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Noncellular screening for the discovery of protein–protein interaction modulators

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Teaser: We discuss which tests to choose for the screening of small molecules targeting protein–protein interactions.

Author biographies

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Charline Kieffer studied pharmacy at Henri Poincaré University (Nancy, France), and went to Aix-Marseille University to complete her pharmacy residency (Marseille, France). In 2014, she received her PharmD and Pharmacy Residency Diploma and was awarded her PhD in Organic Chemistry (School of Pharmacy, Marseille, France). After completing a postdoctoral period in medicinal chemistry in CERMN (Caen, France), she was appointed lecturer in medicinal chemistry in the School of Pharmacy at the University of Caen Normandy in 2017. Her research interests include medicinal chemistry in the field of protein–protein interactions, to design and evaluate abiotic compounds for use in oncology and neurosciences.

Jean-Pierre Jourdan

Jean-Pierre Jourdan is a hospital pharmacist university teaching assistant. He was awarded his PhD and PharmD in 2015. Since then, he has worked in the University of Caen Normandy toward the discovery of new small molecules potentially useful against Alzheimer's disease. His hospital practice is oriented towards pharmaceutical care of older patients in the Caen University Teaching Hospital.

Anne Sophie Voisin-Chiret

Anne Sophie Voisin-Chiret was awarded her PharmD (2002) and received her PhD from the University of Caen (2005); after completing a postdoctoral period, she was appointed lecturer in medicinal chemistry in the School of Pharmacy at the University of Caen (2007). Since September 2015, she has been a professor of medicinal chemistry. Her research interests include medicinal chemistry in the field of protein–protein interactions to design oligo(het)aromatic compounds that mimic proteins to fold into well-defined conformations, such as helices and β -sheets. Since 2012, she has led a research studying protein–protein interfaces to design drug-like modulators of protein–protein interactions, particularly in oncology and neurodegenerative diseases.

Protein–protein interactions (PPIs) constitute many potential therapeutic targets for the discovery of new drugs. Given their specificity, PPIs are more challenging to target than other ligands. Thus, finding the best screening process can be difficult. Moreover, PPIs often have no direct accessible activity readout. Therefore, it can be unclear which test to choose for the screening of small molecules targeting PPIs. Given that noncellular assays are the most suitable both as first screening assays and for high-throughput screening (HTS), here we focus on noncellular screening assays. For each assay, we discuss the principles and advantages/drawbacks and provide a recent example. We also highlight the crucial parameters to take into account to select the most suitable assays to screen PPI modulators.

Introduction

PPIs (see Glossary) have an important role in the regulation of biological pathways, and PPI disorders are involved in the progression of many diseases [1,2]. According to Azzarito *et al.* [3], the PPI network is estimated to involve >650 000 interactions, but only a small part of this complex network has been characterized so far (www.interactome-atlas.org). The interactome constitutes many potential therapeutic targets to discover new drugs with original mechanisms of action [4–7].

Targeting PPIs is a challenge: the surfaces involved are large and flat [8], featureless, and often lacking in suitable pockets for small molecules to bind to [6]. Recent progress was made to target challenging PPIs with synthetic molecules [9–11], and some inhibitors are now in oncology clinical trials. For examples of molecules in clinical trials, see [12,13] and <https://clinicaltrials.gov/ct2/show/NCT02503423>.

To access new **PPI modulators** (mPPIs), several drug discovery strategies have been used, and can be mixed [14]. HTS is the most common methodology that can rapidly identify small molecules as potential mPPIs [15]. HTS includes the screening of not only classical molecule libraries, but also **fragment** libraries, and the **fragment-**

based drug design (FBDD) strategy is being increasingly used to identify new mPPIs [16,17]. However, the success of a HTS depends on whether the library contains putative hits, and it is well established that the chemical space of mPPIs differs from that of other ligands: many mPPIs do not fit with the Lipinski's rule of five and have higher molecular weight (MW) and higher hydrophobicity [18–20]. Therefore, mPPIs libraries are more diversified. Villoutreix *et al.* [21] showed that mPPIs have a cumulated proportion of **pan assay interference compounds** (PAINS) equal to 23%, whereas it is only 5% for enzyme inhibitors. This leads to potentially finding more false positives in screening. The complexity of the screening process is also increased if the protein function is unknown or if no direct activity readout is accessible. Moreover, with the expansion of the FBDD method in the design of mPPIs, the screening of fragments, which have a low affinity for the target, requires the use of sensitive methods to detect and measure the interactions [22].

The screening of mPPIs involves the use of noncellular *in vitro* assays using purified proteins, to determine the affinity of molecules. The hits are then confirmed by a screening on a cellular model, to check the disruption of the PPI in a cellular environment. In this review, we particularly focus on noncell-based *in vitro* screening assays, used for first-line screening (Figure 1 and Table 1). Cell-based assays are reviewed elsewhere [23]. Given that most mPPIs are developed against cancer, we provide examples of mPPIs in oncology to determine how to choose the most suitable noncell-based assay for the screening of small molecules targeting PPIs.

High-throughput assays

Mix-and-read assays

Fluorescence polarization assay First described in 1926 [24], the fluorescence polarization assay (FPA) is based on the ability of a small fluorescent molecule (ligand A), excited by a polarized light, to emit a depolarized light depending on the rotational diffusion. Free in solution, ligand A rotates rapidly, resulting in low polarization of the emitted light. When ligand A interacts with protein B, the heavier AB complex rotates more slowly and results in high polarization of the emitted light (Figure 2a). The FPA for PPI inhibitor screening tends to be carried out in a competitive inhibition mode. The presence of a mPPI releases the fluorescent ligand A from its binding site and a decrease in the emitted light depolarization is then recorded. This technique, based on rotational diffusion, is closely linked to the mass difference between the free fluorescent ligand A and the AB complex. Therefore, it requires the use of a fluorescent ligand A, small enough for the recorded polarization difference to be significant. For this, ligand A, which is fluorophore labeled, is often a truncated protein containing 'hot-spots', capable of interacting with B. FPA can be implemented using a microplate reader that includes polarizing filters.

