Biophysical Reviews
https://doi.org/10.1007/s12551-026-01429-8

REVIEW

Precision by design: Surface plasmon resonance and the integration of biophysics in fragment-based drug discovery

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Received: 7 January 2026 / Accepted: 12 March 2026

Abstract

Surface plasmon resonance (SPR) is a widely adopted and highly informative biophysical technique in fragment-based drug discovery, enabling the detection and characterization of weak and transient molecular interactions that initiate the progression from fragments to lead compounds. Owing to its sensitivity, low sample consumption, and compatibility with both direct-binding and functional assay formats, SPR plays a central role within integrated fragment discovery workflows, supporting affinity estimation, hit validation, and mechanistic interrogation when applied with appropriate experimental design. Recent advances in sensor-chip chemistries, buffer and solvent optimization, clean-screening strategies, and high-throughput instrumentation have further enhanced the robustness and reliability of SPR-based fragment assays. When combined with complementary biophysical and structural approaches, including thermal shift analysis, calorimetry, mass spectrometry, thermophoresis, NMR spectroscopy, X-ray crystallography, and cryo-electron microscopy, SPR contributes to a multidimensional validation framework that enables informed hit triage, structural interpretation, and structure-guided optimization. This review provides a practical guide to the use of SPR in fragment-based discovery, covering conceptual principles, experimental design, data analysis strategies, and best practices for minimizing artifacts and improving data quality. Beyond technical considerations, SPR illustrates a broader principle of molecular discovery: even weak interactions, when measured rigorously and interpreted in context, can yield insights that guide the transformation of fragment hits into biologically meaningful and therapeutically relevant molecules.

Keywords FBDD · Biophysics · Structural biology · Screening · SPR

Introduction

Fragment-Based Drug Discovery (FBDD) emerged in the 1990 s from the recognition that low molecular weight compounds can bind weakly yet specifically to biological targets and serve as efficient starting points for structure-guided optimization (Hajduk et al. 1997; Hajduk and Greer 2007). The publication of the "Rule of Three" (Congreve et al. 2003) formalized rational fragment selection criteria, enabling systematic library construction favoring low molecular weight, low lipophilicity, and minimal hydrogen bonding.

Advances in synthetic chemistry, including cross-coupling reactions, microwave-assisted synthesis, flow chemistry, and diversity-oriented strategies, expanded fragment throughput and structural diversity (Ley et al. 2015; Murray and Rees 2009; Spring 2003). Parallel improvements in computational design and *in-silico* filtering further broadened accessible fragment space (Igashov et al. 2022), enabling scalable and chemically diverse fragment libraries across academia and industry (Bon et al. 2022; Keseru et al. 2016; Pedawi et al. 2022).

The conceptual consolidation of FBDD in the early 2000 s was marked by industrial implementation of structure-based screening approaches, notably by Astex Therapeutics using X-ray crystallography to detect low-affinity fragment binding (Carr et al. 2005; Jhoti et al. 2007). From 2005 to 2015, academic laboratories significantly advanced

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Published online: 25 March 2026

biophysical screening technologies for fragment detection and validation. SPR was refined for sensitive, label-free measurement of low-affinity interactions (Giannetti 2011), isothermal titration calorimetry (ITC) enabled thermodynamic characterization (Holdgate and Ward 2005; Velazquez-Campoy and Freire 2006), and mass spectrometry (MS) approaches expanded detection of weak, non-covalent complexes (Chen et al. 2015; Maple et al. 2012; Pedro and Quinn 2016; Woods et al. 2016). Together, these advances facilitated robust hit triage and orthogonal validation, supporting integration of FBDD into pharmaceutical discovery pipelines (Erlanson et al. 2016). With the approval of multiple fragment-derived drugs and the advancement of numerous fragment-origin clinical candidates, FBDD has now demonstrated its capacity to deliver clinically validated therapeutics, establishing the approach as a bona fide drug discovery methodology.

Importantly, the relatively small size of fragment libraries makes it feasible for academic laboratories and small biotechnology companies to curate and maintain their own collections. In contrast to high-throughput screening (HTS) libraries, which typically require substantial financial resources and centralized infrastructure, fragment libraries can be assembled, managed, and screened within modest institutional settings. This practical accessibility represents a major operational advantage of fragment-based approaches over traditional HTS paradigms. Rather than screening large collections of fully elaborated drug-sized molecules, FBDD interrogates chemical space using smaller fragments that typically bind in the micromolar to millimolar range but provide high ligand efficiency and broad chemical space coverage (Congreve et al. 2003; Erlanson et al. 2016). Because fragment interactions are inherently weak and transient, their detection and characterization require highly sensitive and information-rich biophysical techniques.

