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# High throughput methods to study protein-protein interactions during host-pathogen interactions

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ABSTRACT

The ability of a pathogen to survive and cause an infection is often determined by specific interactions between the host and pathogen proteins. Such interactions can be both intra- and extracellular and may define the outcome of an infection. There are a range of innovative biochemical, biophysical and bioinformatic techniques currently available to identify protein-protein interactions (PPI) between the host and the pathogen. However, the complexity and the diversity of host-pathogen PPIs has led to the development of several high throughput (HT) techniques that enable the study of multiple interactions at once and/or screen multiple samples at the same time, in an unbiased manner. We review here the major HT laboratory-based technologies employed for host-bacterial interaction studies.

## 1. Introduction

The cellular proteome reflects the functional status of a cell, with well-orchestrated interactions between multiple proteins in a cellular pathway, and with changes to a single protein or the level of a protein impacting the output of that pathway. Such a disruption occurs when a foreign protein for e.g., a pathogen protein, interacts with a protein in a host cell pathway. Host-bacterial interactions typically are multifactorial, but such protein-protein interactions (PPI) between the host and bacterial proteins can play a key role in determining infection outcomes (Crua Asensio et al., 2017). The ability of a pathogen to rewire and hijack host proteins has been reported to directly correlate with the number of interactions between the pathogen and host proteins, and has a higher impact on fitness of some pathogens during infection (Crua Asensio et al., 2017). Studying PPI can provide valuable insight into mechanisms underlying disease pathogenesis and potentially lead to identification of drug or vaccine targets.

While pathogens elicit PPIs within the host in order to infect and survive in the host, proteins from commensals also interact with host proteins and modulate host pathways (Balint and Brito, 2023; Zhou et al., 2022). Many of the bacterial proteins that interact with host proteins are also conserved between commensals and pathogens (Lebeer et al., 2010). Additionally, evolution has led to the phenomenon of molecular mimicry, where the pathogen has evolved similar motifs and structures to host proteins (Doxey and McConkey, 2013; Via et al., 2015). Indeed, most of such interactions are not simple one protein bait to one protein target interactions but involve the formation of multi-protein complexes. Probing complex host-pathogen PPIs, is quite challenging, especially during infection, and is dependent on the type of microbe and host cell types that the interactions occur in.

Typically, techniques to identify PPIs are multi-disciplinary. Although current techniques can identify host-microbial PPIs, given the added complexity of cross-kingdom interactions, several advanced experimental methodologies are being developed. High throughput (HT) assays are of interest due to its potential to identify multiple targets at the same time and/or screen large numbers of samples in parallel, saving effort, time and resources compared to the traditional one sample at a time approach (Xiao et al., 2015). Such methods would give us a

*Abbreviations:* SLiMs, short linear motifs; HPLC, high performance liquid chromatography; LC/MS, liquid chromatography/mass spectrometry; 2D-DIGE, 2D difference in-gel electrophoresis; PIR, Protein interaction reporter; SUMO, Small Ubiquitin-like Modifier; AP-MS, affinity purification- mass spectrometry; TAP, tandem affinity purification; NHS, N-hydroxysuccinamide; BDP-NHP, Biotin-Aspartate Proline-PIR n-hydroxyphthalimide; APEX, ascorbate peroxidase; BLOC-2, biogenesis of lysosome-related organelles complex-2; SPR, surface plasmon resonance; Y2H assay, Yeast 2-hybrid assays; DB, DNA binding domain; AD, Activator domain; GAL4, galactose-responsive transcription factor 4; GFP, green fluorescent protein; ESCRT, endosomal sorting complex required for transport; HIV, human immunodeficiency virus; HP1, Hepatocyte binding peptide 1; ELISA, enzyme-linked immunosorbent assay; LESA, liquid extraction surface analysis; TMT, tandem mass tags; iTRAQ, isobaric tags for relative and absolute quantification; ICAT, isotope-coded affinity tag.

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better understanding of host-pathogen protein complexes during infection and hence provide a broader perspective on microbial pathogenicity. The purpose of this review is to give an overview of HT methods to identify protein interaction partners between the host and pathogens. We have focused on laboratory techniques employed for bacterial pathogen interactions as viral host PPIs have been reviewed extensively elsewhere (Brito and Pinney, 2017; Farooq et al., 2021; Mendez-Rios and Uetz, 2010) and there are several recent reviews summarising numerous bioinformatic tools available to study PPIs (Durham et al., 2023; Iuchi et al., 2023; Nourani et al., 2015).

Protein interactions can be classified based on their strength/persistence (permanent and transient), specificity (specific or nonspecific), similarity between interacting subunits/interaction surfaces (homo and hetero oligomer) and stability (obligate or nonobligate) (Poluri et al., 2021). Most studies examine permanent PPIs that form complexes (Table 1), but bioinformatic studies have revealed that transient PPIs are also important (Ghadie and Xia, 2022). Host short linear motifs (SLiMs) (Van Roey et al., 2014) play important roles in host-pathogen interactions, as bacterial proteins are able to mimic these motifs and block host interaction networks (Sámano-Sánchez and Gibson, 2020). There are broadly two types of HT methods to identify PPIs: binary PPI assays, to identify direct pairwise interactions, and affinity purification with mass spectrometry (MS), to identify direct and indirect protein associations in co-purified complexes. We have broadly classified these methods into MS-based and non-MS-based methods.

## 2. Mass spectrometry based methods

There are different approaches used in high-throughput MS: A top-down proteomic approach where proteins are identified through full-length protein fragments or ions, and a bottom-up approach, where proteins are chemically or enzymatically digested into peptides and these peptides are analysed in a mass spectrometer (Cupp-Sutton and Wu, 2020). Shotgun proteomics is a type of bottom-up method where complex mixture of whole proteins are broken down into smaller peptides and separated by high performance liquid chromatography (HPLC) and identified by MS, to get an global overview of the sample (Cui et al., 2022). Different traditional methods used to detect interactions have been combined with LC/MS to enable identification of multiple proteins within a complex (Fig. 1).

## 3. 2D polyacrylamide gel electrophoresis and MS

Two-dimensional gel electrophoresis involves separation of proteins based on two different properties of proteins, the charge (isoelectric point, pI), which is the first dimension and the mass of the protein, the second dimension. Since proteins are separated based on two parameters, they are separated all across the gel (Saraswathy and Ramalingam, 2011). This is a HT protein separation technique that can separate up to 10,000 proteins at the same time in a single gel. Each protein spot on the gel is characterised by specific pI and relative molecular weights and can be excised, processed and analysed using a LC/MS. 2D gel electrophoresis has also been employed to detect post-translational modifications like glycosylation, phosphorylation, etc (Hameed and Fatima, 2021).