The use of FPA in drug discovery targeting PPIs has been widely developed since the 2000s (e.g., see [25–27]). Nikolovska-Coleska *et al.* [28] have participated extensively in FPA deployment in PPI screening [29]. Given that it is readily versatile to low volume and requires simple procedures without separation or washing steps, FPA is particularly suitable for HTS [30–33]. Inexpensive, FPA is also mostly used by academic labs. Recently, in addition to existing methods, Zhou *et al.* [34] developed a FPA to screen YAP–TEAD interaction small inhibitors, as potential anticancer drugs targeting the Hippo pathway, specifically binding to the Ω -loop region on TEAD.

Among its drawbacks, FPA needs the introduction of fluorophore tags on the ligand, which can also induce non-native binding properties. FPA is also limited by the aggregation of tested compounds, especially of compounds with hydrophobic moieties [35]. The biggest hurdle to FPA is fluorescence interference [36]. Using longer wavelength fluorophores are pertinent options for reducing fluorescence interference and light scattering [37].

Amplified luminescent proximity homogeneous assay screen **Amplified luminescent proximity homogeneous assay screen** (AlphaScreen®) is a chemiluminescent technology, first licensed by PerkinElmer® for drug discovery applications in 1999 [38]. AlphaScreen® uses a donor bead (DB) and an acceptor bead (AB). Excited at 680 nm, DB releases a singlet oxygen (1O_2). If AB and DB are close enough (<200 nm), this singlet oxygen excites AB, which emits a signal between 520 and 620 nm. Protein A is captured on the DB and protein B is captured on the AB. When A interacts with B, DB transfers the singlet oxygen to AB and the luminescent signal is registered (Figure 2b). At the 'hook' point, either the donor or the acceptor component is saturated with the target molecule and a maximum signal is detected. For screening, AlphaScreen® is used as a competitive binding assay: the presence of a mPPI prevents the interaction and induces a decrease in the signal.

Compared with the fluorescence (or Förster) resonance energy transfer (FRET) assay, AlphaScreen® detects advantageously the interaction between two proteins at a longer distance (200 versus 10 nm). Technically, this sensitive assay is easily performed in a microplate format without any wash or filtration steps, adaptable to many assay types. Therefore, it is well suited for HTS-compatible assays [39,40]. In a recent example [41], AlphaScreen® was used in a screening campaign of a chemical library containing ~90 000 compounds (associated with TR-FRET), to discover novel small molecules that disrupt the CD47–SIRP α interaction, involved in cancer as an innate immune checkpoint. Given that AlphaScreen® is an easy and adaptable method, it is also widely used in academic research, even for low throughput screening. For the discovery of XIAP inhibitors, Seigal *et al.* [42] used AlphaScreen® to assess the affinity of compounds for the XIAP-BIR2 domain, as an alternative to FPA, which is less sensitive [43] and requires larger protein amounts [44].

From a practical point of view, the technique needs a specialized plate reader, and the signal is temperature sensitive. DMSO (usually used to solubilize the molecules) also disturbs the signal, limiting its use [45]. Moreover, the signal is produced by a chemical reaction, leading to false positive, called 'hitters'. Frequent AlphaScreen® hitters were identified by Schropp *et al.* [46] and are listed in a specific open-access database

(<http://ochem.eu/alerts>). After a structural analysis of >100 000 compounds from four HTSs using AlphaScreen®, the authors showed that hitters were often chelating agents, which contain elements that might quench excitation/emitted radiation or singlet oxygen, such as 8-hydroxyquinolines, picolylamines, or pyridine moieties.

Even though this technique is relatively expensive, mainly because of the cost of the beads, such costs can be greatly reduced after optimization of their concentration [47].

Fluorescence (or Förster) resonance energy transfer The basic principle of resonance energy transfer (RET) was first described by Förster in 1948 [48], and comprises nonradiative energy transfer between an excited probe, called the ‘donor’ and an accepted probe, called the ‘acceptor’. This energy transfer, called FRET, occurs when the donor and the acceptor are <10 nm from each other, which results in a reduction in donor emissions and an increase in acceptor emissions. FRET can occur if the emission spectrum of the donor fluorophore overlaps with the absorption spectrum of the acceptor fluorophore. In a screening assay, each protein partner, A and B, is fused with a fluorescent protein [i.e., Cyan Fluorescent Protein (CFP) and Yellow Fluorescent Protein (YFP), respectively]. If A interacts with B, CFP and YFP are close enough for FRET to occur, leading to an emission in the YFP wavelength (530 nm). The presence of a mPPI abolishes the FRET signal by dissociating the AB complex, resulting in an emission at the CFP wavelength (480 nm) (Figure 3a). A and B can be directly labeled with a FRET donor and acceptor, using reactive functional groups on the fluorophore (i.e., amine, carboxylic acid, or thiol), or by using specific fluorescent conjugated antibodies or fluorescent fusion proteins [49]. Similar to other **mix-and-read assays**, FRET methods are miniaturized and automated assays [50]. Many FRET pairs are available and, in contrast to FPA, FRET can be used with a range of protein sizes [51].

For example, Schaarp *et al.* [52,53] developed a FRET-based assay to identify inhibitors of the Keap1–Nrf2 interaction involved in oxidative-stress defence, using a CFP–Nrf2-derived peptide and a YFP–Keap1 domain. The FRET assay was adapted to a multiwell plate format, and the competition assay conditions were validated by screening a series of previously described inhibitors. The authors monitored the decrease in the donor emission to minimize fluorescence interferences. Compared with isothermal titration calorimetry (ITC; see later), FRET has higher sensitivity and throughput and does not require sample immobilization, which is needed for surface plasmon resonance (SP; see later). Compared to FPA, FRET assays can be applied using less sophisticated plate readers with simpler optics. However, both FRET and FPA can be confounded by fluorescence interference from inhibitors and, thus, a second confirmation of hits is required in the context of HTS.

Frequently, because of fluorescence interference (i.e., strong background autofluorescence of biological media or tested compounds), the FRET signal can be significantly contaminated and lead to false positive readouts. Donor fluorophores with long emission half-lives, such as cryptates of lanthanides ions (europium or terbium) can be used to reduce FRET signal contamination. This modification of FRET is known as time-resolved (TR) FRET (also called HTRF). This long emission results in a time delay between the donor excitation and the recording of the acceptor emission [54].

