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Review Article

Protein microarrays, biosensors, and cell-based methods for secretome-wide extracellular protein–protein interaction mapping

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ABSTRACT

Approximately one quarter of all human genes encode proteins that function in the extracellular space or serve to bridge the extracellular and intracellular environments. Physical associations between these secretome proteins serve to regulate a wide range of biological activities and consequently represent important therapeutic targets. Moreover, some extracellular proteins are targeted by pathogens to allow host access or immune evasion. Despite the importance of extracellular protein–protein interactions, our knowledge in this area has remained sparse. Weak affinities and low abundance have often hindered efforts to identify these interactions using traditional methods such as biochemical purification and cDNA library expression cloning. Moreover, current large-scale protein–protein interaction mapping techniques largely under represent extracellular protein–protein interactions. This review highlights emerging biosensor and protein microarray technology, along with more traditional cell-based techniques, that are compatible with secretome-wide screens for extracellular protein–protein interaction discovery. A combination of these approaches will serve to rapidly expand our knowledge of the extracellular protein–protein interactome.

1. Introduction

From high-affinity cytokine–receptor interactions to low-affinity cell–cell adhesion receptor interactions, the diversity of extracellular protein–protein interactions (ePPI) has been well documented [1–5]. Standard methods for intracellular protein–protein interaction mapping have proven challenging for ePPIs [1,2]. The yeast-2-hybrid (Y2H) system functions through an intracellular mechanism and therefore the proper folding and post-translational processing of secreted or membrane proteins are less likely to occur. Affinity purification–mass spectrometry (AP–MS) depends on co-purification of interacting partners from a single cell line; therefore, weak interactors and binding partners expressed on different cellular sources will be missed. Furthermore, solubilization of plasma membrane proteins under conditions that can preserve active conformations and lower-affinity interactions remains a challenge for AP–MS. Consequently, there is a pressing need for new and improved methods that can help dissect the extracellular interactome.

Historically, biochemical approaches were employed to isolate receptors through the identification of specific cell binding and crosslinking of Iodine-125-labeled ligands, followed by co-purification and later cloning. Receptors for PDGF, EGF and insulin, for example, were discovered in this manner [6–11]. In some cases, interacting molecules were inferred from genetic phenotypes or functional data [12–15]. Antibodies that block a specific interaction have also been useful in identifying target receptors [16–18]. Although these classical techniques are effective, they are often laborious and time consuming. The advent of cDNA cloning in the 1980s led to a dramatic expansion of our knowledge of ePPIs [19,20]. Unfortunately, cDNA library screening has often been challenging, likely because of limitations due to appropriate library construction, transcript abundance and in some cases low-affinity binding.

Today, with the completion of the human genome and subsequent improvement in gene annotation, it is now practical to define the "secretome" (i.e. all secreted, single transmembrane and multi-transmembrane containing proteins) and employ high-coverage, secretome-wide clone or protein libraries for tackling ePPI discovery. This review provides examples of methodologies that have proven successful for ePPI discovery and are potentially compatible with large-scale library screening. Also, emerging technologies that will likely impact the field of ePPI mapping will be considered, and early efforts at constructing secretome-wide libraries will be highlighted. Taken together, this information offers a broad range of methodology for the development of the next-generation ePPI screening platforms.

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2. Cell-based ePPI mapping

2.1. Single-clone expression cloning

With the completion of the human genome we might expect an increased use of individual clone libraries for screening ePPIs yet surprisingly only a few efforts have been described. One early example by Lin et al. described a screen of 300 secreted or transmembrane (TM) human proteins, fused at the C-terminus with the dimeric Fc portion of IgG (Fc), against a library of 400 cell-surface proteins transiently expressed on COS7 cells [21]. NGL-1 was reported to be the only protein that bound to COS7 expressing netrin-G1, having an affinity (K_D) of 1.6 nM. These results are encouraging and suggest this approach might easily be adapted to a secretome-wide scale. However, in order to achieve this, it is important to evaluate the degree of false positives that such screens might generate. Moreover, the affinity of the interaction is an important consideration in cell-binding assays with soluble bait proteins [5]. Unfortunately, the authors did not report the raw data set, so the overall performance of the assay is unclear.

We can evaluate more carefully a focused screen, by Bossen et al., where the complete interaction data set is provided [22]. This study probed the ePPI network between all available TNF ligands and TNF receptors in mouse and human. They utilized 96-well format flow cytometry to interrogate transiently transfected 293T cells expressing TNF receptors linked to a C-terminal glycosylphosphatidyl inositol (GPI) anchor sequence. Binding of soluble recombinant Fc- or FLAG-tagged protein to these cells was detected via PE-coupled anti-tag antibodies. A plot of ligand binding vs. expression (via EGFP co-transfection) mean fluorescence intensity allows unambiguous identification of positive interactors by an upward curving profile (Fig. 1). Encouragingly, only a few plots showed ambiguous profiles, and overall, the authors were able to detect more than a hundred specific interactions. Only a handful of interactions were missed and the authors attribute this to tag or steric interference. In several of the missed cases, reversing the orientation, by expressing untagged full-length TNF ligand on the cell surface, resulted in positive interactions as expected. Importantly, false–positive interactions within the human–human and mouse–mouse interacting sets were largely absent. The only apparent false–positive interaction was a weak interaction between mAPRIL and mBAFFR, an interaction that was not observed between the human proteins. Interestingly, the flow cytometry binding profiles for BAFF-Fc ligand binding to BAFFR- and BCMA-transfected cells look quite similar despite two orders of magnitude difference in their monovalent affinities as measured by surface plasmon resonance (SPR) (16 nM vs 1.6 μM, respectively) [23]. This may be explained by an avidity effect of the trimeric TNF ligands that likely form hexameric structures due to the Fc tag [24]. Indeed, subnanomolar affinities were measured by SPR for both these receptors binding to BAFF-Fc fusion protein [23]. This observation suggests that the assay may be capable of detecting low affinity ePPIs if ligand multivalency is employed.

2.2. Multivalent microbead-based cell binding

An older but potentially useful and scalable approach for lower-affinity ePPI mapping utilizes multivalent microbeads. Microbeads have been used extensively to investigate the properties of ePPI and to enhance activity assays such as T cell proliferation [25–33]. Weak and transient ePPI with fast dissociation rates can achieve high physiological affinities through avidity effects by lateral diffusion and receptor interactions across apposing membranes [34,35]. Similarly, multivalent microbeads provide a 2-dimensional surface capable of presenting proteins in a multivalent fashion. Indeed, CD48-coated microbeads specifically bound cells transfected with CD2 despite an affinity of tens of micromolar [30]. Microbead-based cell binding assays are also capable of detecting homotypic interactions on cells or between the microbeads themselves by following their aggregation over time [26–29]. More recently, Wojtowicz et al. made extensive use of microbead-binding assays to characterize the interaction specificity of Dscam isoforms, showing that each isoform can bind itself but not to other isoforms [32]. The Groves lab has developed a microbead coated with a fluid phospholipid bilayer membrane [36]. By incorporating GPI-anchored neuroligin-1 onto these microbeads, the authors were able to demonstrate specific neuron binding and activation [37]. The GPI anchor, which is embedded in the outer leaflet of the membrane bilayer, allows for 2-dimensional diffusion and presumably efficient multivalent interaction at the cell surface [38]. In some cases, particularly where cis-interactions are also important for avidity, these membrane-coated particles may be useful.