Within this landscape, orthogonal biophysical methods including SPR, ITC, differential scanning fluorimetry (DSF), native and ligand-detected MS, microscale scanning thermophoresis (MST), and nuclear magnetic resonance (NMR) collectively provide quantitative and mechanistic insight across fragment screening, hit validation, and hit-to-lead progression (Gossert and Jahnke 2016; Jerabek-Willemsen et al. 2011; Niesen et al. 2007; Pellecchia et al. 2002; Shuker et al. 1996; Vedadi et al. 2006). Their integration minimizes false positives and supports structure-guided optimization (Erlanson et al. 2016).

Structural biology further anchors FBDD workflows. X-ray crystallography provides atomic-level binding poses (Blundell et al. 2002; Congreve et al. 2003), while cryo-electron microscopy (Cryo-EM) expands applicability to large and heterogeneous assemblies (Merk et al. 2016; Renaud et al. 2016; Saur et al. 2020; Van Drie and Tong 2020). Complementary approaches such as hydrogen–deuterium exchange MS (HDX-MS) and diethyl pyrocarbonate MS (DEPC-MS) capture conformational and allosteric effects often inaccessible to static structural methods (Engen 2009; Limpikirati et al. 2019, 2020; Masson et al. 2017). Computational tools including docking, molecular dynamics, and AI-guided modeling further accelerate interpretation and optimization (Ghanakota and Carlson 2016; Igashov et al. 2022).

As of 2025, FBDD has matured into a productive source of approved medicines and clinical candidates targeting diverse protein classes, including kinases, GPCRs, PPIs, and covalent binding sites (AlKharboush et al. 2025; Fragments 2024; Woodhead et al. 2024; Xu and Kang 2025). This progress reflects the deliberate integration of fragment library design, sensitive biophysical detection, and structural guidance across academic and industrial settings.

Within this integrated framework, SPR has emerged as a widely adopted, label free technique for real time analysis of fragment-protein interactions. When applied with appropriate experimental design and interpretative caution, SPR supports fragment screening, steady-state response-based affinity estimation, and mechanistic interrogation throughout multiple stages of drug discovery and development. However, the interpretation of kinetic parameters at the fragment stage requires particular care, as rapid dissociation rates often favor steady-state analyses over detailed kinetic resolution.

In this review, we examine the role of SPR within modern FBDD workflows, discussing fragment library considerations relevant to SPR screening, direct and functional assay formats, data analysis strategies, and practical measures for minimizing artifacts and improving reliability.

Historical evolution of FBDD and enabling technologies

The development of FBDD has been shaped by parallel advances in structural biology, synthetic chemistry, and sensitive biophysical detection methods. Early demonstrations using NMR and X-ray crystallography established that weakly binding fragments could be detected and structurally characterized, providing a foundation for rational optimization (Congreve et al. 2003; Hajduk et al. 1997; Jhoti et al. 2007). Subsequent progress in SPR, ITC, DSF, MS, and computational modeling expanded the practical feasibility of fragment screening and validation across academic and industrial environments (Chen et al. 2015; Giannetti 2011; Igashov et al. 2022; Rich and Myszka 2000; Velazquez-Campoy and Freire 2006).

The major milestones that collectively shaped the emergence and maturation of FBDD are summarized in Table 1.

Table 1 Chronological milestones in fragment-based drug discovery

Year(s)	Milestone	References
1980s-1990s	X-ray crystallography begins enabling structure-based fragment optimization	(Blundell et al. 2002; Jhoti et al. 2007)
1996–1997	First application of NMR to fragment screening	(Hajduk et al. 1997; Shuker et al. 1996)
1999	Astex Therapeutics founded, pioneering commercial structure-based FBDD	(Carr et al. 2005)
2000–2002	Early adoption of computational docking in fragment hit evaluation	(Klebe, 2006; Pfaff et al., 2015)
2003	"Rule of Three" published—framework for fragment library design	(Congreve et al. 2003)
Early–Mid 2000s	SPR adapted for fragment screening (biosensor-based interaction profiling)	(Giannetti et al., 2008; Rich and Myszka 2000)
Late 2000s	ITC becomes standard for fragment binding thermodynamics	(Klebe, 2015; Ladbury, 2010)
~2001 onward	DSF applied to screen fragments via thermal shift assays	(Kranz and Schalk-Hihi, 2011; Pantoliano et al., 2001; Vedadi et al. 2006)
2008–2013	Multiple fragment-derived compounds advance into clinical development (e.g., AT9283, Vemurafenib)	(Erlanson et al. 2016; Murray and Rees 2009; Rees et al. 2004)
2011	FDA approval of Vemurafenib, widely recognized as an early fragment-derived oncology drug	(Erlanson et al. 2016)
2012–2016	Native mass spectrometry adapted for weak binder detection in FBDD	(Chen et al. 2015; Maple et al. 2012; Pedro and Quinn 2016)
2022–2025	Cryo-EM and AI gain traction in structure-guided FBDD innovation	(Igashov et al. 2022; Robertson et al., 2022; Rubach et al., 2025; Saur et al. 2020)

Fragment libraries in FBDD: chemical space efficiency, biophysical screening, and advantages over HTS

Fragment libraries are typically composed of 500–3,000 chemically tractable compounds designed according to the "Rule of Three," which emphasizes low molecular weight (MW < 300 Da), limited hydrogen bonding capacity, and moderate lipophilicity to ensure solubility and synthetic expandability (Congreve et al. 2003). In contrast to high-throughput screening (HTS) libraries, which often contain hundreds of thousands to millions of drug-sized molecules, fragment collections prioritize chemical efficiency and broad coverage of chemical space with relatively few compounds (Erlanson et al. 2016).