TR-FRET is largely used as a mPPI screening method. For instance, in the search for new Mcl-1 inhibitors, Du *et al.* designed and developed a HTS technology that allows the generation of two HTS readouts from one reaction by combining FRET and FPA technologies into one platform [55]. The test, called a ‘dual-readout F2 assay’, was validated in an HTS campaign of 102 255 compounds [56]. Of these, 36 molecules were selected and characterized using FP- and SPR-based competitive binding assays. Guided by chemoinformatics, the authors highlighted a new family of Mcl-1 inhibitors, and performed a structure–activity relationship (SAR) study.

Another variant of FRET is BRET, which substitutes the initial excitation of the system by an enzymatic reaction, using bioluminescence as the energy source. Although BRET is widely used for the study of PPIs [57], it is rarely used for the screening of molecules. A model was recently developed by Corbel *et al.* to screen inhibitors of the p53–MDM2 interaction, involved in apoptosis evasion [58].

Differential scanning fluorimetry Differential scanning fluorimetry (DSF), also called a thermal shift assay (TSA) or Thermofluor®, was first described in 2001 as a method for drug discovery [59]. The thermal denaturation of a folded protein B leads to an unfolded protein B, which exposes some hydrophobic parts, which are usually unreachable. A hydrophobic fluorescent dye (the most popular being Sypro Orange®) [60], is able to bind to the hydrophobic parts of proteins. The more the temperature increases (usually from 25°C to 95°C), the more the protein B is unfolded, the more the Sypro Orange® binds, and the higher the fluorescence is important. The melting temperature (T_m) is then recorded. The presence in the medium of a ligand L of the protein B stabilizes the LB complex and increases the melting temperature (T_m'). The calculated thermal shift ΔT_m ($T_m' - T_m$) is proportional to the ligand affinity (Figure 3b).

DSF does not require specific equipment, and can be performed in real-time PCR machines. It can also be adapted to a large number of label-free proteins, and requires no specific knowledge of the protein. Thus, it is particularly suitable for HTS [61–64] dedicated to targeting PPIs [65] and is widely used to screen fragment libraries in FBDD.

However, DSF uses a fluorescent dye. As a consequence, because of a high fluorescence background, some proteins might not be amenable to DSF screening by using Sypro Orange® [66]. Moreover, the PPI binding sites are particularly hydrophobic [67], leading to potential competition between the compounds and the dye. However, binding of small molecules to the protein surface is usually so weak that no significant T_m change can be obtained. As a consequence, the compounds are screened at relatively high concentrations (1–10 mM), which is incompatible with low-solubility compounds [60,68].

Complementarily to classical FPA, Wan *et al.* used DSF as a secondary tool to screen small-molecule inhibitors of Bel-xL, which is involved in apoptosis evasion [69]. In this study, the binding profile results of peptide ligands were found to be consistent with results obtained through SPR and FPA. Nevertheless, results obtained with abiotic ligands, such as ABT-737, were not concordant with those obtained by FPA. The advanced hypothesis was that the chemical inhibitors could induce a global change in the protein structure, unlike peptides, which induce a moderate change. This highlights the importance of coupling two different screening techniques in the drug discovery of mPPIs.

Recently, Tran *et al.* used TSA to study modulators of the Nrf2–Keap1 interaction [70], a promising target to combat oxidative stress implicated in cancers [71]. The authors compared 21 known small inhibitors using a three **orthogonal assay** (FPA, TSA, and SPR). Results were confirmed for the half of the compounds. The others were false positive compounds, with PAINS moieties or fluorescence interference. This study also highlighted the advantage of combining several screening methods to obtain relevant results.

Nuclear magnetic resonance

In the mPPI drug discovery process, NMR assays are usually the most adapted for hit validation and binding studies [72]. Recently, NMR-based approaches have also found application in mPPI screening processes. These approaches, termed in general ‘HTS by NMR’, are particularly suitable for fragment screening. Highly valuable, effective, and sensitive, NMR spectrometry directly identifies and characterizes the protein–ligand binding. This method is less prone to artefacts compared with biochemical HTS (reviewed in [73,74]).

Two main strategies stand out regarding screening by NMR to detect mPPIs, 1D ¹H-NMR and 2D ¹H-NMR (Figure 4). The first and simplest is the measurement of 1D ¹H-aliphatic NMR shift spectra. This method represents the portion of the protein 1D ¹H-NMR spectrum having a chemical shift <0.7 ppm. This spectral region is typical of the chemical shift of methyl groups from several proteins because protons of small peptides or molecules generally do not provide signals in this area. Hence, the comparison of 1D ¹H-aliph NMR spectra of the target protein in the absence and presence of the ligands allows the detection of ligand binding (Figure 4). This label-free method mainly concerns small to medium-sized proteins (<30 kDa) and needs relatively low protein concentrations (1–10 μM). As illustration, a ¹H-NMR experiment was used by Astex Pharmaceuticals as the first step to identify new hits targeting the BIR3 domain of IAP family proteins [75]. They screened a fragment library containing 100 fragments selected by virtual screening. In this study, other ligand-detected NMR methods, such as LOGSY or STD-NMR commonly used to detect fragment binding, demonstrated lower sensitivity because of the small size of the protein.