2.3. Cell–cell interaction-based expression cloning

Crocker and colleagues have extended and optimized this multivalent binding approach by developing a cell–cell interaction assay based on a C-terminal multi-purpose tag [39]. As with the TNF receptor–ligand screen described above, a GPI anchor sequence derived from hTRAILR3 was used. In addition to cell-surface mobility, using an ECD–GPI tag fusion is expected to provide high cell-surface expression and removes the chance that the intracellular regions of TM proteins will restrict diffusion via cytoskeletal interactions or retention within the cell [40]. The authors also use the TRAILR3 GPI sequence to confirm cell surface expression by immunostaining. The rest of the multi-purpose tag design allows for cleavage from the cell surface for the generation of a soluble protein reagent and an Avitag motif for biotinylation and an EGFP domain for detection. The adhesion receptors JAM-B and JAM-C were used in a cell–cell mixing experiment with cells differentially labeled with DiI or DiD phospholipid binding dyes. Heterogeneous cell clusters were analyzed by flow cytometry, and specific heterotypic interactions were successfully detected with a signal just over twice the background. Because more interactions were not tested and the low signal-to-noise observed in this example, it remains unclear whether heterogeneous cell–cell binding, at least as employed here, has utility for larger scale ePPI screens. Cell–cell binding studies are often difficult to perform because of the narrow window of mixing and washing stringency and the potential for non-specific cell–cell clustering. Nevertheless, because of the possibility for detecting avid cell–cell ePPIs, without the need for purified proteins, this method clearly warrants further investigation. Evaluation of a panel of interactions along with different detection modalities would be useful and help determine if the relatively large multi-purpose tag can be detrimental to certain ePPIs.

Alternatively, reports have described yeast and baculovirus "display" as potential multivalent constructs for activity and binding screens. Using yeast expressing high cell-surface levels of an anti-TCR (scFv) antibody, the authors were able to demonstrate efficient and specific T-cell activation, while monomer antibodies were unable to trigger activation [41]. Unfortunately, direct cell–cell binding was not evaluated in this study. Baculovirus (BV) particles acquire a phospholipid membrane as they bud from the surface of insect cells. If TM-containing proteins are over expressed at the cell-surface, BV will incorporate these proteins into the virion and act as multivalent binding reagents. Sakihama et al. evaluated BV display by testing several receptor–ligand pairs (CD2-CD58, CD40-CD40L and GITR-GITRL) with an ELISA-type assay, demonstrating specific BV (receptor expressing) to BV (ligand expressing)

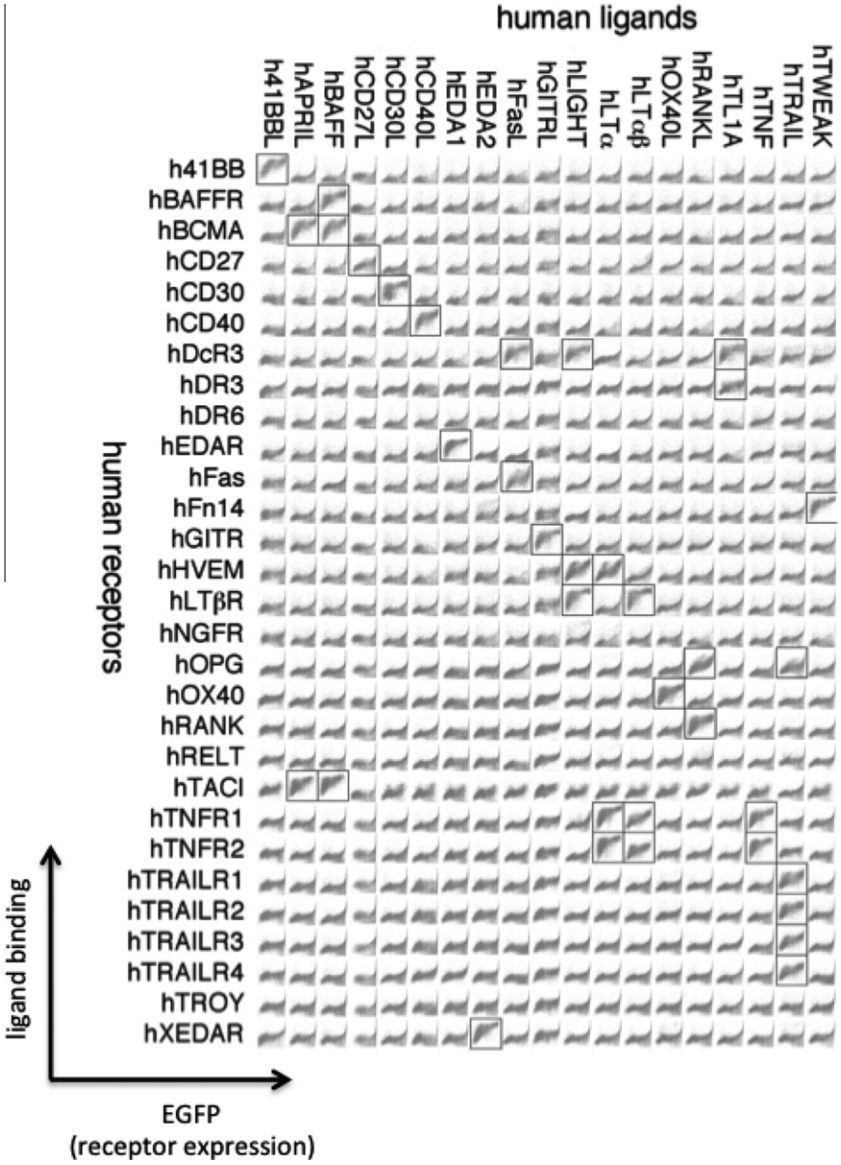

Fig. 1. Ligand–receptor interaction screen of the human TNF and TNFR families. Cells were analyzed by two-color flow cytometry, and the scattergrams obtained for each receptor–ligand combination are shown. Data are displayed with mean fluorescence intensity on a logarithmic scale (10^0–10^4), with ligand binding on the *y*-axis and EGFP fluorescence on the *x*-axis. Receptors are listed on the side and ligands at the top of the figure. Positive interactions are boxed and are characterized by the fact that cells transfected to a high level bind more ligand than cells with low levels of transfection. Reproduced by permission from Bossen et al. [22].

binding [42]. Flow cytometry was also used to demonstrate specific BV particle binding to ligand-expressing cells. As with the membrane-coated microbeads, these systems provide 2-dimensional mobility in the context of a native-like phospholipid membrane surface.