Because the number of theoretically possible organic molecules increases exponentially with molecular weight, fragment libraries enable efficient sampling of relevant chemical space using smaller, more manageable collections (Dobson 2004; Reymond et al. 2010). A comparatively small fragment library can therefore span a region of chemical space similar to that accessed by much larger HTS collections, reflecting the inherent efficiency of fragment-based approaches (Erlanson 2012; Murray and Rees 2009). This concept is illustrated in Fig. 1.

Advances in synthetic chemistry, including cross-coupling reactions, microwave-assisted synthesis, flow chemistry, and diversity-oriented strategies, significantly improved fragment throughput and diversity Ley et al. 2015; Murray and Rees 2009) (Spring 2003). The integration of AI and in silico filtering tools further expanded accessible fragment space while maintaining physicochemical tractability (Igashov et al. 2022). These developments enabled scalable, diverse, and target-relevant fragment libraries across academia and industry (Bon et al. 2022; Keseru et al. 2016; Pedawi et al. 2022).

Variants of fragment libraries include diversity-oriented collections, covalent fragments targeting nucleophilic residues, fluorinated libraries optimized for ^{19}F NMR detection, and target-focused subsets tailored to specific protein classes (Cox et al. 2016; Erlanson et al. 2004; Vulpetti and Dalvit 2013). Although these classifications reflect distinct design philosophies, their practical relevance to SPR-based screening lies primarily in physicochemical control. Fragment solubility, chemical stability, limited aggregation propensity, and controlled lipophilicity directly influence assay robustness, nonspecific surface interactions, and artifact suppression in immobilization-based biosensor systems.

Fragment stocks are typically stored at concentrations of 10-100 mM in 100% DMSO for biophysical applications and may exceed 500 mM for structural screening workflows. Prior to screening, these concentrated stocks are diluted into assay-compatible buffers to achieve final working concentrations, typically in the range of 0.5–2 mM fragment with 0.5–5% DMSO, depending on target stability and instrument tolerance. Careful control of DMSO content is essential in