Without labeled proteins or ligands, the intermolecular environment using homonuclear bi-dimensional proton [¹H, ¹H] Nuclear Overhauser Effect Spectroscopy (NOESY) can be detected [76]. The assay time is longer (12–48 h), depending on the ligand concentration (500 mM–2 mM). However, coupled with the ¹³C spectra, this approach is useful to detect intermolecular distances (via NOE) and to study the docking of ligands [77,78]. A faster method using [¹H, ¹H] NOE spectroscopy combined with Heteronuclear Single Quantum Correlation (HSQC) is also available. Increasing the analysis time, the 2D band-Selective Optimized-Flip-Angle Short-Transient-HSQC (2D SOFAST-HSQC) is also promising [79]. This fast process was used to identify, among 24 scaffolds, a novel inhibitor of the CXCL12–CXCR4 interaction, involved in tumor progression [80,81].

mPPI studies can also rely on the detection of 2D heteronuclear experiments by using uniformly or selective ¹⁵N and/or ¹³C-labeled proteins. These 2D NMR methods are not suitable for HTS, but those combinatory experiments, called chemical-shift mapping, are widely used for the characterization of protein–ligand binding. The target proteins have to be ¹³C or ¹⁵N labeled and the acquisition is developed with and without ligands. As described for aliphatic assays, the protein size has to be small or medium (<25–30 kDa) and the protein concentration has to be increased (10–50 mM). Pfizer® [82] used 2D [¹H,¹⁵N] NMR to develop disruptive PDK1–downstream kinase interactions for cancer research. This method was also applied by Wyeth® [83], combined with 1D ¹H-aliph NMR assay, to identify new ligands against the binding domain of EphA, a tyrosine kinase receptor involved in some cancers, among more than 10⁵ compounds.

One general limit of NMR spectroscopy is the solubility of proteins and ligands. Given that fragments present generally a better water solubility, NMR spectroscopy is particularly suitable for fragment screening, enabling the detection of fragment hits of weak binding affinity (K_a values up to mM) without ambiguity [84].

Concluding remarks on high-throughput assays

Fast, relatively simple, and miniaturizable, high-throughput methods are widely preferred in HTS campaigns. Less costly than the techniques described later, they also make it possible to conduct low- and medium-throughput screening campaigns at a lower cost. The major drawback of these techniques (outside of NMR), remains the high rate of false positive results, especially with methods using fluorescence.

Low-throughput assays

Methods with immobilization

Surface plasmon resonance The optical technique SPR detects the interaction between two partners: one (protein A) is fixed on a sensor chip and the other one is mobile (ligand L). The sensor chip comprises a gold thin film, and a reaction section enables the immobilization of A (via noncovalent immobilization using carboxymethylated dextran matrix, or a covalent immobilization using a specific matrix). Upon binding of L to A, the mass fluctuation directly

changes the refractive index of polarized light close to the gold surface, which is then measured (Figure 5a). The variation of the refractive index is proportional to the ligand concentration and enables the K_d value to be determined (for a guide to the set-up of SPR experiments in PPI drug discovery, see [85]).

Among its advantages, SPR is a fast and highly sensitive technique requiring minimal sample quantities. SPR provides access to kinetic, affinity, and thermodynamic data for analyzing biomolecular interactions without using any labels. For these reasons, SPR is widely used to characterize PPIs and mPPIs and, therefore, is considered to be a 'gold standard'. SPR can also be used for screening of mPPIs, including fragment screening [86]. Given the low fragment affinity (in the millimolar range), higher fragment concentrations (up to five to tenfold of the K_D) should be used.

In a recent example, a SPR-based fragment screening assay against BCL6 was performed by Kamada *et al.* [87]. A selection of 64 fragments was made from a library (1494 compounds) using a primary SPR-based screening. For SPR assay development, mutated BCL6 was used, which contained fewer cysteine residues, to prevent both protein aggregation and false positives. A second screening using STD-NMR highlighted one fragment as a starting point for a medicinal chemistry study using FBDD with fragment growing.

However, SPR needs the immobilization of one of the binding partners, which can change the native configuration of the protein and access to the active binding site [88]. SPR assay conditions have to be adapted according to the chemical series to which the ligands belong. Furthermore, to decrease the rate of false negatives, a selectivity test should be performed in parallel on the main target, with a protein bearing the binding site mutated or blocked with a low off-rate ligand. SPR also requires the use of a dedicated and expensive device and sensor chips [89].

Advances in technology, including miniaturization of devices and the ability to perform measurements in parallel in the same device, increased the screening throughput even though the cost remains a significant brake to their use. Nevertheless, SPR is widely used as a secondary assay to confirm and profile results from biochemical or biophysical screening. For example, Jiang *et al.* [90] used SPR as a second screening method to identify new Keap1–Nrf2 PPI inhibitors. A library of 569 compounds was first screened in a FPA, and a SPR competition assay was then used to confirm the interaction of the hits with Keap1.

Bio-layer interferometry First described in 2005 by ForteBio® (www.fortebio.com/applications/bli-technology), biolayer interferometry (BLI) is a label-free optical technology that analyzes the interference pattern of white light reverberated from two surfaces: an internal reference layer, and a layer of immobilized protein A on the biosensor surface. The binding between a ligand L and A induces an increase in optical thickness at the biosensor tip. The length of the reflected light path increases, leading to a shift in the interference pattern [91,92]. A sensorgram is registered, which enables the kinetic parameters of the interaction to be determined (Figure 5b).

Compared with SPR, BLI is compatible with a 96-plate format and has a better throughput capacity. Wartchow *et al.* [93] used BLI to screen a fragment library of 6500 fragments targeting Bcl-2 and eIF4E proteins, modulators of disease progression in oncology. The results were compared with those obtained in a FRET-HTS. The overlap between the hits identified in the two assays was 52%. In addition to the hits observed in the biochemical screening, 68% of the hits from the BLI screening were unique and were not found in the biochemical screening. These results are encouraging and show the advantage that BLI can represent in the screening of mPPIs, although additional investigations are required to better understand the differences observed. In parallel, BLI can also be used in complex media, because refractive index changes in the medium do not affect shifts in the interference pattern. The BLI technique is also more tolerant of samples containing a high level of solvent, in particular DMSO.

However, BLI is less sensitive than SPR. Given the limited temperature control of the samples, a thermodynamic characterization of ligand binding is impossible. In addition, because SPR uses a continuous flow system, it is more repeatable than BLI. Yet, because BLI can be used in mix-and-read format, this makes it a promising technology in future mPPI screening.

Immobilization-free methods

Isothermal titration calorimetry ITC measures quantitative energy changes that are associated with the binding reaction of two components. An adiabatic system is used, containing two cells: a reference cell and a cell with the protein A, maintained at strictly identical temperatures. The ligand L in the syringe is introduced in several portions into the sample cell. When L binds to A, a heat change occurs, resulting in a temperature difference between the two cells. The energy (DP for differential power in $\mu\text{cal}\cdot\text{s}^{-1}$) necessary to maintain a zero temperature difference is then recorded (Figure 6a) [89].