In an alternative format, the recombinant bait can be directly bound to a plate followed by panning of cells expressing a candidate protein. Such an avidity-based cell binding approach was used to discover the co-receptor interaction between the immune regulatory proteins PD-L1 and B7-1 [43]. Because of the relatively low affinity (K_D) derived from SPR, between 1.5 and 1.9 μM, this particular assay format was likely critical in the success of the experiment. Binding of a soluble monomeric or dimeric ligand (Fc-fusion) would have been challenging to detect, even with fast washing and fixation. In this example, a cDNA library was used, but conceptually, an individual clone library can be screened in this format as well.

2.4. Functional assay-based expression cloning

An alternative cell-based approach to identify novel receptor–ligand pairs is exemplified by the activity-based screen used by Lin et al. [44]. A proprietary secretome-wide clone collection representing secreted, extracellular regions of single TM and N-terminal domains from multi-TM proteins was used to generate conditioned media in 96-well format and applied to a panel of cell-based activity screens. A novel cytokine, IL-34 was identified that increased monocyte viability, and by screening for the ability of conditioned media from the ECDs of the single TM proteins to block this activity the receptor was identified. The IL34-CSF-1R binding affinity (K_D) as measured by SPR was 1.0 pM. This high affinity was likely essential for identification of the receptor, because, although a considerable fraction of proteins successfully expressed in 293T cells (>90%), the estimated median concentration was only 75 ng/ml. Thus, the concentration of CSF-1R (ECD)-Fc fusion protein in the

media would have been approximately 500 pM. Therefore, unless higher protein concentrations can be attained, this approach will likely miss low-affinity ePPIs. An advantage of this approach is that no protein purification is required and direct evidence of functionality is provided by the activity-based assays.

Taken together, these examples show that there are a variety of cell-based ePPI screening approaches that when combined with multivalent binding can be quite powerful. Moreover, clone libraries obviate the need for large-scale protein purification. However, despite the larger investment required to generate a protein library, there are a number of biosensor and microarray technologies that have been shown to work well for ePPI mapping. Ultimately, applying diverse approaches utilizing both clone and protein libraries will likely increase the chances of identifying novel ePPIs.

3. Sensor-based ePPI mapping

3.1. Surface plasmon resonance

Label-free kinetic binding analyses of protein–protein interactions became possible with the introduction of surface plasmon resonance (SPR) in the early 1990's. Barclay and colleagues first demonstrated that SPR was well suited for detecting low-affinity interactions between receptors of the immunoglobulin superfamily by characterizing the interactions of rat CD2-CD48 (K_D 60–90 μM) and CD2-CD58 (K_D 10–20 μM) [5,45–47]. They discovered that weak affinities were largely due to fast off rates. More recently Barclay's group combined SPR with bait-coated microbeads to enhance ePPI detection [48]. They used mCD200-coupled microbeads to bind sensors immobilized with mCD200R (K_D ~4 μM), mCD200RLa (K_D > 500 μM), and mCD200RLb (no binding). Interestingly, both mCD200R and mCD200RLa showed roughly equivalent binding sensograms and essentially no dissociation, while no binding was detected to mCD200RLb. This observation experimentally reinforces the concept that enhanced binding through multivalency for weak interactions is possible without promoting spurious non-specific interactions. Multimerization has also been achieved using the pentamerization domain from COMP [49]. The measured off rate for mCD200-COMP was over twice as slow ($t_{1/2}$ = >3,000 s) compared to mCD200-Fc ($t_{1/2}$ = 1,500 s). As a second example, CD48-COMP also showed enhanced binding to CD2 by both SPR and cell-binding experiments. Wright's group has taken advantage of COMP fusions to generate soluble multivalent baits for an ELISA style ePPI screen they developed called AVEXIS (avidity-based extracellular interaction screen) [50]. AVEXIS has been used for large-scale screens, including 150 zebrafish IgSF and/or lucine-rich repeat containing receptors [51,52].

Although well suited for detection of weak interactions, the use of SPR for global ePPI mapping has not been widely employed. A major limitation is the available throughput. The most widely used Biacore systems have 4 flow cells, allowing the parallel screening of up to three immobilized or captured proteins with one flow-cell as reference. Analyte proteins are injected one at a time via an auto-sampler. Despite this low-throughput, a library of over 2,000 secreted proteins and receptor extracellular domains was utilized for SPR screening and led to the discovery of the interaction between HVEM and BTLA [53,54]. A significant challenge with this approach, however, stems from the fact that some protein samples will invariably bind to the sensor surface irreversibly, necessitating the use of a new sensor and re-immobilization of the bait, itself a low-throughput process. Consequently, a SPR format that instead allows immobilization of the library and injection of the bait as an analyte would be better suited for large-scale ePPI screens.

Encouragingly, instrumentation advances continue to push SPR towards higher throughput. The ProteOn™, for example, offers a 6-by-6 channel microfluidics-based sensor, allowing for detection of up to 36 different interactions in a single assay. As proof of concept, Jiang and Barclay used this system for a small-scale ePPI mapping experiment with 36 members of the IgSF family, again using the multivalent bead approach described above [55]. For global secretome screens, however, even this level of throughput is severely limiting.

3.2. SPR imaging microarrays

Grating-coupled SPR instruments, such as the Biacore Flexchip, allow for a larger sensor surface where up to 400 samples can be immobilized. Such a system has been used to map an endostatin ePPI network of over 30 proteins [56]. Similarly, SPR imaging (SPRI), also termed SPR microscopy, has potential as a secretome-wide screening platform [57–63]. Technical limitations, however, have hindered full development of SPRI for this application. While conventional SPR monitors a change in the angle of minimum reflection of p-polarized visible light due to protein binding on the gold sensor, the larger detection surface employed in SPRI generally requires a set incident angle with the change in intensity of the reflected light measured instead [62]. Ultimately, this difference results in diminished sensitivity for SPRI platforms compared to conventional angle-shift SPR [64]. Uniform illumination of a relatively large area also introduces challenges for large microarray applications [62]; consequently, the sensor size and the number of elements that can be printed is smaller than conventional glass slide microarrays. With current microarrayer technologies, SPRI sensors should be able to hold up to 2,000 elements, depending on the spotting parameters. Improvements in instrumentation, surface chemistries and signal amplification may enable SPRI for secretome-wide ePPI studies, but clear demonstrations have yet to be published.