A combination of 2D gel electrophoresis and mass spectrometry has been widely employed in understanding host changes induced by several bacterial pathogens (Backert et al., 2000; Wong et al., 1999). Host protein changes were identified post exposure of U937 cells to *Yersinia pestis* and *Y. pseudotuberculosis* (Zhang et al., 2005). 16 differentially abundant human proteins were identified from *Y. pestis* infection, and 13 from *Y. pseudotuberculosis* infection; these proteins were involved in a spectrum of cellular functions and host defence mechanisms like apoptosis, cytoskeletal rearrangement, protein synthesis and degradation, protein folding and cell signalling (Zhang et al., 2005). In

**Table 1**
Some examples of use of high throughput methods to identify host pathogen interactions.

| Host | Pathogen | Method | No. of proteins identified | Human/ pathogen | References |
|---|---|---|---|---|---|
| U937 | *Yersinia pestis* | 2D PAGE and | 16 | human | (Zhang et al., 2005) |
| Human monocytes | *Y. pseudotuberculosis* | MS | 13 | human | |
| Bovine milk | *Streptococcus uberis* | 2D PAGE and MS | 136 | human | (Smolenski et al., 2014) |
| Rat peritoneal implant | *Leptospira interrogans* | 2D-DIGE | 202 | pathogen | (Nally et al., 2017) |
| HeLa and RAW264.7 | *Salmonella enterica serovar* Typhimurium | AP-MS | 15 | pathogen | (Walch et al., 2021) |
| | | | 446 | human | |
| A549 | *Legionella pneumophila* | TAP-MS | 8 | human | (So et al., 2016) |
| Human lung epithelial cells | | | | | |
| H292 | *Acinetobacter baumannii* | Cross-linking | 46 | human | (Schweppe et al., |
| Human lung epithelial cells | | MS | | | 2015) |
| HEK293T Human embryonic | *Salmonella enterica serovar* Typhimurium | BioID | 381 | human | (D'Costa et al., |
| kidney cells | | IP-MS | 106 | human | 2019) |
| HEK293 | *Chlamydia psitaccii* | BioID | 22 | human | (Mojica et al., 2015) |
| HeLa | *Chlamydia trachomatis* | APEX-2 | 452 | human | (Dickinson et al., 2019) |
| HFF human foreskin fibroblast | *Toxoplasma gondii* | BioID and | 46 | human | (Pan et al., 2018) |
| cells | | APEX | | | |
| AGS human epithelial cells or | *Enteropathogenic Escherichia coli* | SILAC | 2 | human | (Selbach et al., |
| HeLa cells | *Helicobacter pylori* | | 4 | human | 2009) |
| | *Bartonella henselae* | | 7 | human | |
| | *Chlamydia trachomatis* | | 5 | human | |
| Caco-2 human epithelial cells | *Listeria monocytogenes* | TMT with SILAC | 100 | human | (Birk et al., 2021) |
| HFF human foreskin fibroblast cells | *Toxoplasma gondii* | iTRAQ | 665 | human | (He et al., 2019) |
| Human ORF | *Mycobacterium tuberculosis* | Yeast 2-hybrid | 53 | pathogen | (Mehra et al., 2013) |
| | | | 63 | human | |
| *Saccharomyces cerevisiae* | *Salmonella enterica serovar* Typhimurium, *Coxiella burnetii, and Brucella melitensis* | E-MAP | 43 | pathogen | (Patrick et al., 2018) |
| Human isolated neutrophils | *Staphylococcus aureus* | Phage display | 2 | human | (Fevre et al., 2014) |
| | | | 2 | pathogen | |
| Human serum | *Mycobacterium tuberculosis* | SPR | 9 | pathogen | (Hsieh et al., 2012) |

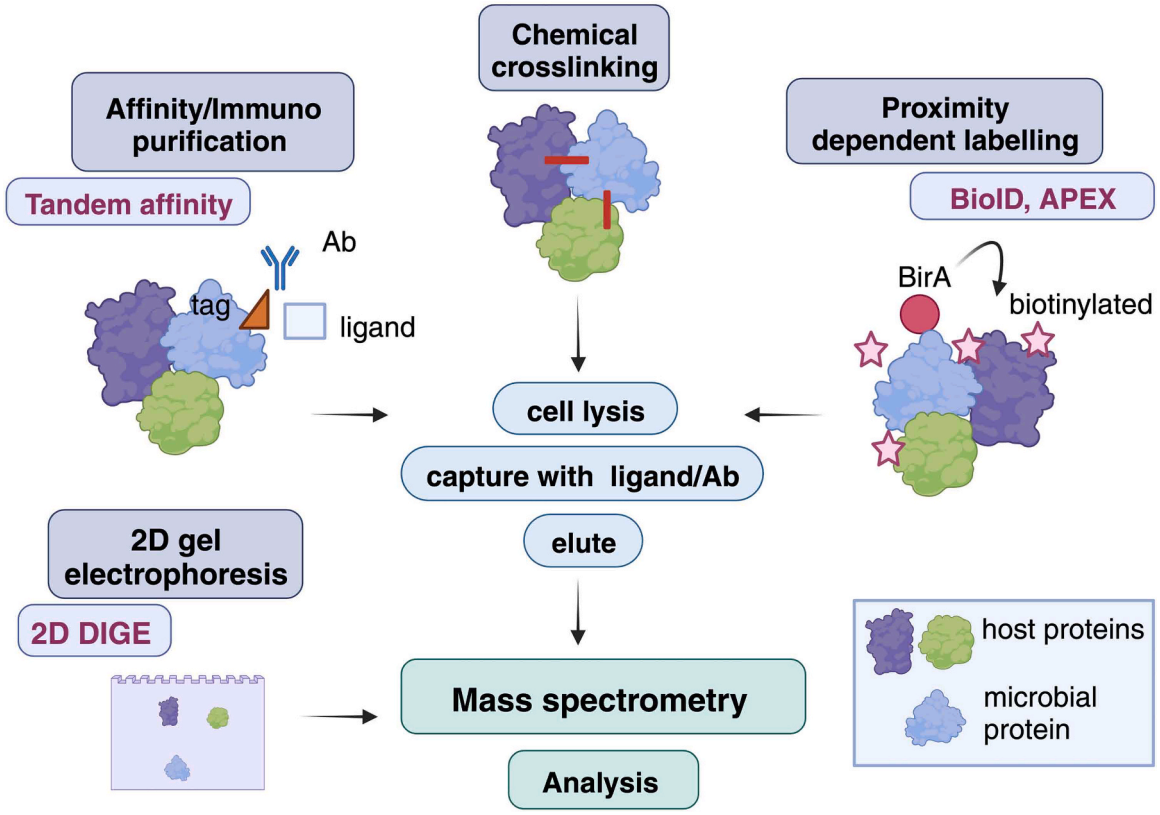


**Fig. 1.** Schematic diagram summarising main steps in MS-based approaches for studying protein interactions between host and microbial proteins (Created using BioRender).