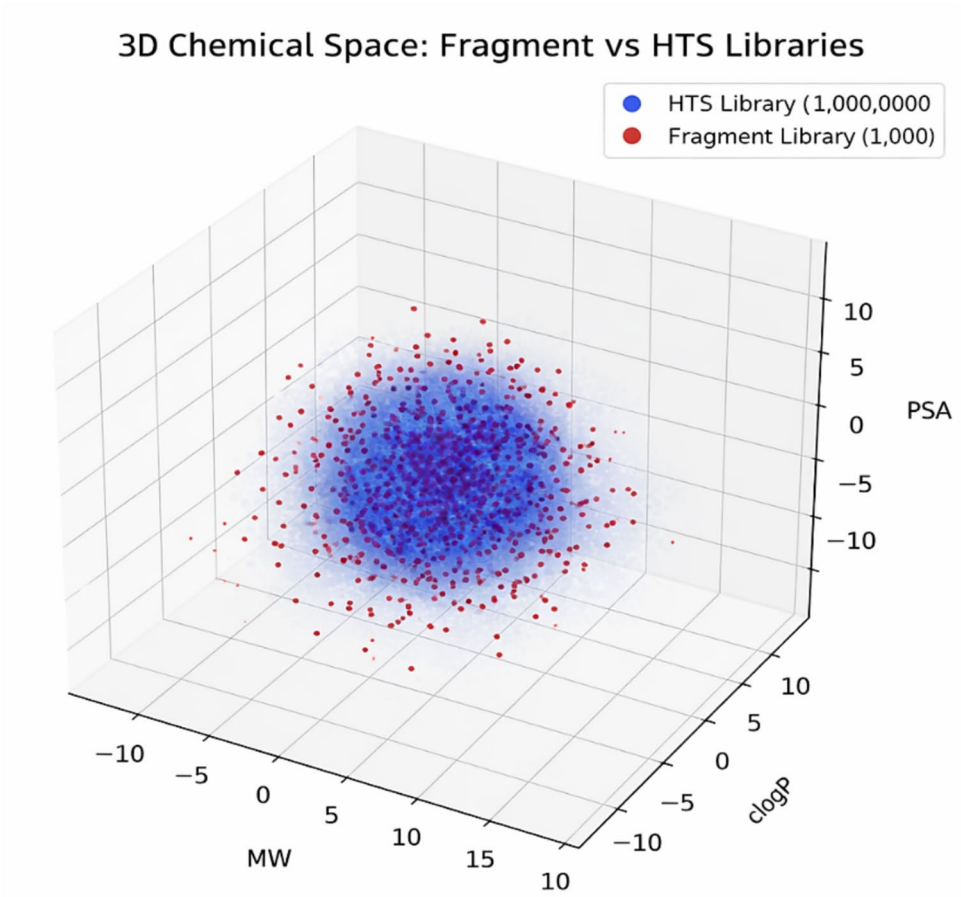

Fig. 1 Efficient Chemical Space Coverage by Fragment Libraries Compared to HTS Libraries. 3D plot illustrates the chemical space occupied by a 1,000-compound fragment library compared with a 1,000,000-compound HTS library. Despite its smaller size, the fragment library spans a comparable region of chemical space, reflecting the efficiency of fragment-based coverage. Axes represent molecular weight (MW), lipophilicity (cLogP), and polar surface area (PSA). Descriptor values were scaled and centered for visualization purposes to enable comparative representation of chemical space distributions

SPR experiments because variations in solvent concentration directly affect the refractive index and can introduce bulk-shift artifacts. Accordingly, DMSO matching between sample and running buffer, as well as solvent correction procedures, are standard practices in SPR-based fragment screening campaigns (Karlsson and Falt 1997; Mauriz et al. 2007; Myszka 1999).

For SPR in particular, fragment library composition directly affects signal-to-noise ratio, solvent tolerance, surface binding artifacts, and the reliability of steady-state affinity estimation. Accordingly, fragment library design is not merely a chemical consideration but a determinant of experimental interpretability and data quality in fragment-based workflows.

Biophysical techniques in fragment-based drug discovery

Because fragment–protein interactions are typically weak and transient, their detection requires sensitive and information-rich biophysical methods. No single technique is sufficient to fully characterize fragment binding; instead, orthogonal approaches are routinely combined to validate interactions, quantify affinity, and guide optimization (Erlanson et al. 2016; Jhoti et al. 2013; Murray et al. 2012).

SPR, ITC, DSF, MS, MST, and NMR spectroscopy each contribute complementary information, ranging from equilibrium affinity estimation to thermodynamic profiling and structural mapping (Chen et al. 2015; Giannetti 2011; Gossert and Jahnke 2016; Holdgate and Ward 2005; Jerabek-Willemsen et al. 2011; Niesen et al. 2007). The typical application of these techniques across different stages of FBDD workflows is summarized in Table 2.

Structural biology in fragment-based drug discovery

Structural biology plays a central role in FBDD by providing atomic or near-atomic resolution insight into fragment binding modes. Because fragments typically bind with low affinity, direct structural visualization is often essential for guiding fragment growing, merging, or linking strategies (Blundell et al. 2002; Congreve et al. 2003; Murray and Rees 2009).

X-ray crystallography has historically been the dominant structural method in FBDD, enabling high-throughput

Table 2 Biophysical techniques used for FBDD for fragment screening, hit validation, hit-to-lead and lead optimization

Technique	Fragment Screening	Hit Validation	Hit-to-Lead Optimization	Lead Optimization	References
SPR	✓ Measures affinity ✓ Moderate throughput	✓ Validates binding	✓ Kinetics of analogs	✓ Selectivity profiling	(de Esch et al., 2022) (Acharya et al., 2024)
ITC	✓ Thermodynamic profiling Low throughput	✓ Confirms binding ✓ stoichiometry	✓ Guides enthalpy-driven interactions	✓ Optimization balance	(Holdgate and Ward 2005; Klebe, 2015; Ladbury, 2010)
DSF	✓ Screening-compatible ✓ High Throughput	✓ Quantifies K_D	✓ Stability indicator	✓ Detects aggregation	(de Esch et al., 2022)
Native-MS	✓ Complex detection ✓ Moderate throughput	✓ Conformational analysis	✓ Allosteric detection	✓ High-mass resolution	(Chen et al. 2015; Maple et al. 2012; Pedro and Quinn 2016)
MST	✓ Detects binding in solution ✓ Moderate throughput	✓ Quantifies K_D	✓ Detects affinity improvements	✓ Validates SAR in solution	(Jerabek-Willemsen et al. 2011; Wienken et al., 2010)
NMR (LO/PO)	✓ Detects weak binders ✓ Low throughput	✓ Maps binding site	✓ Tracks SAR progression	✓ Monitors dynamics	(Pellecchia et al. 2002; Shuker et al. 1996)

fragment soaking and precise mapping of binding interactions within defined active sites (Blundell et al. 2002; Congreve et al. 2003). More recently, cryo-EM has expanded applicability to large, flexible, and heterogeneous protein assemblies that are challenging for crystallographic approaches (Merk et al. 2016; Renaud et al. 2018; Saur et al. 2020; Van Drie and Tong 2020). NMR spectroscopy continues to provide valuable structural and dynamic information, particularly for weak binders and conformationally flexible systems (Gossert and Jahnke 2016; Pellecchia et al. 2002; Shuker et al. 1996). Complementary structural proteomics approaches, including HDX-MS and covalent labeling MS such as DEPC-MS, offer insight into conformational perturbations and allosteric effects that may not be directly visible in static structures (Engen 2009; Limpikirati et al. 2019; Masson et al. 2017).