Multivalent microbeads may improve SPRI sensitivity for ePPI applications in more ways than one. For example, Corn and colleagues have developed a highly sensitive approach termed nanoparticle-enhanced diffraction gratings (NEDG) [65,66]. In this system, planar surface plasmons on gold diffraction gratings are coupled to localized surface plasmons on specifically bound gold nanoparticles. These gold nano-particles could conceivably be coated with bait proteins to allow for improved sensitivity for ePPI detection through both avidity and SPR signal enhancement.

3.3. GMR sensors

Significant progress has been made recently in giant magnetoresistive (GMR) biosensors for ultrasensitive and multiplexed interaction detection [67–71]. Based on technology used in computer hard drives, GMR biosensors rely on the quantum mechanical magnetoresistance effect, which is induced when iron-based nanoparticles are brought into close proximity (within 150 nm) of the sensor surface. In a magnetic field these nanoparticles are magnetized and induce a change in the resistance of a current applied through the sensor. In practice, a capture antibody, receptor, or ligand can be immobilized on the sensor surface and probed with 50 nm nanoparticles coated with a detection antibody or a binding partner. Real-time signal detection is measured in solution and without washing. When directly compared to a standard sandwich-ELISA format using the same antibody reagents, GMR sensors have exceeded sensitivity by at least two orders of magnitude [68]. Moreover, an analytical model has been developed that allows estimation of kinetic rate constants (k_{on} and k_{off}) for high-affinity interactions [71]. This technology is also theoretically compatible with very high sensor densities. Indeed, the Wang lab has

generated sensors that are 100 × 100 μm, similar in size to traditional microarray spots, that could ultimately allow for many thousands of sensors on a single chip [71]. Currently an 80-sensor array is being developed for market, which is a more advanced version of the previously published 64-sensor array [67] (Magarray, Inc., personal communication). This nanoparticle-based approach incorporates multivalent binding with the potential for very high sensitivity and real-time binding in a scalable format, all ideal for ePPI discovery.

3.4. Bio-layer interferometry

Bio-layer interferometry (BLI) is an established biosensor technology that also has strong potential for ePPI screening. While providing similar kinetic binding data as SPR, the platform commercialized by ForteBio uses cost-effective, single-use fiber optic sensors enabling parallel detection on up to eight channels. These features allow for real-time and label-free screening of a protein library in a 96-well plate format. A SPR screen of 2,000 samples taking several months could be completed within a week using this BLI platform. The co-receptor interactions between TIGIT-PVR, and Robo4-UNC5B were discovered by this approach [72,73]. In these examples, a Fc-fusion bait was captured to saturation on anti-human Fc sensors, and the sensors were then sampled column-wise across 96-well plates containing candidate proteins at 5 μg/ml (Fig. 2). The reported affinities (K_D) for these interactions were on the order of 3–12 nM, representing strong interactions. Because of the low protein concentrations used, this assay may have missed weaker affinity interactions.

Recent advancements in BLI instrumentation from ForteBio have doubled the throughput by incorporating 16 parallel sensors, each with an independent detector to increase the signal-to-noise ratio, and a 384-well plate option that allows for sample volumes as low as 40 uL. These smaller volumes can translate into higher sample concentrations when protein quantity is limited. This second-generation instrument could be used to screen a bait protein against a 2,000-sample library in one day, excluding the time to prepare the source plates. Importantly, because sensors are simply "dipped" in each well, protein samples are not consumed and plates can be recovered and reused.

As with SPR, early efforts have been made to adapt interference optical sensors into a microarray format. The Interferometric Reflectance Imaging Sensor (IRIS) [74–76], Arrayed Imaging Reflectometry (AIR) [77,78], and Molecular Interferometric Imaging (MI2) [79] all use variations of interferometry to measure binding to the sensor surface. Similar to SPRI, the sensor surface is illuminated and detected though a CCD camera capturing time-resolved changes in signal. These interferometric microarray technologies, however, have yet to be commercialized and their sensitivities relative to other approaches have not been demonstrated.

3.5. Back-scattering interferometry

Back-scattering interferometry (BSI) is an emerging technique for measuring molecular interactions in solution without the need for labeling or immobilization [80]. The platform consists of a collimated coherent light source (HeNe laser), a capillary or microfluidic chip containing the sample, and a CCD array detector. The light beam passing into the capillary or microfluidic chip results in a reflection/refraction phenomenon that creates a series of high contrast fringes derived from classical constructive and deconstructive interference. These fringes, detected in the back-scatter direction, are highly sensitive to the refractive index of the sample [81,82]. The refractive index in turn is dependent on the conformation and association state of the constituent proteins and molecules within the capillary/chip. With this relatively simple set up, BSI can be used to evaluate ePPIs in solution. Interactions between soluble extracellular proteins and membrane-associated receptors, incorporated into small unilamellar vesicles, have also been demonstrated [83]. Despite the advantage of being a label free, in-solution technique, the approach is currently not suitable for high-throughput screening. Nevertheless, because of the more native-like context, BSI may prove very useful for secondary validation and characterization of ePPIs after initial discovery.

4. Protein microarray-based ePPI mapping

Protein microarrays for detecting protein–protein interactions were first demonstrated over a decade ago [84,85]. Large-scale yeast and human proteome microarray chips have been generated from proteins expressed in yeast and baculoviral systems, respectively, and purified using GST tags [85,86]. These microarrays have been commercialized (ProtoArray, Life Technologies™) and used for profiling studies, including kinase phosphorylation, ubiquitination, biomarker and autoantibody characterization [86,87]. Despite these developments, few large-scale protein–protein interaction mapping studies using protein microarrays have been reported. One likely reason is the difficulty and expense of generating large plasmid and/or protein libraries. Also, techniques like yeast-2-hybrid (Y2H) and affinity-purification mass spectrometry (AP–MS) do not require laborious library constructions and have emerged as powerful alternatives for intercellular PPI mapping. However, because Y2H and AP–MS data sets under represent ePPIs [1,2], there is renewed interest in developing microarrays as a platform for ePPI studies.

4.1. In vitro transcription-translation-based microarrays

In vitro transcription-translation (IVTT) systems for high-throughput protein production have been described and used to generate high-content microarrays. LaBaer's group has developed an innovative method called Nucleic Acid Programmable Protein Arrays (NAPPA) where DNA plasmids are directly spotted onto the microarray itself, followed by cell-free protein expression [88]. A C-terminal GST-tag is used to directly capture proteins *in situ* via an immobilized anti-GST antibody. The ability to express proteins immediately before an assay is performed minimizes potential issues associated with long-term protein storage and stability.