another study, bovine milk protein composition was analysed using 2D gel electrophoresis and LC/MS during *Streptococcus uberis* infection. Protein identification revealed that 68 proteins were associated with host defence with 27 involved in modulation of immune or inflammatory signals, 18 proteins with direct antimicrobial function and 23 with a pathogen recognition function (Smolenski et al., 2014).

A modification of 2D electrophoresis, 2-D difference in-gel electrophoresis (2D-DIGE), is a method where 2–3 samples can be compared on the same gel and proteins that are differentially present are identified using MS (Viswanathan et al., 2006). A study comparing proteomes of *Leptospira interrogans* derived from an in vivo peritoneal implant model, to leptospires cultured in vitro, identified 20 isoforms of a leptospiral outer membrane protein LipL32 using 2D-DIGE, indicating host induced post-translational modifications (Nally et al., 2017).

Key issues associated with 2D gel electrophoretic methods is variation between gels and the poor separation of proteins, particularly acidic, basic or hydrophobic proteins, which may contribute to low reproducibility.

## 4. Affinity and immuno purification MS

An affinity epitope tag is added to a bait protein followed by purification in one or two affinity or immuno purification steps, with a control protein run in parallel under the same reaction conditions. Non-covalently bound interacting individual proteins or protein complexes are purified along with the bait and identified using bottom-up proteomic methods. Proteins specific to the tagged bait proteins are identified through comparison with the proteins bound to the tagged control protein. A range of tags have been used for affinity purification such as the traditionally used glutathione-S-transferase, poly-histidine tags, short epitope tags like Flag, c-myc, HA, and newer affinity tags like the Halotag (Promega), streptavidin-derived Strep-tag (which binds to Streptactin)(Schmidt and Skerra, 2007), Small Ubiquitin-like Modifier (SUMO) tags (Kimple et al., 2013). Newer short tags like the Spot-tag (Chromtech) have been used along with Spot single peptide nanobodies, which display high affinity to these tags (Braun et al., 2016).

Affinity purification (AP) MS can be performed with host cells expressing the tagged pathogen protein, on infected cells or using the immobilised recombinant bacterial effector protein on beads and then flowing through mammalian cell lysates (Dunham et al., 2012). Expressing a single tagged pathogen effector protein and probing for host protein interaction partners gives us a glimpse of complex interactions, although identifying such interactors during infection can provide a more accurate picture of host-bacterial PPIs (Nicod et al., 2017). AP-MS analysis was used to identify host protein interactions of a number of pathogens including *Coxiella burnetii*, *Salmonella* and *Citrobacter* effectors (Fu et al., 2022; Sontag et al., 2016). *Salmonella* expressing 32 chromosomal tagged effector proteins were used to infect epithelial cells or macrophages, followed by AP-MS, which resulted in the identification of 446 host interaction partners. 13 of these interactions were confirmed by reciprocal co-immunoprecipitation (Walch et al., 2021).

***Tandem affinity purification (TAP)*** is an improved AP method where multiple tags are added onto the protein of interest, with multiple rounds of purification carried out to improve the specificity of its interacting partners (Bailey et al., 2012; Goodfellow and Bailey, 2014; Williamson and Sutcliffe, 2010). Since the purification is more stringent, there is a high probability that the interaction partners are not false positives (Walch et al., 2021). Using a TAP approach with hexahistidine and BirA-specific biotinylation (Bio) tags, *Legionella* effector protein interaction partners were identified from A549 epithelial cells expressing the biotin ligase, BirA; single effectors like LidA were shown to bind to 8 Rab GTPases during intracellular infection (So et al., 2016).

AP methods also have the capability to be HT. In a HT AP screen *Saccharomyces cerevisiae* 2708 different tagged proteins could identify 7123 protein-protein interactions (Krogan et al., 2006). Although this

study did not examine host-pathogen interactions, it highlights the potential of applying HT AP in larger scale identification of host interactors.

Although tag-based methods are widely used to identify host/pathogen interactors, they have many drawbacks (Table 2). A key issue is that the tag may interfere with biological functions of the protein. Additionally, there is risk that transient interactions are not identified. As tagged proteins are typically overexpressed, there is a likelihood that non-specific interactors are pulled down, increasing the false positive protein interactions detected. However, the identification of indirect interactions can be significantly reduced with TAP methods, as it involves multiple steps of purification.

## 5. Chemical cross-linking MS

Typically, in chemical cross-linking MS (abbreviated as CL-MS, XL-MS, CX-MS or CXM), a proximal reactive side chain is chemically cross-linked between protein residues, adding new covalent bonds between proximal residues (O'Reilly and Rappsilber, 2018).

CL-MS does not need the protein of interest to be tagged and can cross-link interactions within cells prior to cellular disruption. Hence this method can identify weak and transient interactions and interaction of proteins that are not soluble (Guerrero et al., 2006; Sinz, 2018). This cross-linking reaction can be performed either by soluble cross-linkers or by photoactivatable amino acids such as photo methionine and photo leucine. The cross-linker is typically made up of two elements: a spacer and a reactive group on each end (Leitner et al., 2016). The reactive end group provides information of the interacting residues, based on the amino acid targeted by the reactive end group. Common cross-linkers include homo-bifunctional cross-linking agents which react with primary amines such as N-hydroxysuccinamide (NHS) esters, imidates, diazo compounds which cross-link acidic residues and other hydroxyl group and thiol reacting cross-linkers (Yu and Huang, 2018). Use of multiple combinations of reactive end groups that target multiple amino acid residues provides better predictions of structural interactions within a protein or a protein complex. The spacer region connects the two proximal interacting peptides together. Large spacers allow identification of proteins in proximity and smaller spacers help in getting information regarding the 3D structure conformation of the interacting proteins (Lenz et al., 2021). Spacers can contain cleavable or enrichable groups, which determine cleavability or enrichability of the cross-linker (Belsom and Rappsilber, 2021). The cross-linking reaction is followed by enzymatic digestion (e.g. trypsin) of the cross-linked proteins, enrichment of the cross-linked peptides (if the spacer is enrichable, for e.g. biotin), MS-based data acquisition and peptide identification (Fig. 2). One of the bottlenecks with CL-MS is that cross-linkers may block the enzymatic digestion sites leading to increased peptide size and faulty MS detection (Matzinger and Mechtler, 2021). Cleavable cross-linkers can help overcome this and can generate specific fragmentation ions, enhancing identification (Piersimoni et al., 2022).