Both an immobilization-free and label-free method, ITC is the only technique that determines directly all the thermodynamic parameters of ligand binding: affinity (K_d), stoichiometry, enthalpy change (ΔH), and calculation of both free energy change (ΔG) and entropy change (ΔS). Given that all these parameters can be determined in a single experiment [46], ITC is a suitable tool to both characterize mPPI binding mode [94,95] and guide molecular design [96,97].

However, ITC method dedicated and expensive equipment. Sample consumption (protein and ligand) can be relatively high and limits its application for proteins that are difficult to produce. As a second consequence, a large quantity of ligands and high ligand solubility are required. Thus, ITC can be challenging for weak binding of mPPIs. Given that the affinities are weak, ITC is also challenging for fragment screening [88]. During optimization,

it is also important to coordinate buffers in the syringe and in the sample cell to eliminate heat of dilution (for a guide to data interpretation, see [98]).

Recently, Gal *et al.* [99] used ITC to identify small-molecule inhibitors of the p53–MDM2 interaction. First, a virtual screening extracted 80 hits from a library of 15 million compounds. ITC was then used to test these 80 molecules to identify a hit, confirmed by FPA and NMR. The authors developed an original ITC protocol assay, where the targeted protein MDM2 was injected into a mixture of four compounds placed in the ITC cell, limiting the number of injections required and making a binary decision concerning the change in released or absorbed heat. Finally, a FPA measurement and NMR experiments confirmed that the protein competed with p53.

Microscale thermophoresis Developed by NanoTemper®, microscale thermophoresis (MST) monitors fluorescence. In a typical MST experiment, protein A is covalently labeled with a fluorescent dye. A solution of labeled-protein A is placed in a transparent capillary tube, part of which is illuminated with an infrared (IR) laser, thus establishing a stable thermal gradient. Given ‘thermophoresis’ (Soret effect), A migrates along this gradient, and its movements can be monitored using a fluorescence microscope focused on the IR-illuminated region. The thermophoretic movement of A leads to a decrease in the fluorescence signal. In the presence of a ligand L, the thermophoretic movement induced by IR irradiation of the LA complex differs from the movement of A alone. The decrease in registered fluorescence is less important, enabling the affinity of the interaction to be estimated. However, MST can be performed in a label-free manner, in which the intrinsic ultraviolet (UV)-fluorescent signal of the aromatic amino acid residues is detected (Figure 6b) [100,101].

Easy to handle, MST is a fast and precise way to measure binding affinity. Compared with ITC, it is highly sensitive, has low sample consumption, and measures interactions with no limitation on molecule size or weight. Moreover, MST is able to analyze interactions of small molecules with proteins in complex media (i.e., cell lysates or biological liquids) [102] (for a guide to data interpretation, see [103]).

Currently, MST is mainly used to characterize the binding mode of mPPIs. Gibaud *et al.* [104] used MST to characterize the binding mode of inhibitors of the YAP–TEAD interaction (implicated in the Hippo pathway, which is deregulated in cancer processes). The experiments were successfully carried out on cell lysates. For another example, Rainard *et al.* [105,106] used MST for the triage and characterization of small-molecule inhibitors of the Nrf2–Keap1 interaction. Among 318 132 compounds tested by a FPA, eight compounds were selected for further characterization using a label-free MST assay.

Although MST is not yet used for HTS in drug discovery of PPIs, its automation will push this technology toward a higher throughput, rendering it more promising as a drug discovery technology.

Concluding remarks on low-throughput assays

Compared with high-throughput assays, the techniques described generally require more expensive equipment. More sophisticated, they allow access to a large amount of data, which is particularly interesting for characterizing protein–ligand interactions, but often require dedicated know-how to interpret the data. Recent technological advances in equipment, particularly in terms of automation, will make it possible to consider the use of these methods for higher-throughput campaigns.

Concluding remarks

Targeting PPIs in drug discovery is specific and challenging. As a consequence, finding the best screening processes can be difficult. Moreover, PPIs often have no directly accessible activity readout. Thus, it can be unclear which test to choose for the screening of mPPIs. Putting throughput considerations aside, to answer this question, several parameters must be taken into account: (i) laboratory experience and available budget. Some technologies, such as SPR and ITC, require dedicated and expensive equipment and trained staff. By contrast, DSF, for example, is one of the most accessible techniques because it can be performed with a simple PCR device. Other experiments giving rapid results, such as FPA are also suitable with a small budget; (ii) nature of the library. On the one hand, autofluorescent molecules can create interference with fluorescent-based assays, particularly FPA or FRET. On the other hand, because hydrophobic molecules tend to precipitate or aggregate especially in aqueous buffers, DSF, ITC, or NMR are poorly suited for such uses. Conversely, with libraries of fragments that are smaller and more soluble in aqueous media, NMR will be particularly suitable, including for high throughput; and (iii) nature of the protein partners involved in the interaction. If the targeted protein is difficult to purify, MST and BLI will be particularly suitable, because they allow measurements in complex media. With unstable proteins, SPR or BLI, which require immobilization, should be avoided. If two protein partners are not available, competitive methods, such as FPA, FRET, or AlphaScreen®, cannot be used. Likewise, if a label partner is not available, it will be necessary to use label-free methods. If the proteins are poorly soluble, NMR and ITC will be poorly adapted. Finally, if the interaction is weak, sensitive techniques, such as SPR or AlphaScreen®, are preferred.

The optimal approach is to use two complementary techniques in an orthogonal screening test. With a limited screening capacity, it is also possible to use *in silico* screening to reduce the size of the library. Janzen showed that the less complex the test, the higher the rate of hitters [107]. This is a parameter to take into account when choosing the test. Having the appropriate screening process is the key to success, because the more appropriate the process is, the more relevant the hit selected, and the shorter the hit-to-lead process. Chemist–biologist collaborations in both the choice of the screening test and the triage process are essential for a successful drug discovery campaign. An advantage of noncellular-based assays is that they can be used on isolated proteins to

characterize the protein–ligand interaction without cellular interferences. Nevertheless, the subsequent validation of the identified hit compounds on a cellular model remains a key step in the hit-to-lead process.