Unfortunately, although IVTT systems allow high-throughput protein production they are generally not optimized for producing secreted mammalian proteins. An oxidizing environment along with specialized chaperones, a glycosylation machinery and post-translational processing are often required for proper folding and functional activity of extracellular proteins. Although, mammalian cell lysate-based IVTT systems may overcome some of these limitations by providing relevant chaperones and glycotransferases [89], careful functional studies demonstrating correctly folded and active proteins have yet to be described.

Interestingly, studies clearly suggest that IVTT systems are capable of generating TM-containing proteins in soluble form. A large-scale *in vitro*-expression study, using a wheat germ cell-free system, showed that 97% of 13,364 human proteins were expressed as soluble proteins (defined as protein remaining in solution after a high speed spin), as detected via anti-tag western blots [90]. Because over 3,000 of these proteins (24%) were predicted to contain at least a single TM domain, it is apparent that many TM-containing proteins were generated in soluble form. Similarly, with NAPPA, using a rabbit reticulocyte lysate IVTT system, 253 out of 272 (93%) of TM-containing proteins were expressed, as judged by antibody binding to their C-terminal GST tags on the microarray [88]. The possibility remains, however, that

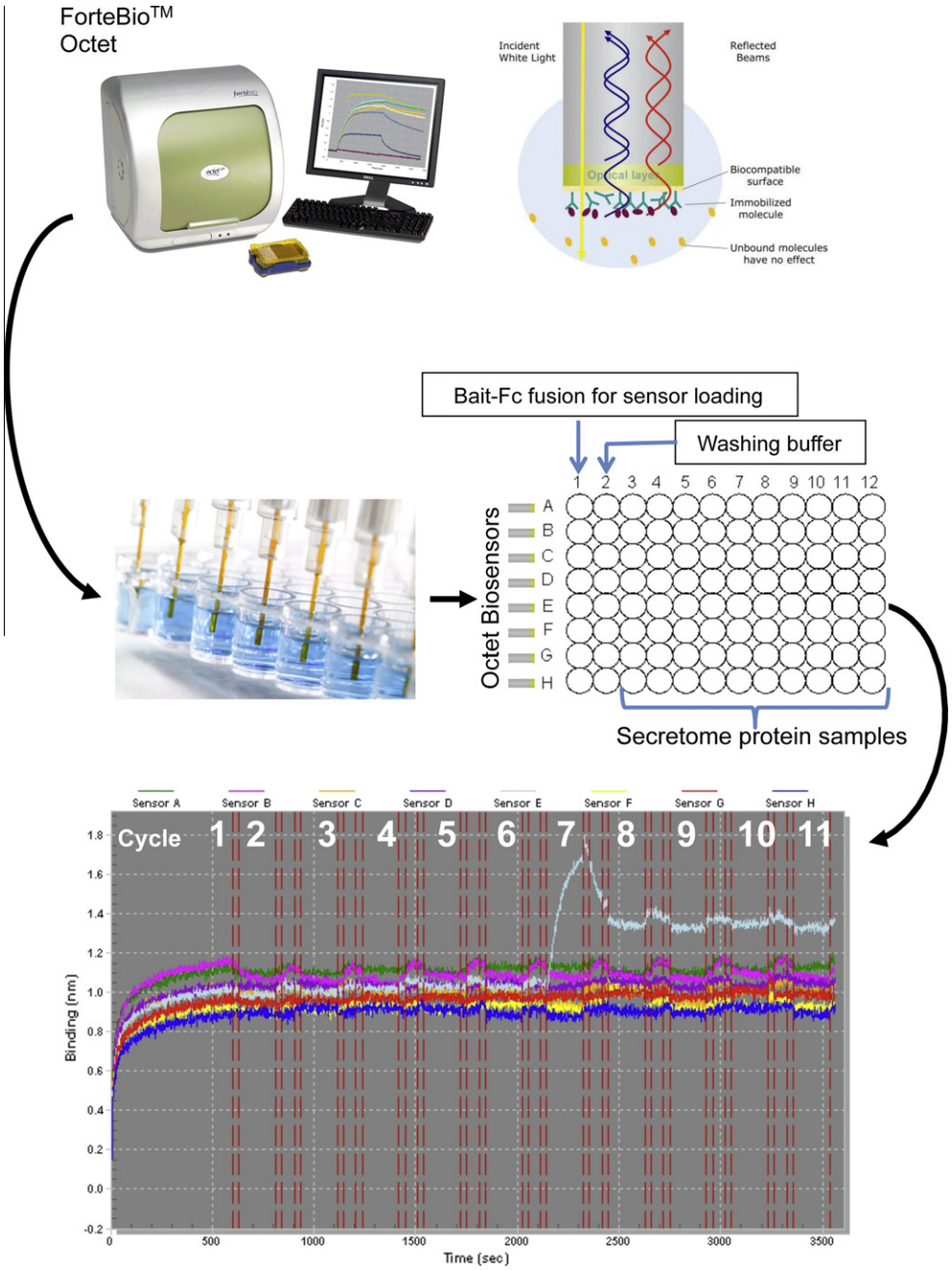

Fig. 2. Screening ePPI by bio-layer interferometry (BLI). Using a ForteBio Octet system a Fc-fusion bait protein is captured from wells in column 1 of a 96-well plate onto eight parallel fiber-optic sensors coated with anti-human Fc antibody until saturation is reached (cycle 1). The sensors are then briefly rinsed in buffer (column 2) and sequentially applied to samples wells (columns 3 to 12; cycles 2–11) with brief rinses (column 2) in between. In this example, each sensor is represented by a different color sensorgram. A hit was identified in cycle 7 in the cyan sensor as evidenced from the spike in signal over baseline.

these secreted or TM-containing proteins are largely misfolded or form soluble aggregates.

With the NAPPA format, intracellular protein–protein interactions were tested for Fos, Jun and p53, clearly identifying the target interacting proteins with apparently very little background or non-specific binding [88]; however, no ePPI's were tested. Goshima et al. used the wheat germ cell-free expression system along with optimized non-reducing conditions (by omitting dithiothreitol and including reduced and oxidized glutathione as a redox system and adding protein disulfide isomerase) to evaluate three secreted proteins containing disulfide bridges, tumor necrosis factor-α (TNF-α), interferon-β (IFN-β) and oncostatin M (OSM) [90]. IFN-β and OSM require proper disulfide link formation for full functional activity. IL-1β was also included as a post-translational processing control. The biological activity of each protein, along with recombinant protein controls, was evaluated by gene expression profiles using endothelial cells (HCAEC). Although biologically activity was detected for these proteins, the assay unfortunately does not quantitate the percentage of active material. Because each of these cytokines binds their respective receptors with very high affinity, very small amount of active material would likely have been sufficient to fully stimulate these cells.