CL-MS has been utilised to study *Acinetobacter baumanni* host interactions (Schweppe et al., 2015). 3076 total cross-linked peptide pairs and 46 specific PPIs were identified from lung epithelial cells infected with *A. baumannii* cross-linked with Biotin-Aspartate Proline-PIR (Protein interaction reporter) *n*-hydroxyphthalimide (BDP-NHP). OmpA, a major *A. baumannii* virulence factor, was shown to be cross-linked to human lung epithelial desmosome proteins which control host cell-cell contact. PIR-CL-MS was also applied to identify in vivo protein complexes in pathogens. 626 cross-linked peptide pairs indicated new interactions between membrane proteins, and structures of membrane proteins SecD-SecF, OprF, and OprI were predicted using in vivo cross-linked sites (Zheng et al., 2011).

A major disadvantage of this method is the high rate of nonspecific interactions due to proteins in proximity of the bait protein getting picked up rather than the ones physically interacting with it. In a complex system to identify protein interaction in situ, the proteins

**Table 2**
Summary of advantages and disadvantages of main techniques to study host-pathogen interactions.

| Technique | Advantages | Disadvantages |
|---|---|---|
| **2D Chromatography-MS** | • Can separate thousands of proteins in a single gel.<br>• Differentially expressed proteins can be easily catalogued and identified.<br>• Cost-effective method | • Poor reproducibility, due to inefficient protein separation and gel-to-gel variation<br>• Elaborate sample processing by dialysis to make it free of ions and salts. |
| **Affinity purification and immune purification /MS** | • Very sensitive technique<br>• IP/MS – Protein can be purified in their natural form from cell or tissue lysates, preventing issues associated with protein tagging; multiple isoforms can be studied simultaneously.<br>• AP/MS – Tagging allows for studying proteins for which native antibodies are not available; many proteins can be tagged with a single epitope. | • High false positive rate<br>• High and unnatural expression levels of tagged proteins may lead to spurious interactions or disruption of interactions.<br>• Inability to identify low-abundance complexes or weak/transient associations.<br>• Data analysis of the results requires expertise with MS and bioinformatic tools Dunham et al., 2012) |
| **Tandem affinity purification** | • Due to multiple steps of purification, the specificity and stringency is high.<br>• Good reproducibility between experiments | • Cannot identify interactions that occur only in specific physiological conditions or transient interactions.<br>• TAP tag may impair function and complex assembly of the protein of interest (Xu et al., 2010). |
| **CL-MS** | • Does not require protein to be tagged.<br>• Can identify low-affinity PPIs.<br>• Can be performed in situ or in vivo.<br>• Provides information for structure validation and structural modelling of interactions. | • High probability of detecting non-specific interactions.<br>• Low abundance of cross-linked proteins and complexity of down-stream process like MS may make protein identification tedious.<br>• Inefficiency of cross-linking chemistry makes it difficult to identify partner proteins (Lam et al., 2002). |
| **Proximity dependent labelling methods BioID, APEX** | • As biotinylation occurs in cells prior to lysis, interactions occur in their natural cellular context.<br>• Excellent for identification of weak and transient interactions.<br>• Can detect interactions between low abundance proteins (Lambert et al., 2015). | • Fusion tags with BirA lead to addition of significant size to the protein and can compromise its targeting and function (Roux et al., 2012).<br>• False negatives due to low expression levels.<br>• Data analysis of the results requires expertise with MS and specific bioinformatic tools. |
| **Two-hybrid methods** | • Established method, molecular systems available.<br>• Cost-effective<br>• Can be used in both large-scale and small-scale studies.<br>• As assays are carried out in an in vivo condition, avoids artifacts | • High false positive rate<br>• Labour intensive screening<br>• High false negatives due to non-physiological expression system.<br>• Lack of or inappropriate post translational modification, cofactors, |

(*continued on next page*)

**Table 2** (*continued*)

| Technique | Advantages | Disadvantages |
|---|---|---|
| | associated with cell lysis.<br>• Best suited to identify binary interactions (Ferro and Trabalzini, 2013; Hamdi and Colas, 2012; Petschnigg et al., 2014) | or other binding partners. |
| **Protein microarray technology** | • Ease of screening<br>• Effective HT method as several proteins can be simultaneously assayed.<br>• Biochemical properties can be assayed (Hall et al., 2007). | • High false positive rate<br>• Libraries of expression clones may not be available.<br>• Requires robust high throughput protein production.<br>• 3D structural orientation of the protein during immobilization must be considered. |
| **Phage display** | • Very good for HT screening, due to the use of large libraries, library with diversities as high as $10^{10}$ can be constructed (Hoogenboom et al., 1998).<br>• Highly flexible and can be performed in vivo and in vitro (Johns et al., 2000).<br>• *In vitro* inorganic targets can also be screened (Whaley et al., 2000). | • If a random phage library is used, peptide sequences not found within the antigen or intact pathogen can be displayed, leading to false positives (Lundin et al., 1996; Mullen et al., 2006). |
| **SPR** | • The method is very fast, requiring only about 10 min for a typical run.<br>• Interactions characterized in real time with thermodynamic and kinetic constants obtained for a wide range of affinities, protein concentrations, molecular weights, and binding rates (Jönsson et al., 1991).<br>• High sensitivity (picomolar range).<br>• Requires low amounts of label free samples.<br>• Amenable to HT. | • High cost of SPR chips and instrumentation<br>• *In vitro* method may not mimic i in vivo/ cellular conditions.<br>• Fusion tags may hinder interaction with bait.<br>• Non-specific interactions between analyte and sensor surface (Homola, 2003). |
| **Spatial Proteomics** | • Highly sensitive<br>• Heavily automated<br>• Provides spatial information. | • Labour intensive and time consuming<br>• Expensive equipment<br>• Requires specialist personnel (Brožová et al., 2023; Gustafsson et al., 2011) |

identified are highly biased towards the most abundant proteins (Lee and O'Reilly, 2023). Another major limitation of this technique is the type of cross-linker that is used. The type of cross-linker impacts aspects of downstream processing like the enrichment of the cross-linked peptides or the MS identification of peptides. The cellular permeability of the cross-linker also has to be considered while doing CL-MS in situ, as different concentrations of cross-linker were shown to associate with varying amounts of interacting proteins (Fürsch et al., 2020). Hence, rigorous optimization of each step of the MS workflow is critical for reliable results with this method.