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Figure 1. Use of screening assays in a hit-to-lead strategy applied to small molecules targeting protein-protein interactions (PPIs). Abbreviations: AlphaScreen, amplified luminescent proximity homogeneous assay screen; BLI, biolayer interferometry; CETSA, cellular thermal shift assay; FPA, fluorescence polarization assay; FRET, fluorescence (or Förster) resonance energy transfer; ITC, isothermal titration calorimetry; NMR, nuclear magnetic resonance; SPR, surface plasmon resonance; TSA, thermal shift assay; X-ray, X-ray crystallography.

Figure 2. High-throughput assays in a mix-and-read format (Part 1). Principles of (a) fluorescence polarization assays (FPA) and (b) amplified luminescent proximity homogeneous assay screen (AlphaScreen) assays.

Figure 3. High-throughput assays in a mix-and-read format (Part 2). (a) Fluorescence resonance energy transfer (FRET) assays and (b) differential scanning fluorimetry (DSF) assays.

Figure 4. Principle of nuclear magnetic resonance (NMR) screening assays.

Figure 5. Principles of low-throughput assays: Methods requiring immobilization of a partner: (a) surface plasmon resonance (SPR) assays and (b) biolayer interferometry (BLI) assays.

Figure 6. Principles of low-throughput assays: Immobilization-free methods: (a) isothermal titration calorimetry (ITC) and (b) microscale thermophoresis (MST).

Glossary

Fragment: small organic molecules that are small in size, low in molecular weight (MW), and have a weak affinity for the targeted protein. Sometimes, in analogy to the 'rule of five', it has been proposed that an ideal fragment should follow the 'rule of three' (MW <300, logP <3, and number of hydrogen bonds <3).

Fragment-based drug design (FBDD): in a hit to lead strategy, the screening of a fragment library enables the identification of hit fragments presenting a weak affinity for the targeted protein (in the micromolar range). Pharmacomodulation studies are then conducted to produce a lead with an affinity in the nanomolar range. In FBDD, three main strategies are used: fragment growing, fragment merging, and fragment linking.

Hot-spots: in some cases, PPI surfaces contain subsets of a few amino acids, which are called hot-spots, where most of the PPI binding energy is localized [108]. Sometimes, the design of mPPIs is possible by targeting these hot-spots.

Mix-and-read assay: simple procedure without separation, filtration, or washing steps; often used in HTS.

Modulators of PPIs (mPPIs): compounds that disrupt a PPI by inhibition (with a PPI inhibitor) or stabilization (with a PPI stabilizer) of the interaction.

Orthogonal assay: experiment combining two or more assays that use different technologies to limit false positive screening results (i.e., FPA and SPR, or AlphaScreen® and NMR).

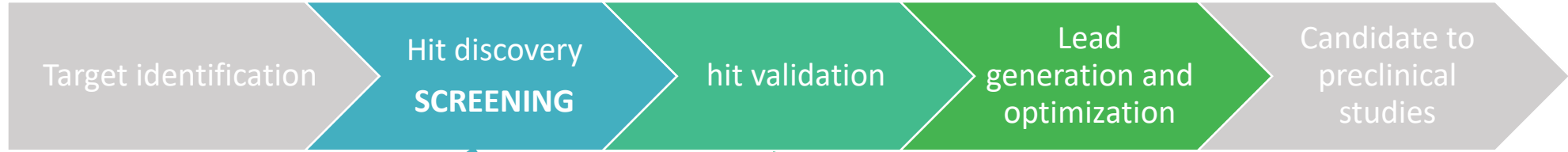
Pan assay interference compounds (PAIS): also called 'hitters'; designate compounds that generally interact nonspecifically with numerous biological targets rather than specifically affecting one desired target [109], leading to false positive readouts in many different biological assays

Protein-protein Interactions (PPIs): physical interaction between two proteins in a living organism implicated in a biological process. The PPI network of an organism constitutes its interactome.

Table 1. Noncellular-based assays for targeting PPIs used in drug discovery

Assay	Throughput	Mix and read	Biochemical/biophysical	Advantages	Drawbacks
FPA	High	Yes	Biochemical	Very simple; low cost; adaptable to low volumes	Needs fluorescent-tagged proteins; interferences in fluorescence
AlphaScreen	High	Yes	Biochemical	Detection of interaction until 200 nm; very sensitive; label-free proteins	High cost (beads); sophisticated plate reader; temperature sensitive; high rate of 'hitters'
FRET/TR-FRET	High	Yes	Biochemical	Sensitive; adaptable to low volumes; low-cost plate reader	Interaction <10 nm; needs two fluorescent-tagged proteins (acceptor and donor); fluorescence interferences
DSF	High	Yes	Biochemical	No specific equipment; low cost; label-free proteins	High fluorescent background; competition between compounds and dye; high concentration of compounds
NMR	1D	High	No	Label free; low cost; immobilization free	High protein concentration; solubility incompatibilities; ¹ H aliph 1D NMR: media interferences; ¹⁵ N-labeled protein
	2D	Low	No	Epitope mapping; immobilization free	² D NMR: needs ¹⁵ N-labeled protein

SPR	Low	No	Biophysical	Kinetics parameters; label free	Expensive equipment; needs protein immobilization
ITC	Low	No	Biophysical	Label free; immobilization free; thermodynamics parameters	High protein consumption; difficult data interpretation; expensive equipment
MST	Low	No	Biochemical	Easy to handle; fast measurement; compatible with complex media; lower sample consumption than ITC; thermodynamic parameters	Needs labeled protein
BLI	High	Yes	Biophysical	Label free; compatible with complex media; kinetics parameters	Needs protein immobilization; less sensitive than SPR