The application of these structural approaches across different stages of FBDD workflows is summarized in Table 3.

Institutional adoption and translational maturity of FBDD

Over the past two decades, FBDD has transitioned from a proof-of-concept methodology to a widely adopted discovery paradigm across both academic and industrial

Table 3 Structural biology techniques in FBDD for fragment screening, hit validation, hit-to-lead and lead optimization

Technique	Fragment Screening	Hit Validation	Hit-to-Lead Optimization	Lead Optimization	References
X-ray Crystallography	✓ High-throughput soaking	✓ Atomic binding pose	✓ Iterative structure-guided design	✓ Off-target validation	(Blundell et al. 2002; Congreve et al. 2003)
Cryo-EM	✓ Binding validation rather than screening	✓ Large complex elucidation	✓ Suitable for flexible targets	✓ Conformational insights	(Cebi et al., 2024; Robertson et al., 2022; Rubach et al., 2025; Zhu et al., 2023)
Computational Modeling	✓ Library docking	✓ Pose prediction	✓ AI-based linker building	✓ Multitarget modeling	(Igashov et al. 2022)
HDX-MS	✓ Detects conformational perturbations	✓ Maps dynamic interfaces	✓ Allosteric effect mapping	✓ Monitors structural stability	(Engen 2009; Masson et al. 2017)
DEPC-MS	✓ Labels accessible residues	✓ Identifies protected sites	✓ Conformational tracking	✓ Interface resolution	(Limpikirati et al. 2019, 2020)
NMR (Structure-focused)	✓ Detects structural changes	✓ Epitope mapping	✓ Orientation refinement	✓ Monitors conformational dynamics	(Mureddu and Vuister, 2022; Wang et al., 2022)

environments. Multiple pharmaceutical companies and research institutions have integrated fragment screening into their early discovery pipelines, leveraging complementary biophysical and structural approaches to support hit validation and optimization (Erlanson et al. 2016).

The institutional landscape reflects this maturation, with dedicated fragment platforms implemented across industry and academia. Selected examples of programs, platforms, and enabling technologies are summarized in Table 4.

SPR and related immobilization-based biosensor techniques in FBDD

SPR is a powerful label-free biophysical technique for real time analysis of biomolecular interactions. It is based on the principle of plasmonic resonance, where polarized light striking a thin metal film (typically gold) under total internal reflection conditions excites surface plasmons, collective oscillations of free electrons at the metal–dielectric interface. This phenomenon is highly sensitive to changes in the refractive index at the surface, enabling precise detection of molecular binding events (Homola, 2003, 2008; Rich and Myszka 2000).

SPR has evolved into a foundational technology in FBDD, offering unparalleled sensitivity in detecting weak interactions with real-time kinetic profiling. Next, in this review we outline the historical development, instrumentation advances, and methodological innovations that have positioned SPR as both a primary screening and validation platform in FBDD. Particular attention is given to the utility of SPR in both direct-binding and functional assays, culminating in practical guidelines and future perspectives. In Table 5 we present the several kinds of SPR instruments available, vendors and pros and cons of each one of them.

Table 4 Leading institutions advancing FBDD programs

Institution	Country	Notable Programs/ Contributions	Biophysical Techniques	Structural Biology Techniques	References
Astex (Otsuka)	United Kingdom	Ribociclib, Erdafitinib, Capivasertib (Pyramid™ platform collaborations),	NMR, SPR	X-ray crystallography	(Astex 2026; Erlanson et al. 2016)
Plexxikon	United States	Vemurafenib, Pexidartinib	SPR, NMR	X-ray crystallography	(Erlanson et al. 2016)
AbbVie	United States	Venetoclax (BCL-2 program)	NMR, SPR	X-ray crystallography	(Erlanson et al. 2016)
Novartis	Switzerland	Asciminib (ABL allosteric inhibitor)	NMR, SPR	X-ray crystallography	(Erlanson et al. 2016))
BioCryst	United States	Berotralstat	NMR, SPR	X-ray crystallography	(Erlanson et al. 2016)
Pfizer	United States	IRAK4 fragment series; internal FBDD integration	SPR, NMR	X-ray crystallography	(Pfizer 2026)
Amgen	United States	KRAS^G12C covalent fragment programs	SPR, MS	X-ray crystallography	(Erlanson et al. 2016)
Vernalis	United Kingdom	Chk1, Hsp90, Bcl-2 fragment campaigns	NMR, SPR, ITC	X-ray crystallography	(Vernalis 2026)
University of Cambridge	United Kingdom	Structure-guided fragment campaigns (oncology, antimicrobials)	ITC, NMR, SPR	X-ray crystallography	(Blundell et al. 2002; Congreve et al. 2003)
University of Oxford	United Kingdom	Bromodomain fragment programs	NMR, SPR	X-ray crystallography	(Filippakopoulos and Knapp 2014)
UCSF Small Molecule Discovery Center	United States	Kinase and PPI fragment libraries	DSF, SPR	Crystallography, Cryo-EM	(UCSF 2026)
Utrecht University (Bijvoet Center)	Netherlands	ssNMR-based fragment studies (GPCR, amyloid)	HDX-MS, ssNMR	Cryo-EM, solid-state NMR	(Bijvoet 2026)
Monash University	Australia	Structure-based FBDD (anti-infectives, oncology)	SPR, MST	X-ray crystallography	(Monash 2026)