## 6. Proximity dependent labelling – mass spectrometry

Proximity labelling methods for identifying PPIs are broadly based on enzyme-mediated labelling of interacting proteins based on its proximity to the bait. The two main proximity labelling methodologies include BioID, based on a bacterial biotin ligase mutant and APEX, based on an engineered soybean ascorbate peroxidase. Both methods are effective for detecting transient and weak PPI.

BioID involves the fusion of the bait protein with a biotin ligase BirA and is coupled with affinity purification (Roux et al., 2018). BioID2 is an improved version where a mutated promiscuous biotin ligase BirA* is fused to the bait (Kim et al., 2016). Turbo ID and miniTurbo are newer engineered mutants which label with much greater efficiency than BioID or BioID2 (Branon et al., 2018). When mammalian cells expressing this fusion construct are exposed to high biotin concentration, neighbours of the bait-BirA* get biotinylated in a proximity-dependent manner. Potential direct or indirect interactors are purified using streptavidin-coated affinity matrices and identified using MS. It is essential to have multiple control conditions to eliminate the nonspecific interactors of bait proteins. Even though endogenous biotinylation is a rare modification, the background must be accounted for by proper control design. The controls might differ based on the nature of the experiment, but the simplest ones include cells transfected with the BirA*, expressed similar to the BirA*-fusion protein, mock-transfected cells not expressing any BirA* or the parental cell line for experiments with stable cell lines (Firat-Karalar and Stearns, 2015).

A comparison of BioID and anti-FLAG immunoprecipitation for identifying host interactors of *S*. Typhimurium type 3 secretion system effectors showed that BioID identified 381 human proteins versus 106 proteins by anti-FLAG IP, which included 8 known interactors of the effectors. The *Salmonella* virulence protein SifA was demonstrated to interact with the BLOC-2, which controls the Sif-A mediated positioning of *Salmonella* containing vacuole, thereby affecting intracellular *Salmonella* replication (D'Costa et al., 2019). BioID has been used in other intracellular pathogens like the avian pathogen *Chlamydia psitaccii* where 22 host proteins controlling the nuclear structure were shown to interact with a type III effector SINC; SINC was further shown to target the nuclear membrane, altering nuclear envelope functions (Mojica et al., 2015).

One of the disadvantages of using BioID is that it can alter the localization of tagged proteins and hence this method is not reliable to examine dynamics of intracellular trafficking pathways (Jorgenson et al., 2021). Additionally, BioID and BioID2 take a long time (18–24 h) to generate enough biotinylated material, which prevents its use for identifying PPI dynamics occurring at shorter timescales. However, these are overcome in the newer TurboID and mini Turbo which demonstrate 10 min proximal labelling in cells,with low toxicity (Branon et al., 2018).

Ascorbate peroxidase (APEX) is an enzyme which, in the presence of hydrogen peroxide oxidizes biotin-phenol into biotin-phenoxyl radical that biotinylate electron rich amino acids within a radius of several nanometres (Nguyen et al., 2020; Rhee et al., 2013). This enables biotinylation of proteins proximal to the APEX. Subsequently, biotinylated proteins are enriched by streptavidin and identified by mass spectrometry. APEX can be targeted to a specific cell compartment either through fusing with the protein of interest or through adding a localisation signal. Due to its shorter labelling time, it is particularly useful to study dynamic PPIs especially in subcellular locations including membrane bound organelles (Hung et al., 2017, 2014; Jing et al., 2015; Kim and Roux, 2016). APEX-2 is a mutant that has enhanced activity for both proteomic and electron microscopy mapping applications (Lam et al., 2015). APEX-2 labelling systems have been used to identify host interactors in the intracellular pathogen *Chlamydia trachomatis*. Dickinson et al. developed a proximity-based labelling system based on APEX-2 by fusing flag-APEX-2 to an inclusion membrane protein IncA, which localises to inclusion membranes during infection enabling visualisation

