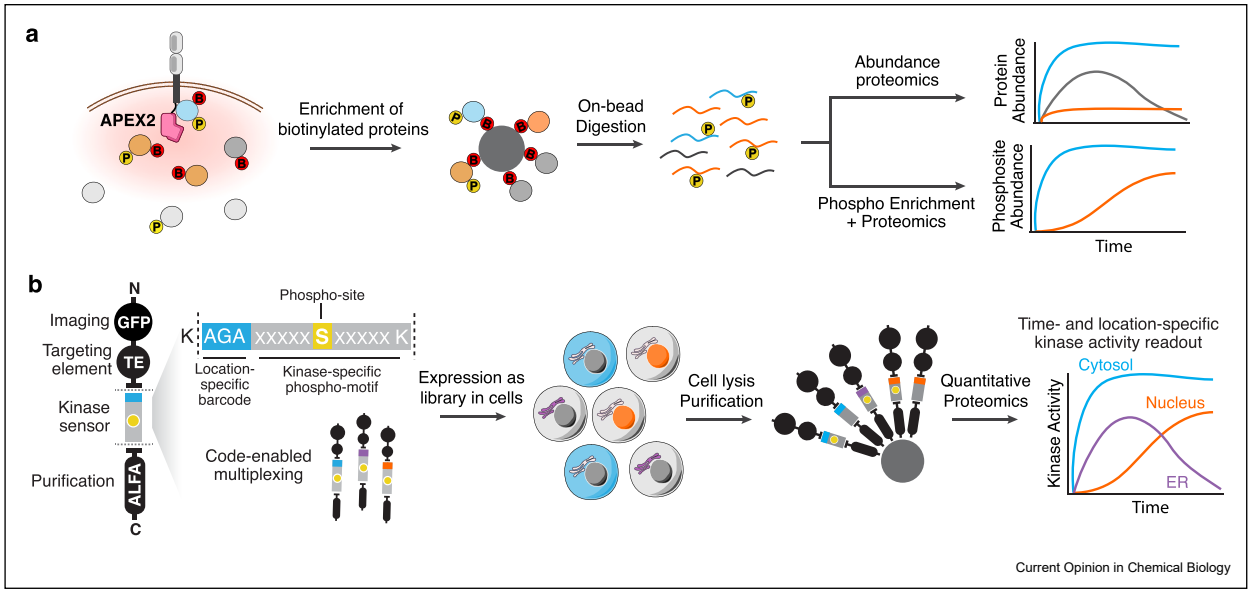
Building on these kinase libraries datasets, kinase activity inference methods have focused on improving both coverage and accuracy by integrating prior knowledge with data-driven models. For example, PhosX uses kinase-substrate sequence specificity derived from these libraries to test whether predicted substrates of a given kinase are preferentially enriched among regulated phosphopeptides, using a gene set enrichment analysis (GSEA)-like enrichment framework with

significance assessed by rank permutation. This reduces the reliance on curated kinase-substrate associations, thereby expanding kinase coverage and reducing bias toward well-characterized signaling pathways [49]. Other kinase activity prediction strategies incorporate protein network information instead of solely relying on substrate sequence motifs of kinases. Jiang et al. constructed a coregulation network of the human phosphoproteome called CoPheeMap from large-scale human phosphoproteomic data and incorporated the network embedding features from CoPheeMap into the machine learning model CoPheeKSA to improve predictions of kinase activities including that of understudied kinases [50]. Another example which incorporates protein network information is PhuEGO: it reconstructs active signaling pathways by performing network propagation of phosphoproteomic data on protein interaction networks thereby boosting the signal-to-noise ratio of phosphoproteomic data, enriching functional phosphosites, and extracting active signaling network signatures [51]. Finally, systematic benchmarking efforts have highlighted substantial variability across inference methods and underscored the importance of method selection and validation for robust biological interpretation [52,53].

With improvements in both experimental and computational workflows for phosphoproteomics, the next frontier is now resolving *where* phosphorylation events occur within the cell. To obtain spatial resolution, subcellular fractionation has been combined with phosphopeptide enrichment to quantify compartment-specific phosphorylation dynamics. Earlier work by Krahmer et al. in mouse liver during the development of high-fat diet-induced insulin resistance demonstrated that phosphorylation is distributed across organelles in a highly structured manner rather than uniformly across the proteome [54]. More recent studies have extended this framework to perturbation-resolved datasets, showing that epidermal growth factor receptor (EGFR) signaling responses are spatially partitioned across subcellular compartments and dynamically rewired upon receptor activation [9].