Table 5 Comparative summary of major SPR instrumentation platforms used in FBDD applications (as of 2025)

Model	Vendor	Pros	Cons	Reference
Biacore T200	Cytiva	High sensitivity for small molecules, robust kinetic tools, widely validated	Medium throughput, costly maintenance, discontinued (support until 2030)	(Cytiva, 2025b; Karlsson and Falt 1997; Rich and Myszka, 2008)
Biacore X100	Cytiva	Compact, user-friendly, ideal for routine fragment screening	Only one flow cell, limited throughput	(Cytiva, 2025e)
Biacore 1 K	Cytiva	Bench-top SPR system with simplified interface, suitable for routine kinetic assays	Not suitable for high-throughput or fragment libraries	(Cytiva, 2025c)
Biacore 8 K	Cytiva	High-throughput (96–384), parallel kinetics	Requires advanced data handling	(Cytiva, 2025a, d)
Biacore 8 K +	Cytiva	Enhanced throughput and automation over 8 K	Higher complexity and training requirements	(Cytiva, 2025a, d)
Carterra LSA	Carterra	Ultra-high throughput, epitope binning	Very high cost, less optimal for ultra-weak fragments	(Carterra, 2025)
Bruker Sierra SPR-32 Pro	Bruker	4400 + samples/day, 0.02 RU RMS noise, excellent for fragments	High setup cost, requires robotics	(Bruker, 2025)
Delta inQuiQ™	Delta Life Science	Multiplexed label-free, accessible UI	New system; limited market data	(DeltaLifeScience, 2025)
OpenSPR Alto™	Nicoya	Affordable, compact, good for fragment and PPI assays	Lower throughput, limited buffer options	(Nicoya, 2025)
ProteOn XPR36	Bio-Rad	6 × 6 array (36 interactions), mid-throughput	Obsolete fluidics, limited updates	(Abdiche et al., 2008; Bio-Rad, 2025)
Horiba XelPleX	Horiba Scientific	Compact, modular, flexible chip design	Less adopted in FBDD; niche usage	(Horiba, 2017)

Historical evolution of SPR in FBDD

The adoption of SPR for fragment screening was gradual, with early skepticism about its ability to detect weak-affinity interactions. By the early 2000 s, however, successful applications to proteases and kinases proved its efficacy (Giannetti et al., 2008; Murray et al. 2012; Navratilova and Hopkins 2011; Rich and Myszka, 2008). The integration of high-throughput platforms and AI-assisted analysis has further solidified SPR's role in both academic and industrial FBDD workflows (Arkin et al., 2014; Zaheer and Pillai, 2021).

SPR instrumentation and technical advancements

The SPR system typically consists of a light source emitting polarized incident light directed onto a prism that is in contact with a sensor chip coated with a thin gold film. The reflected light is detected and analyzed to determine the resonance angle, which shifts in response to molecular interactions at the sensor surface. A fluidic system allows controlled delivery of analytes across the immobilized ligands on the sensor chip. The sensor surface is typically functionalized with target molecules (e.g., proteins, nucleic acids), and when analytes (e.g., drug candidates or fragments) bind to these targets, the resulting mass change alters the refractive index. This change is detected in real time as a shift in the resonance angle, producing a measurable signal in resonance units (RU), where 1 RU corresponds approximately to 1 pg/mm^2 (Myszka 1999).

Figure 2A illustrates the core components of an SPR instrument. A laser beam is directed through a prism onto a sensor chip, generating a plasmon wave at the gold interface. Binding events between analytes and immobilized targets induce changes in the dip angle and reflected intensity. This optical signal is converted into RU by a detector. The lower part of the figure visualizes the kinetic process, with analyte binding followed by dissociation, resulting in characteristic sensorgrams.

SPR measures changes in refractive index near a sensor surface as analytes bind to immobilized ligands. The response, recorded in RU, is directly proportional to mass changes on the surface. Sensitivity enhancements through microfluidics, image based multiplexing, and low noise optics have expanded SPR's applicability (Rich and Myszka, 2008).