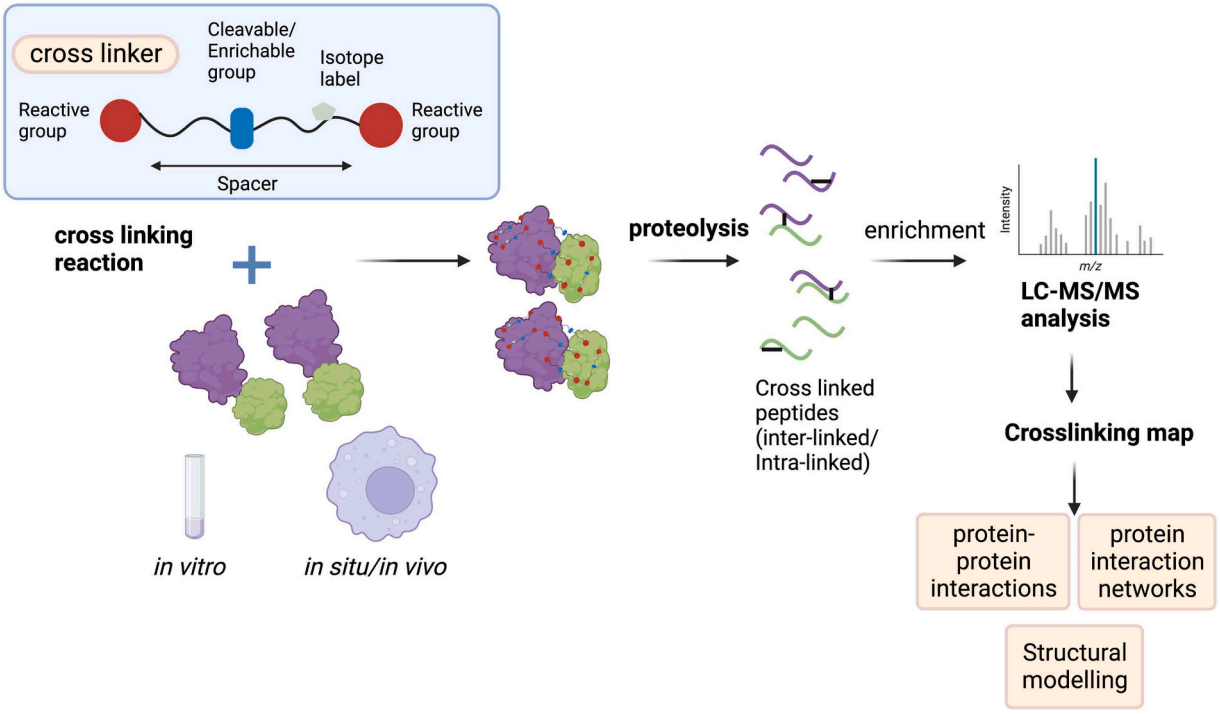


**Fig. 2.** Schematic diagram showing typical cross-linker features and steps in cross-linking mass spectrometry (CL-MS) (Created using BioRender).

in live cells. Mass spectrometry of APEX-2 labelled samples revealed 452 host proteins associated with inclusion membranes at different stages of infection (Dickinson et al., 2019; Rucks et al., 2017). APEX-2 based labelling systems were also employed recently in *Helicobacter pylori* to identify membrane proteins from stomach epithelial cells that interact with the *H. pylori* protease HtrA (Xie et al., 2023). In *Toxoplasma gondii*, both APEX and BioID were used to identify secretory granule proteins (GRA) using tagged GRA1 as a bait which pulled down 46 proteins of which 26 were novel GRA's (Pan et al., 2018).

Overall, although they have some disadvantages related to the size of the large tags, proximity-based systems have excellent scope for global host protein interaction studies during infection, particularly in detecting transient interactions in low abundance proteins.

## 7. Quantitative mass spectrometry approaches

While most MS methods used for bottom-up proteomics to study PPIs are non-targeted and use relative quantification of peptides, targeted quantitative methods provide important information regarding interacting proteins including topology, stoichiometry and dynamic behaviour (Jean Beltran et al., 2017; Meyer and Selbach, 2015; Rozanova et al., 2021). Label free quantification is usually cheaper, easy to perform, and with a larger dynamic range, however, this method has lower reproducibility and is less accurate (Li et al., 2012). Label-based methods are more expensive, but more specific and require optimization (Rozanova et al., 2021). A widely used metabolic labelling method is stable isotope labelling by amino acids in cell culture (SILAC), where cells are fed with metabolites labelled with heavy isotopes. SILAC (Ong et al., 2002) has been used to track proteomes during infection, for example, it was used to study the newly synthesised subcellular proteomes in *S.* Typhimurium-infected cells (Selkrig et al., 2020). Host proteins interacting with tyrosine phosphorylated bacterial effectors were identified by incubating phosphorylated and non-phosphorylated peptides with heavy or light SILAC-labelled HeLa cell lysates (Selbach et al., 2009). In chemical labelling methods, a reagent which contains stable heavy isotopes reacts with peptides causing a shift in the MS spectra. Examples of chemical labelling include isobaric tag-based methods include TMT (tandem mass tags), iTRAQ (isobaric tags for relative and absolute quantification), and non-isobaric tag-based methods include isotope-coded affinity tag (ICAT) (Gygi et al., 1999). TMT labelling has been widely used to study protein level changes in response to bacterial infection over time (Birk et al., 2021), while iTRAQ has been applied to quantify phosphorylated proteins during infection (He et al., 2019) Furthermore, isotope-labelled cross-linkers have been used for quantitative XL-MS (Fischer et al., 2013). Although most label-based methods have been used to map global proteomic profiles of host and/or pathogens during infection (Rozanova et al., 2021), these methods have good scope in identifying specific host-bacterial interactions.

## 8. Non-mass spectrometry based methods

A range of methods from the classical 2-hybrid assays to several new methods including protein arrays, surface plasmon resonance (SPR) and spatial proteomics have been employed in identifying host-microbial PPIs.

## 9. Two-hybrid assays

The 2-hybrid assay is a conventional method used to determine specific interactions, with several applications in both bacterial and mammalian interactor studies. The overall principle of these assays is that the interaction between two proteins results in formation of a functional transcription factor that transcribes a reporter gene (Fields and Sternglanz, 1994; Uetz, 2002). While yeast-2-hybrid assays have been widely used for studying host-microbial PPI, mammalian systems have also been developed alongside (Lievens et al., 2009; Stynen et al., 2012).

### 9.1. *Yeast 2-hybrid assays (Y2H assay)*

In eukaryotes, generally transcriptional activators are made of two domains, a DNA binding (DB) domain, and an activator domain (AD). The DB domain is a folded protein domain that recognises a specific DNA

sequence motif and interacts with it (Lilley, 1992; Ptashne and Gann, 1997). The AD activates transcription by binding to the RNA polymerase. The yeast 2-hybrid assay exploits the fact that these two domains of GAL4 or another transcription factor such as VP16 are not typically covalently associated but need to be in physical contact for activation of transcription of reporter genes such as LacZ, GFP or HIS3 (Lopez and Mukhtar, 2017). Fusions of genes encoding specific proteins or cDNA libraries are constructed with one of the domains, and a successful protein interaction would result in the activation of a suitable reporter gene.

A HT Y2H platform was employed by Mehra et al. to identify the host interactors of the *Mycobacterium tuberculosis (Mtb)* effectors. 99 novel interactions were identified between 53 *Mtb* proteins and 63 human proteins. The study subsequently demonstrated an interaction between the type VII secretion system effector EsxH and a component of the endosomal sorting complex required for transport (ESCRT) pathway, the hepatocyte growth factor-regulated tyrosine kinase substrate (Hgs/Hrs) (Mehra et al., 2013). HT Y2H assays have been recently employed to study PPI in SARS Cov-2 infection, the human protein-protein interactome identified 739 interactions of which 361 were novel (Zhou et al., 2023).