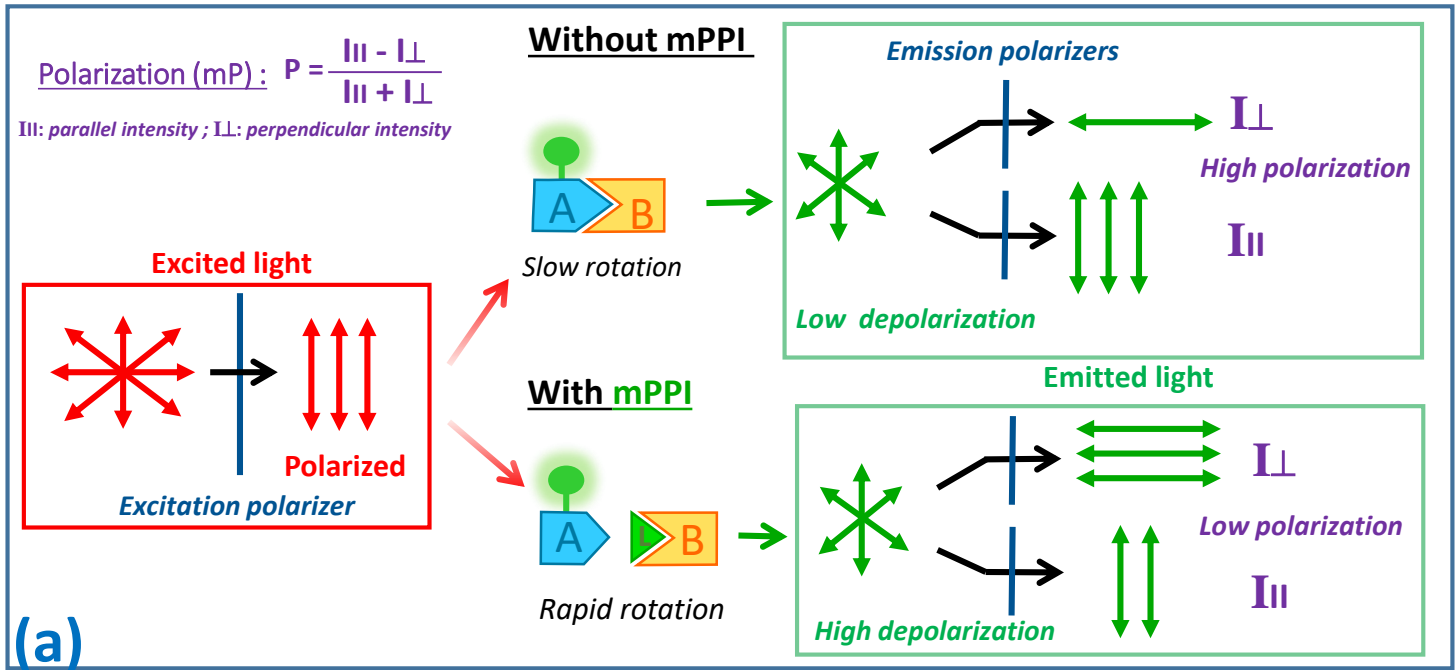
« High » throughput

- **Biochemical assays:** FPA, FRET, and Tr-FRET, AlphaScreen, TSA
- **Biophysical assays:** NMR, BLI

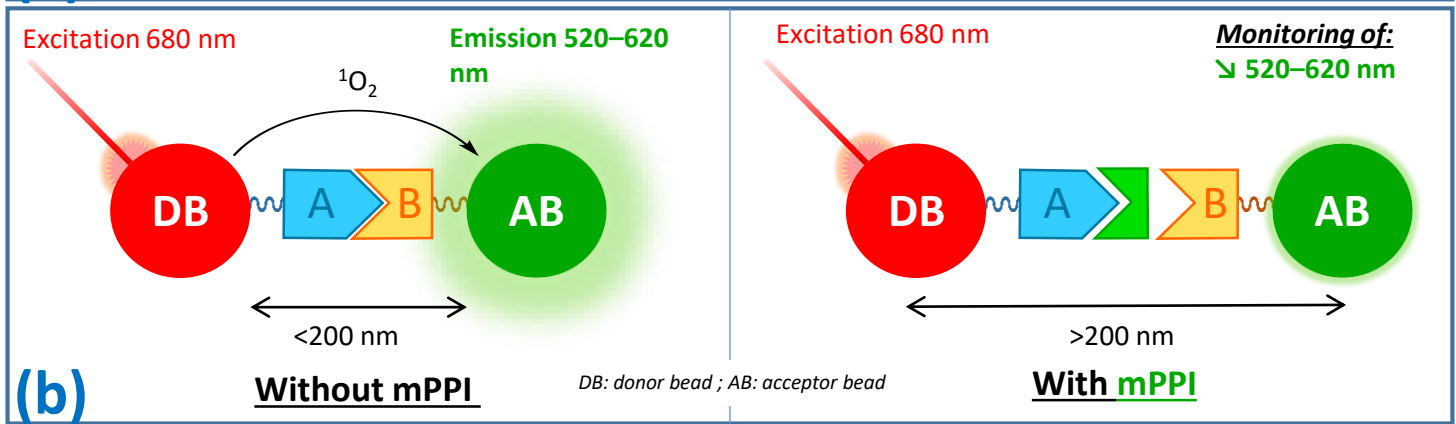
« Low » throughput

- **Biochemical and biophysical assays:** SPR, ITC
- **Structural analysis:** X-ray, NMR
- **Cellular assays:** FRET, CETSA, AlphaLISA...