The IVTT system provides a means for circumventing the significant resource investment required for protein library construction. If IVTT methods can be developed and demonstrated to produce high levels of active, properly folded extracellular proteins

for a large fraction of the secretome, this approach will surely enable much wider use of the microarray format for ePPI mapping. The results outlined above are encouraging, but more studies are necessary.

4.2. Glass slide-based protein microarrays

For the present time, microarray platforms for ePPI mapping will likely require the production of proteins through traditional mammalian or insect cell expression systems and the utilization of a multivalent approach. Towards this end, in 2005 Barclay's group first evaluated low-affinity ePPIs in the protein microarray format by using CD200-coupled multivalent microbeads to demonstrate the efficient binding to its receptor CD200R immobilized on epoxy-coated glass slides [49,91]. Recently our own group has further validated this approach by screening a library of 1,334 arrayed proteins against 89 immunoglobulin superfamily (IgSF) receptor baits using bait-Fc fusion proteins bound on Protein A coated microbeads (Figs. 3 and 4) [92]. An important consideration in utilizing protein microarrays is the degree of non-specific binding that occurs when using a diverse protein library. The large IgSF test set allowed us to develop a statistical scoring scheme used to track non-specific hit rates for each protein on the microarray. Proteins that repeatedly showed binding to non-related baits were considered non-specific. Only five proteins exceeded an empirical 10% non-specific hit-rate, indicating that non-specific interactions (or false positives) were not a major concern for this assay format. Data for screens of TIGIT, CD160, JAM3 and CD2, representing high- and low-affinity interactions, are shown in Fig. 4. This microarray was subsequently used to discover a novel interaction between a small, secreted cysteine-rich protein termed IGFL with the TNF receptor-like protein TMEM149 (renamed IGFLR1) [93].

Wright's group has also recently described an ePPI microarray platform derived from the AVEXIS assay. Here the protein library was generated with a biotinylated C-terminal tag and captured via immobilized streptavidin on polycarboxylate hydrogel glass slides. Potential advantages of this approach lie in the orientation provided through specific capture of the biotin tag [94] and the ability to systematically and uniformly titrate the amount of protein captured. By analyzing a titration series, insights into the strength of the interaction may be possible from primary screens. Further, the possible flexibility of the polycarboxylate matrix may assist in allowing the surface captured proteins to adopt multivalent interactions with multivalent baits. To begin to construct a protein library, they also developed a low-cost platform of their own design with a capacity to purify up to 96 poly-His proteins in parallel from conditioned media samples ranging from 50 to 100 mL. This system utilizes a pneumatic piston to depress a solid aluminum plate over a rack of syringes connected to a 96-well Ni^{2+}-NTA resin filter plate and can achieve a consistent flow rate of 1 ml/min for efficient affinity capture.

These emerging protein microarray platforms have demonstrated encouraging results for ePPI mapping. Currently, they offer the highest throughput for protein library screening and consume very small amount of protein per microarray. The percentage of false positives and false negatives appears to be small, at least for IgSF family baits. Furthermore, printed microarrays, as long as they are maintained hydrated, offer a convenient format to distribute and transport protein libraries among investigators.

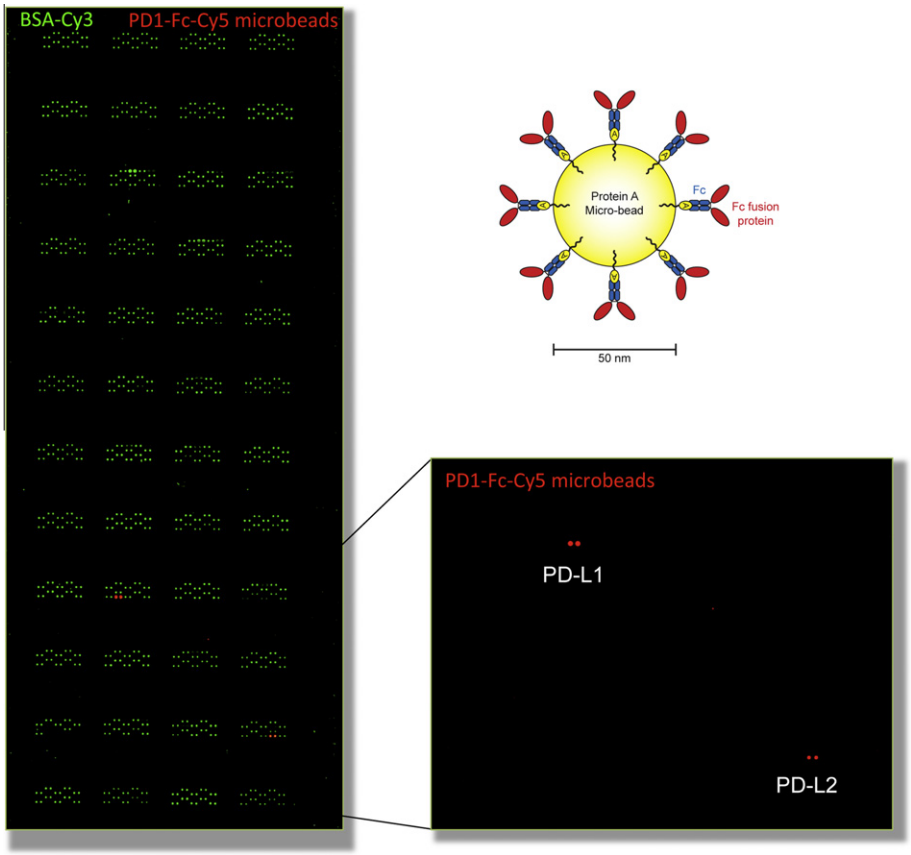

Fig. 3. An example secretome microarray containing 480 proteins. All samples were printed in duplicate spots, with BSA-Cy3 (green) printed between each secretome protein for visualization. Each of the 48 subarrays contains 10 different proteins. A screen of PD1-Fc-Cy5 microbead complex (schematic shown) identified specific interaction with 2 samples on the microarray (PD-L1 and PD-L2).