To define signaling events within a specific cell type, subcellular compartment, or in proximity to a protein of interest, proximity labeling-based strategies have been combined with phosphopeptide enrichment [55–58]. Dumrongprechachan et al. generated a Cre-dependent APEX2 reporter mouse line for cell-type-specific labeling. Using *ex vivo* biotinylation in tissue slices, they mapped both proteome and phosphoproteome changes in corticostriatal projections during development via sequential enrichment of biotinylated proteins and phosphopeptides [57]. Liu et al. engineered a bioorthogonally activatable TurboID with fast turn-on kinetics, enabling subcellular-specific uncaging-assisted biotinylation and phosphoproteome mapping (SubMAPP) [55]. Proximity labeling coupled to phosphoproteomics has also been used to capture signaling

Figure 3



Proteomic strategies for the mapping spatially resolved signaling events and kinase activity. To map spatially defined signaling events, for example, in proximity of a signaling receptor, a combined proximity labeling and phosphoproteomic strategy can capture recruitment of proteins upon receptor activation as well as proximal signaling events **(a)**. To monitor kinase activity with subcellular resolution, proteomic kinase activity sensors equipped with a location-specific barcode and a kinase-specific phospho-motif can be targeted to discrete subcellular compartments, and kinase activity can be determined by purification of the proteomic kinase sensors and measuring the amount of phosphorylated sensor motif **(b)**.

events in proximity of a B-cell coreceptor during B-cell activation [56] and to identify direct kinase-substrate relationships by labeling from kinases themselves [58] (Figure 3a).

Complementing MS-based spatial phosphoproteomics, proteomic kinase activity sensors such as ProKAS provide an orthogonal strategy for measuring where kinase activity occurs within the cell (Figure 3b) [59]. Rather than enriching endogenous phosphopeptides, ProKAS encodes kinase activity into genetically encoded peptide barcodes that are phosphorylated *in situ* and subsequently identified by quantitative proteomics. This approach decouples the inference of kinase activity from the complexity of the endogenous phosphoproteome, enabling direct, spatially resolved readouts of signaling activity linked to specific subcellular compartments or cell types.

Together, these strategies transform phosphoproteomics into a spatially resolved readout of signaling activity, completing the link between subcellular organization, molecular interactions, and functional signaling output.

Conclusions

Recent advances in spatial proteomics are converging to provide an integrated view of cellular signaling across localization, interaction, and activity layers. Organelle-resolved proteome mapping defines the spatial context in which signaling occurs, proximity labeling captures dynamic molecular assemblies within that context, and spatial phosphoproteomics reveals where signaling events are executed. A key shift is the increasing integration of these approaches within unified experimental workflows—enabling reconstruction of signaling networks not as static pathways but as dynamic, spatially organized systems. As throughput increases and sample input requirements decrease, the proteomics methods described here are becoming compatible with complex perturbation designs, primary cells, and tissue contexts. Continued advances in enzyme engineering and low-input proteomics will further enhance spatial and temporal resolution.

Equally important, advances in computational methods and data analysis approaches will be critical to fully exploit the information content of these increasingly rich and multimodal datasets. Neural network classifiers are already improving subcellular localization prediction from fractionation data [15], while network propagation [51] and machine learning models [50] are enhancing kinase activity inference from phosphoproteomic measurements. Looking forward, deep generative models that learn joint representations across data modalities, as demonstrated for multimodal single-cell genomics [60], offer a promising framework for integrating spatial proteomics, proximity labeling, and phosphoproteomic datasets into unified models of signaling organization. However, realizing this potential will require careful benchmarking and validation to ensure that computational predictions translate into biologically relevant insights. In addition, the incorporation of protein structural information, including interfaces and intrinsically disordered regions, will provide a framework for linking spatial organization to molecular mechanisms [61]. Together, these developments will enable predictive models of signaling networks that move beyond descriptive maps toward a mechanistic understanding of how signaling specificity is encoded in space and time.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

No data was used for the research described in the article.

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