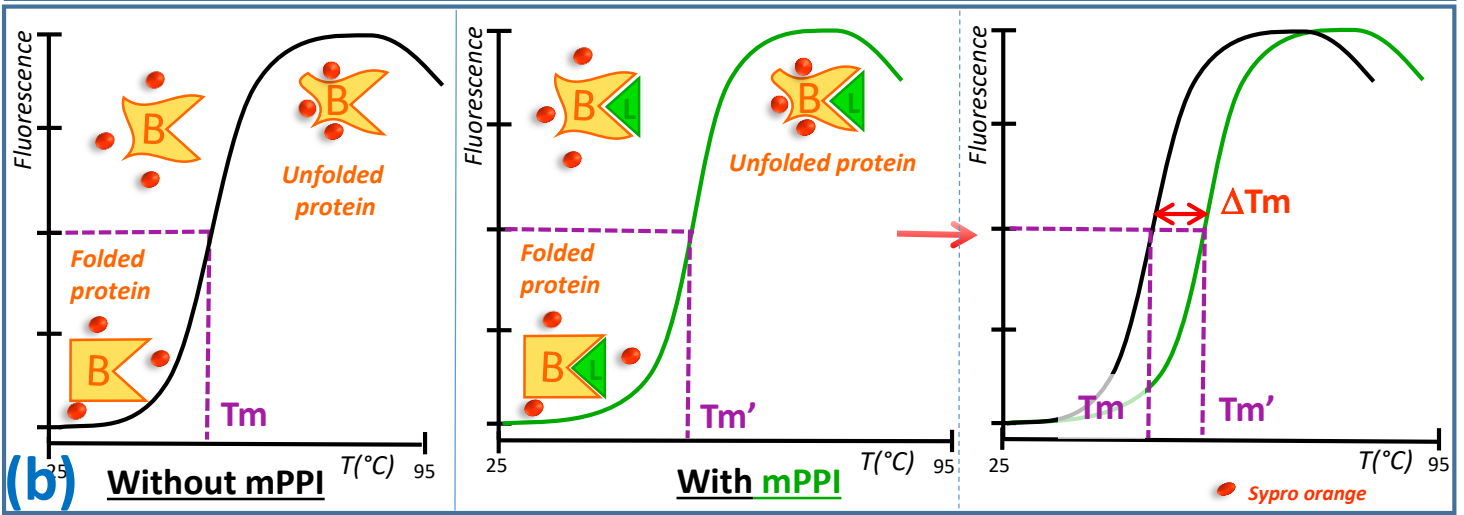
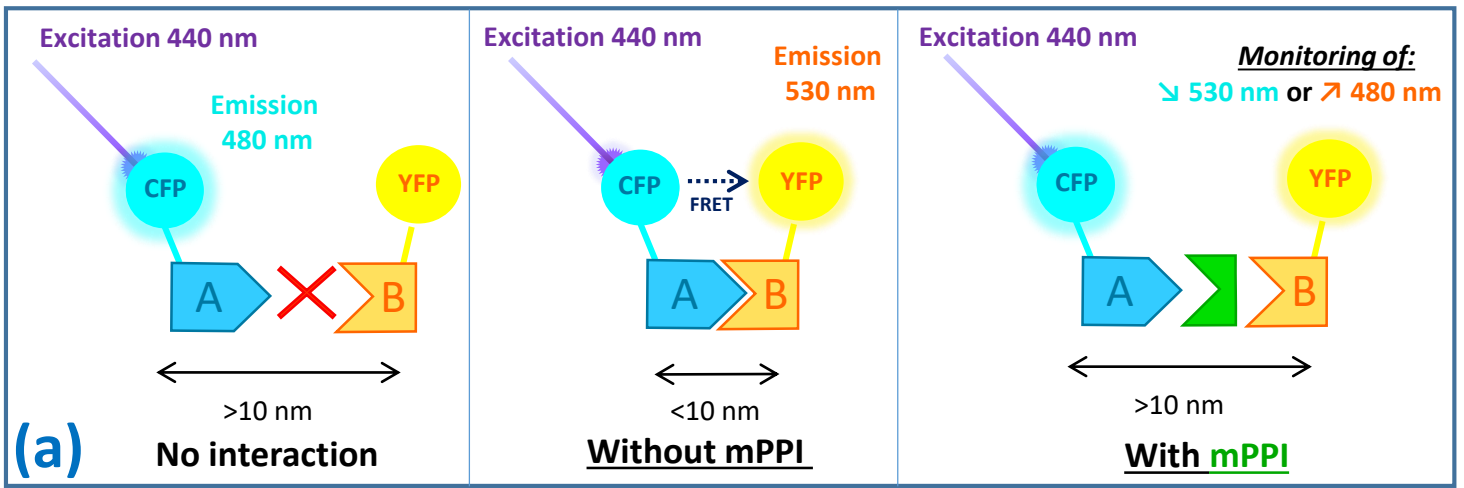
→ Additional data: binding, kinetic...



(a)

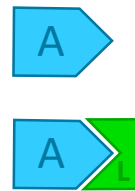
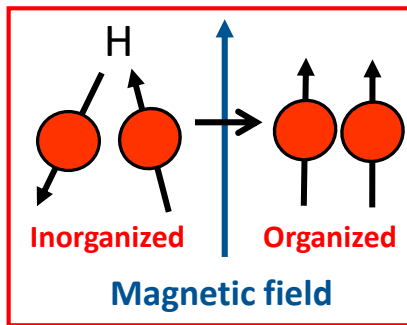


(b)

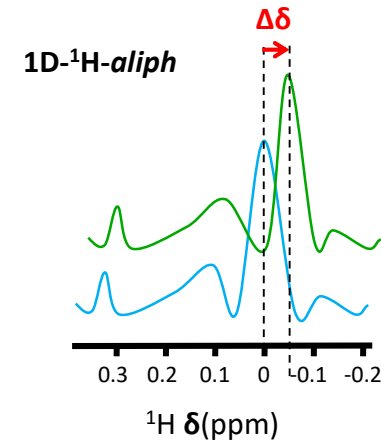


Chemical shift perturbation registration

Deuterated solvent



1D ¹H-aliph NMR spectra

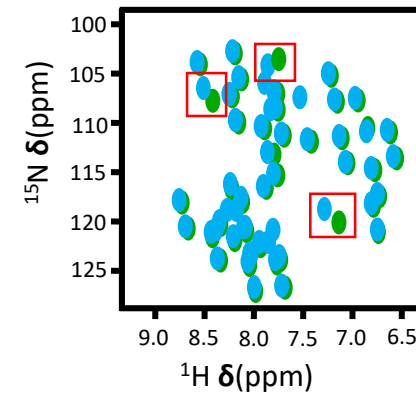


A alone
A with L

2D-[¹⁵N, ¹H] HSQC NMR spectra



2D-[¹⁵N, ¹H] HSQC



A alone
A with L

$$\text{Chemical shift: } \delta = \frac{\left(\text{Frequency of electromagnetic radiation absorbed by sample nucleus} \right) - \left(\text{Frequency of electromagnetic radiation absorbed by TMS standard} \right)}{\text{Spectrometer frequency}}$$