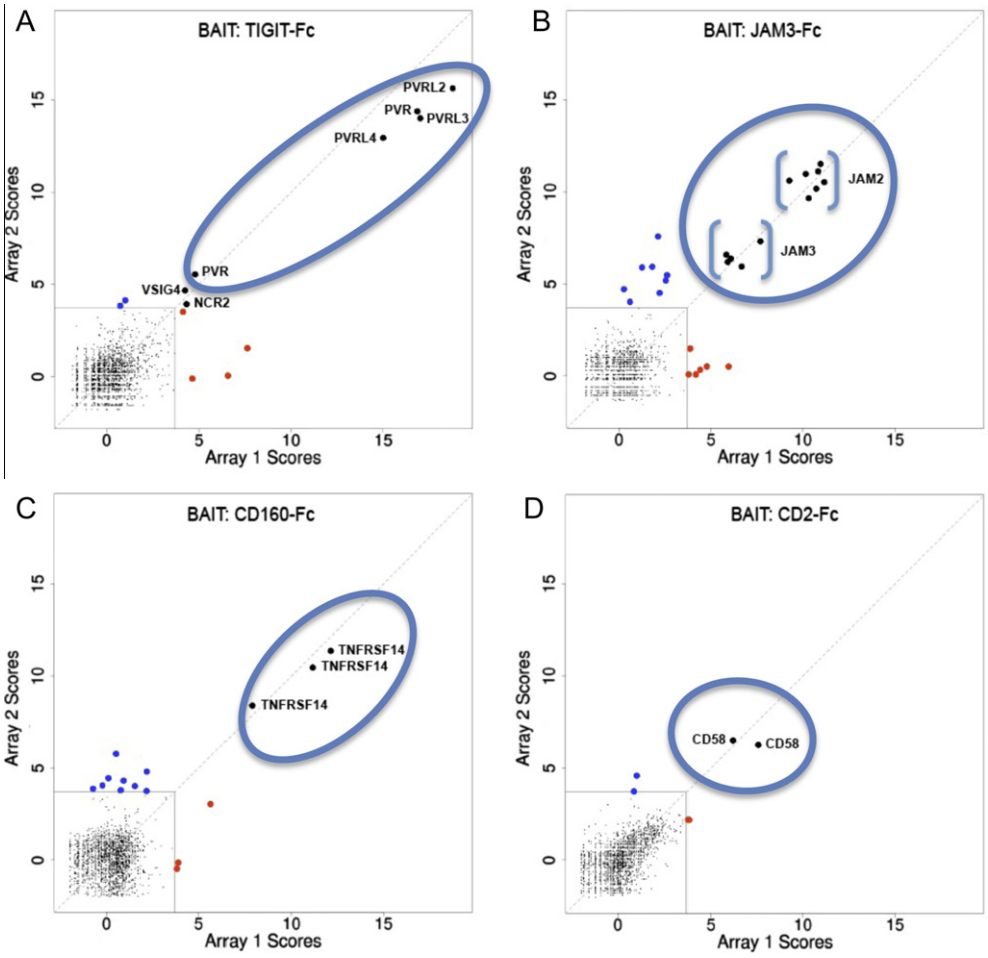

Fig. 4. Secretome protein microarray intersection plots. Black circles (labeled) represent intersection hits from duplicate screens. Red and blue circles represent hits called on only a single array. The expected hits are circled (blue ovals). The lower left square of each plot represents the 0.0001 percentile cutoff and contains all non-hit proteins. Each microarray contains 1,334 secretome proteins. Results from screens of TIGIT-Fc (A), JAM3-Fc (B), CD160-Fc (C) and CD2-Fc (D) are shown. Reproduced by permission from Ramani et al. [92].

5. Clone and protein library efforts

For any of the ePPI mapping methods described here, it is essential to generate quality clone and/or protein libraries for secretome-wide screens. Unfortunately, comprehensive libraries are logistically and resource intensive to construct. With continued advances in automation and development of lower-cost methods, library construction and management will likely become more accessible in the future. Despite this challenge, several organizations have published efforts to establish secretome specific libraries.

The first secretome-based collection was published in 2003 by Genentech, called the Secreted Protein Discovery Initiative (SPDI), with a compilation of 1,021 human extracellular genes [95]. Proteins from the SPDI library were expressed in *Escherichia coli*, insect or mammalian cells with either a poly-His or Fc tag and have been used to identify interactions using SPR, bio-layer interferometry and protein microarrays [53,72,73,92,93].

In 2006, MerckSerono published a 4-year effort to produce over 2,000 human secretome proteins [96]. In this instance, proteins were produced from HEK293-EBNA cells in 100 or 500 mL cultures and purified on an automated platform utilizing affinity chromatography and desalting with a throughput of about 70 proteins per week and a target recovery between 60 and 500 μg. Proteins were aliquoted using robotics and stored at −80 °C.

The National Institute of Advanced Industrial Science and Technology in Japan assembled a versatile Gateway system by creating entry clones suitable for N- or C-terminal tags or untagged constructs [90]. These entry clones, when used in combination with several destination vectors, can be used to generate a variety of different tag options. The proteins were produced by IVTT and left unpurified for microarray applications, which in their estimate constituted only about 1% of the total protein mixture.

Five Prime Therapeutics assembled a nearly comprehensive list of the extracellular proteome and expressed ~3,500 human secreted and extracellular domains of TM proteins from 293T cells using a 96-well high-throughput system [44]. They were able to achieve a 90% expression success rate with a median protein concentration of 75 ng/ml in conditioned media. No purification was done.

The Scripps Research Institute and the Genomics Institute of the Novartis Research Foundation, in a similar effort, assembled a list of ~3,500 human secretome genes and as a pilot effort expressed and purified 529 proteins [97]. Three clones for each gene were generated, representing N- and C-terminal Fc fusions and untagged versions. A fully automated custom robotic system was used to perform mammalian cell culture, transient transfection, protein expression, harvest, and purification. Cell cultures were performed at ~50 mL scale followed by nickel affinity purification, desalting, aliquoting and storage at −80 °C. Average protein yields were ~100 μg with a protein production success rate of 70%.

As these examples show, depending on the downstream application and the resources available, varied approaches have been