Epistatic miniarray profiles (E-MAP) is a type of synthetic genetic array that allows the quantitative measurement of genetic interactions between pairs of mutations in yeast. This allows identification of genetic interactions by comparing the growth rates of gene mutation pairs created in a yeast strain (Collins et al., 2007; Schuldiner et al., 2005). Using E-MAP, 36 bacterial effectors from three pathogens were shown to interact with host targets in conserved host pathways; a novel physical interaction between *Salmonella* effect SseC and a human retromer complex was identified in this study (Patrick et al., 2018).

### 9.2. Mammalian 2-hybrid assays

Mammalian 2-hybrid assays involve transiently transfecting mammalian cells with DNA binding and the transcription activation domains which associate to activate the expression of a reporter gene (Feng and Derynck, 2001).

Inhibitors of p53 interaction with MDM2 was identified using a reverse mammalian 2-hybrid method; 3840 compounds were screened to identify one compound that activated the p53 pathway (Li et al., 2011). To identify the therapeutic target of HIV type 1, the envelope glycoprotein gp41 inhibitor was identified using a large scale mammalian 2-hybrid screening assay (Shui et al., 2011).

While 2-hybrid techniques demonstrate direct interactions, the creation of fusions may impact the biological interaction, leading to false positives, i.e., interactions that do not occur between the corresponding proteins or false negatives, i.e. interactions between proteins that are missed. It is important to consider that steric hindrance, conformational changes and altered localization may impact interactions detected using this method. Additionally, artificial co-expression and over-expression of the tagged proteins may result in false positives (Lievens et al., 2009).

## 10. Protein microarray technology

Protein arrays enable the simultaneous processing of several proteins arranged at a high density on a solid surface. The main types of arrays include analytical arrays, reverse phase array, functional microarrays, carbohydrate/carbohydrate antigen microarray and other arrays like microsphere arrays (Manzano-Román et al., 2013). Among the various methods, functional microarrays are the most commonly used.

Full length proteins or protein domains are immobilized onto a solid support such as a glass slide. Creating a human protein microarray usually involves cloning of all the human ORFs into a yeast high copy expression vector with an appropriate tag (e.g. GST-His6), expression and protein purification from yeast using HT methods. Protein expression and purification can be carried out in *Escherichia coli*, in yeast, insect or mammalian cells or in cell-free transcription-translation systems. Although *E. coli* is easier to lyse for producing recombinant proteins for HT synthesis, a major disadvantage is the absence of co- and post-translational (PTM) modifications of eukaryotic proteins. In comparison, the yeast, insect and mammalian expression systems are able to add PTMs to the protein of interest (Duarte and Blackburn, 2017). Molecular weights of purified proteins are confirmed by immunoblotting before being arrayed on microtiter plates in 96-, 384-, or 1536- well formats (Hu et al., 2009). Labelled pathogen proteins of interest are applied to this protein microarray (Syahir et al., 2015), and binding is detected using a candidate label detection method such as fluorescence or chemiluminescence detection. This technique can be applied the other way around as well, by creating a pathogen protein microarray and applying the labelled human proteins to identify potential interactors (Feng et al., 2018). There are quite a few commercially available protein microarray chips for human (Lueking et al., 2003), *Arabidopsis thaliana* (Popescu et al., 2007), coronavirus (Zhu et al., 2006), yeast (Gelperin et al., 2005) and *E. coli* (Chen et al., 2008).

A major challenge with this method is that the protein structure of the capture protein may not be maintained on the chip. If the interactions between the two partners are dependent on the 3D conformation and orientation, it may show false negative results.

## 11. Phage display

Phage display is a molecular technique in which the DNA encoding the protein/peptide of interest is cloned along with the phage coat protein. Upon phage infection, the phage gene is expressed along with the protein/ peptide of interest in the bacterial host and displayed on the external surface of the phage (phage coat). This method can be HT with a phage display library created by cloning random sequences. Since these DNA clones are stable, they can be stored easily.

Phage library screening has been employed to identify interactions of *Plasmodium* with hepatocytes (Cha et al., 2016; Harris et al., 2005). Hepatocyte binding peptide 1 (HP1) which binds to a hepatocyte membrane protein was identified through screening for phages that bound to the hepatocyte surface and further shown to block hepatocyte sporozoite interactions. HP1 was demonstrated to be a structural mimic of the sporozoite phospholipid scramblase, and direct binding of the recombinant scramblase was confirmed with the putative hepatocyte receptor (Cha et al., 2021). Phage display has been employed to identify secreted bacterial proteins that interact with host immune components. Fusion proteins that were specific to the *S. aureus* secretome were displayed and libraries were screened against neutrophils, identifying two new *S. aureus* proteins, SEIX and SSL6, which were demonstrated to bind glycosylated neutrophil receptors PSGL-1 and CD47 respectively (Fevre et al., 2014).

A key issue with this technique is that the proteins that get displayed are often misfolded. False positive interactions with proteins not encoded by pathogens can be a problem when using random sequence libraries.

## 12. Surface plasmon resonance

When plane polarized light is directed on a metal surface at an angle to allow total internal reflection, surface plasmon resonance phenomenon occurs. This leads to generation of electron charge density waves called plasmons that change the intensity of the reflected light at an angle called the resonance angle (Drescher et al., 2018). The SPR signal (resonance angle) is directly dependent on the refractive index of the medium on the sensor chip. Binding of molecules to the sensor chip leads to changes in the refractive index of the chip surface resulting in a change in the SPR signal.

The ligand is usually immobilized on a sensor chip and the analyte is applied over the surface, under flow conditions, to assess the binding of the two molecules (Fig. 3). The SPR sensor chip contains a glass surface