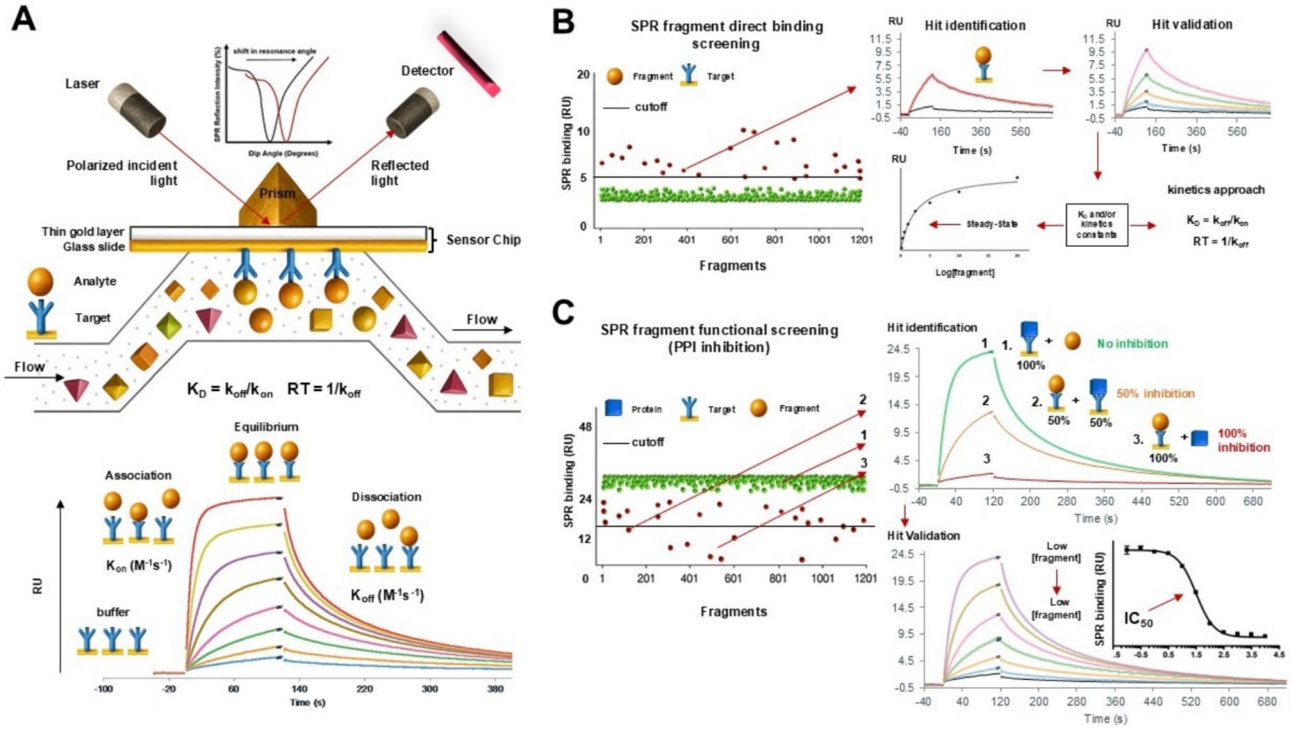

Fig. 2 SPR setup and integrated SPR strategies for FBDD: Direct and functional screening approaches. This figure illustrates the conceptual framework and application of SPR in FBDD, combining direct and functional screening workflows. **(A)** SPR principle and binding kinetics—Schematic representation of the SPR setup, showing a polarized laser beam directed through a prism to a gold-coated sensor chip. The binding of an analyte (e.g., fragment) to the immobilized target induces a refractive index change, resulting in a measurable SPR signal. Sensorgrams display the association and dissociation phases used to calculate kinetic constants: association rate (k_{on}), dissociation rate (k_{off}), equilibrium dissociation constant ($K_D = k_{off}/k_{on}$), and residence time ($RT = 1/k_{off}$). **(B)** Direct binding based fragment screening. Left panel shows a typical primary screen where fragments are ranked by RU *versus* fragment number. Hits exceeding the RU cutoff are selected for further analysis. Middle panel shows sensorgrams from primary hit fragments binding directly to the target, enabling extraction of K_D from either a steady-state fit or kinetic fitting. Right panel confirms reproducibility and validates binding kinetics through multiple fragment concentrations. **(C)** Functional fragment screening via PPI inhibition. In this competitive format, fragments are screened for their ability to inhibit PPI. The top panel shows dose-dependent RU reduction upon fragment addition, indicative of inhibition. Fragments are classified based on the degree of inhibition (e.g. 0%, 50%, 100%). Lower panels validate hits by competition experiments and generate R_{max} for calculation of IC_{50} values. This approach can also be used to screen fragments that enhance the PPI, important for approaches used for molecular glues, typically between a target protein and an E3 ubiquitin ligase. This facilitates ubiquitin tagging of the target, marking it for proteasomal degradation. This integrated approach enables both mechanistic and functional evaluation of fragment hits, offering quantitative insights critical for advancing early drug discovery

Types of sensorgrams

Sensorgrams are plots of RU over time, providing real time profiles of binding interactions. A standard sensorgram consists of several distinct phases: the *baseline phase* (buffer only), *association phase* (analyte introduced and binding occurs), *equilibrium or steady-state phase* (plateau when binding and dissociation rates are balanced), and *dissociation phase* (analyte removed and complex dissociates) (Fig. 2A).