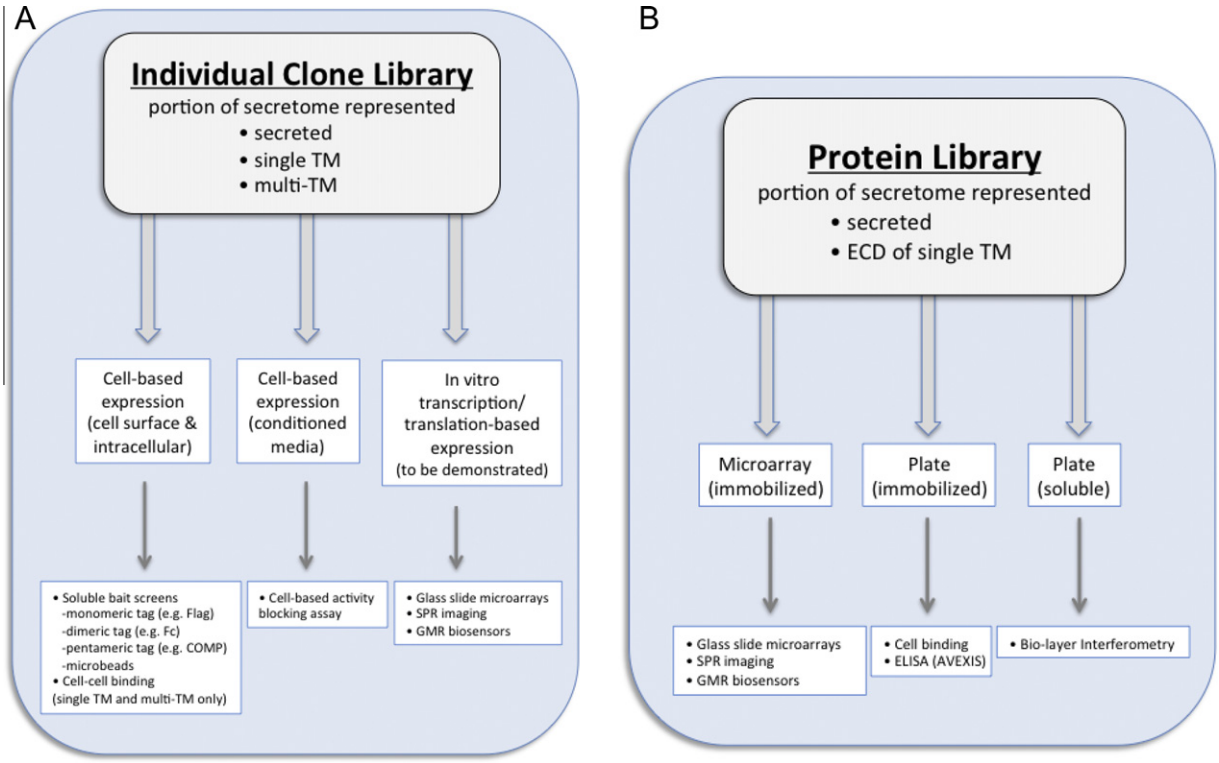

Fig. 5. Outline of individual clone (A) and protein library (B) approaches to extracellular protein–protein interaction mapping. Secretome libraries enable a range of ePPI assays using either cell-based, biosensor or microarray techniques.

taken in constructing secretome libraries. While production success rates of 70% may be possible, protein yields of approximately 100–200 μg are insufficient to run secondary polishing columns such as size-exclusion chromatography or multi-angle laser light scattering for quality control on protein aggregation. Therefore, larger expression volumes will be necessary to allow for improved protein quality. With the availability of automated parallel affinity-based purification systems, however, this increased volume should be manageable.

Formulation of the storage buffer is an important consideration for preserving active material [98]. While a freeze–thaw step may not be detrimental for many proteins stored at −80 °C, freezing is not foolproof and can sometimes lead to protein aggregation or inactivation, depending on the formulation. An alternative storage format is to formulate with 40% glycerol and store at −20 °C to prevent freezing. This glycerol formulation has been used to store 384-well source plates for protein microarray printing [92]. Unfortunately, since most activities are unknown, it will be impossible to evaluate the functionality of the majority of protein samples. Instead, a selected panel of proteins with diverse structures and known activities might serve as a surrogate to represent the overall library stability over time. Clearly there is ample room for improved methodology around protein library production, quality control, and storage.

6. Concluding remarks

The ability to largely define the set of genes that encode the secretome of an organism is now possible. For humans, this set is comprised of roughly 3,500 secreted and single TM proteins, plus approximately 2,000 multi-TM proteins [44,97,99]. Excluding isoform variations and single nucleotide polymorphisms, this number of secretome genes should be manageable as a screening library. In recent years, advances in gene annotation, clone availability and gene synthesis have facilitated assembly of individual clone secretome libraries. As we have seen here, once in hand, both clone and protein libraries can enable a number of different but complementary approaches for ePPI discovery (Fig. 5). No single approach is likely to cover all bases and therefore it makes most sense to apply multiple platforms. Cell-based assays, in any number of formats described here, provide several advantages over protein-based screens, including no need for purification of prey proteins (or bait proteins in some cases), inclusion of multi-TM proteins and a native-like phospholipid environment. Protein libraries, on the other hand, offer a purified system that can be applied to a variety of biosensor technologies, some of which may allow high-throughput detection of very low-affinity interactions without washing.

Clone libraries provide reduced complexity, compared to RNA message derived cDNA libraries, and likely higher levels of expression. Furthermore, depending on the construct design, cell surface expression of each gene can be confirmed, ensuring wide coverage. The cell-based ePPI screening approaches reviewed here suggest that a very low false-positive rate can be achieved, a critical aspect for minimizing the follow up effort required for validation. Interactions with secreted proteins (those without any TM domains) may be challenging to detect in cell-based assays because they are not associated with the outer surface of the cell. In these instances, however, the possibility exists of detecting these interactions using cell permeabilization approaches, as has been widely used in antibody staining protocols. Finally, as we have seen here, there are a number of possible bait formats, including monovalent, dimeric and pentameric tags as well as multivalent microbead assemblies that have been used. Screening untagged proteins is possible, but multivalency is lost and less sensitive direct fluorescent labeling is required for detection, unless an anti-bait antibody is available. Cells expressing the bait can themselves act as multivalent

reagents for cell–cell or cell–plate binding screens; although, additional investigation is needed to validate these particular approaches with diverse types of ePPI.

Protein libraries enable a variety of platforms that offer unparalleled sensitivity for weak interactions. The higher protein concentrations combined with sensitive binding technologies may in certain cases provide an advantage over cell-based binding approaches. A potential downside, however, is the risk of protein inactivation through manipulation during the purification process or long-term storage. Furthermore, activity may be influenced by the choice of tag and immobilization techniques. However, judging from two large protein library ePPI screens [52,92], these risks are probably minimal. Using protein microarrays and AVEXIS, the large majority of ePPI interactions that were expected were successfully identified, indicating that most proteins used in these assays were sufficiently active. The glass slide protein microarray platform has several strengths for ePPI mapping (e.g. minimal protein requirements, high-throughput, and good sensitivity) that will likely make it a useful platform. The incorporation of more advanced technologies, like SPRI and GMR sensors, could in the future provide binding kinetics as well as increased sensitivity for low-affinity ePPI. The 384-well bio-layer interferometry platform is a good compliment to the protein microarray, as the bait protein is immobilized instead of the protein library.

Finally, a major challenge for these approaches is obtaining the reagents themselves. Although resource intensive institutions have been able to compile comprehensive libraries, a public repository for secretome-related plasmid constructs has yet to be established. As a first step, Wright's group at the Wellcome Trust Sanger Institute has begun to deposit constructs used in their ePPI screens with the non-profit plasmid sharing organization Addgene and is in the process of implementing a user group to oversee and manage the collection. Protein library collections are more difficult to manage and distribute compared to plasmid libraries and may require a focused public effort either through a sponsored facility or through a collaborative approach. In the long-term, however, for many of the reasons outlined in this review the field for ePPI discovery looks bright and should contribute substantially to our knowledge of multicellular biology in the coming years.

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