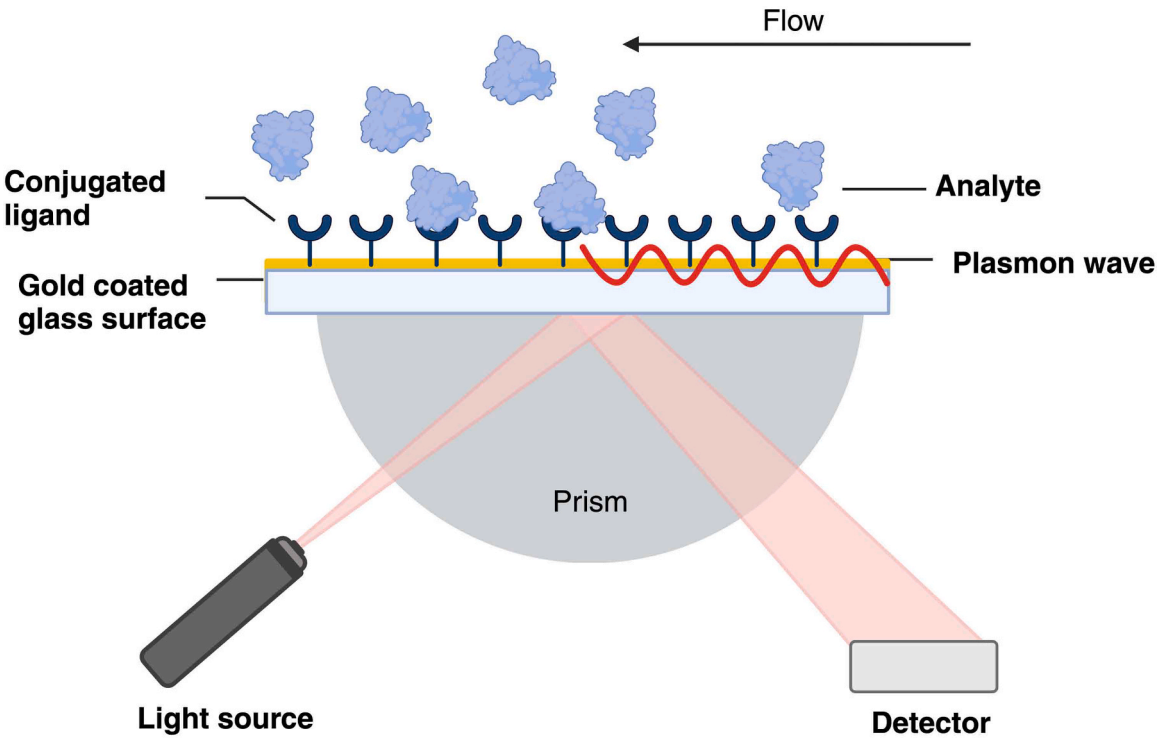


**Fig. 3.** Schematic diagram of the Surface Plasmon Resonance (SPR) based method to detect PPI: Interactions between the analyte molecules in solution with the molecules on the sensor surface (ligand) is monitored in real time by a detector (Created using BioRender).

with a coating of thin layer of gold (50 nm) and an interaction layer most commonly made of carboxymethyl-dextran (~100 nm thick) which is linked to the gold surface (Wilson, 2002). Amine coupling is a method for attaching ligands to the chip; the dextran-carboxyl group on the chip is activated and the amine groups gets covalently bound by an amide linkage to the chip (Lahiri et al., 1999; Wammes et al., 2013). SPR signal measured is proportional to the mass on the surface of the chip at a particular instance and to the number of molecules bound to the surface (Drescher et al., 2018). SPR sensor chips are available in different varieties, including streptavidin, protein A, nickel-nitriloacetic acid (Ni-NTA), based on the method used for immobilization of the ligand of interest to the chip. HT methods are being developed to process more samples with lesser time. Furthermore, advanced methods of making SPR chips are being developed to increase the sensitivity of the instrument (Yang et al., 2017).

Matharu et al. screened and identified diverse therapeutic monoclonal antibodies using this technique (Matharu et al., 2021). Using an array format SPR, rapid identification of *Mycobacterial tuberculosis* infection was reported (Hsieh et al., 2012). Nine TB antigens were immobilized in an array format and multiple antibodies in serum were assessed for binding simultaneously. When compared to the standard immunological methods and ELISA, the SPR method provided real time label-free detection, along with high sensitivity and specificity (Hsieh et al., 2012).

A strength of this highly sensitive method (picomolar range) is that it provides interaction data in real time with details such as equilibrium binding constants, kinetic rate constants, and the thermodynamic parameters that helps understand the mechanism of binding reactions (Huber and Mueller, 2006). However, on the downside, the interaction conditions used may not be physiologically relevant, and the chips and the instrument are highly expensive to use.

## 13. Spatial proteomics

The local environment of protein/s is constantly changing in an infection scenario. Hence, studying temporal and spatial changes in the proteome is essential and provides a better understanding of the molecular mechanisms underlying infection. Spatial information such as changes in a single cell with respect to organelles or changes in infected vs uninfected cells can be identified by appropriate fractioning and labelling methods (Jean Beltran et al., 2017). Individual cell organelles can be isolated using differential or density gradient centrifugation, differential detergent fractionation, biotin labelling and affinity purification (Gudleski-O'Regan et al., 2012; Lempke et al., 2023).

Spatially targeted protein identification involves the extraction of material from tissues surfaces and identifying the proteome at that specific tissue foci. One such technique is liquid extraction surface analysis (LESA) where a small volume of solvent (0.5 - 3 μL) using a glass capillary is dispensed on tissue allowing a liquid microjunction to form between the tip, liquid, and sample. This allows the extraction of proteins and peptides which can be collected for later MS analysis (Ryan et al., 2019). MicroLESA was used recently to spatially analyse specific foci in the abscess formed by *Staphylococcus aureus* to identify the physiological state of bacteria within the abscess and the pathogenic processes at the host-pathogen interface (Guiberson et al., 2021). Spatial methods such as spatial proteomics are key to gaining a holistic understanding of the infection process, although currently they do not detect specific direct interactions between host and pathogen proteins.

## 14. Summary and conclusions

A spectrum of new technologies are now available to study PPIs, which will be critical in defining interactions between the host and the pathogen that control the outcome of an infection (Shah et al., 2015). In contrast to screening single interactions, HT methods have enabled simultaneous identification of multiple binding partners, providing a wealth of new data and increasing the pace at which we can decipher complex biological interactions. Clearly, the technique chosen would depend on the biological systems it is applied to, the tools and expertise available and the costs involved. As all the techniques have their own

merits and demerits, using multiple assays helps to validate interactions. Furthermore, confirming hits from a HT screen using methods demonstrating direct interactions (immunoprecipitation, size exclusion chromatography, directed 2-hybrid screens etc) is also essential, given the possibility of false positive hits.

Thus, with new cross-disciplinary technologies to study host-pathogen interactions, we are well placed to gain a deeper understanding of infection biology, which is crucial for developing effective control strategies. With the newer single cell and spatial proteomics technologies available, we expect that there will be exciting future advances in host-pathogen interaction studies at a single cell level.

## CRediT authorship contribution statement

**Unnikrishnan Meera:** Conceptualization, Writing – original draft, Writing – review & editing. **Chandrasekharan Giridhar:** Conceptualization, Writing – original draft, Writing – review & editing.

## 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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