Beyond the classic kinetic sensorgrams, several other patterns are observed depending on binding kinetics and experimental design. Fast on/off interactions may reach equilibrium rapidly, resulting in sharp curves, whereas slow kinetics yield gradual curves. In some cases, partial dissociation or rebinding artifacts may be seen. Reference sensorgrams obtained from control surfaces (e.g., blank or non-specific ligand) are subtracted to correct for bulk effects and nonspecific binding. Regeneration steps, in which bound analyte is removed using regeneration buffer, restore the surface for subsequent cycles and appear as abrupt signal drops (Karlsson, 2004).

SPR sensor chips: types, composition, and applications in biomolecular interaction studies

Central to the functionality and performance of SPR assays is the sensor chip, which serves as the platform for molecular binding events. The design and composition of

these chips significantly influence experimental outcomes, including sensitivity, specificity, and kinetic resolution.

An SPR sensor chip comprises multiple layers that work in unison to facilitate the detection of binding events. The base of the chip is typically a BK-7 glass substrate, chosen for its excellent optical properties. Over this substrate, a thin layer of gold, generally around 50 nm thick, is deposited using techniques such as sputtering or thermal evaporation. The gold surface serves as the active interface where surface plasmons are excited. To enable the immobilization of biomolecules and to enhance binding capacities, this gold layer is further functionalized with a variety of chemical moieties. These functional coatings include carboxymethylated dextran, nitrilotriacetic acid, streptavidin, or recombinant proteins such as Protein A or Protein G, each tailored to specific experimental needs (Rich and Myszka 2000; Schuck 1997).The variety of available sensor chips allows researchers to select the optimal platform for their experimental design. Table 6 presents a comparative overview of the most widely used SPR sensor chips, detailing their surface compositions, immobilization methods, primary applications, and supporting references (Bergström and Mandenius, 2011; Gao et al., 2022; Karlsson and Falt 1997; Katsamba et al. 2006; Myszka 1999).

Sensor chip selection is crucial for sensitivity and specificity. CM5 (carboxymethyl dextran) is widely used for amine coupling. SA (streptavidin) and NTA (His-tag) surfaces offer site-specific and reversible binding. Optimal immobilization levels should be chosen to minimize mass transport limitation, rebinding, and nonspecific effects (Burris et al., 2024; Katsamba et al. 2006; Myszka 1999; Schuck 1997).

The choice of sensor chip directly impacts the shape and quality of the resulting sensorgrams. For instance, chips with a carboxymethyl dextran matrix, such as CM5 and CM7, provide a three-dimensional hydrogel layer that enhances ligand binding capacity and minimizes steric hindrance. These chips are preferred for complex interactions and high-affinity binding studies. On the other hand, chips like C1, which possess minimal matrix interference, are better suited for small molecule interactions where low noise and baseline stability are critical. Capture chips, including NTA, SA, Protein A, and Protein G, offer reversible immobilization strategies that are ideal for regenerable binding studies, such as in fragment-based screening or repeated kinetic analyses (Abdiche et al., 2009; Katsamba et al. 2006; Myszka 1999).

Recent advancements in SPR sensor chip technology have introduced novel surface chemistries and commercial innovations. For example, Cytiva's Biacore CAPture system allows automated regeneration of the sensor surface in high-throughput workflows, making it suitable for rapid screening applications (Cytiva, 2025f; Papalia and Myszka, 2010). Similarly, Nicoya's OpenSPR platforms have gained attention in emerging markets due to their portability and cost-effectiveness, broadening SPR accessibility beyond centralized laboratories and formats aimed at enhancing sensitivity, specificity, and throughput, and their expansion aligns with efforts to democratize label-free technologies in academic and biotech settings (Cooper, 2002; Homola

Table 6 Comparative matrix of SPR sensor chips: surface chemistries, coupling strategies, and application scope

Sensor Chip	Functional Layer	Immobilization Method	Applications	References
CM5	Carboxymethylated dextran (5000 RU)	Amine coupling, thiol coupling, aldehyde	General protein–protein, protein–ligand, antibody-antigen interactions	(Karlsson and Falt 1997; Myszka 1999)
CM7	High-density carboxymethyl dextran (10,000 RU)	Amine coupling	Suitable for small molecules and low-molecular weight analytes	(Katsamba et al. 2006)
NTA	Nitrilotriacetic acid (chelated with Ni2 +)	Capture of His-tagged proteins	Reversible immobilization for kinetic studies of His-tagged biomolecules	(Abdiche et al., 2009)
SA (Streptavidin)	Streptavidin covalently coupled to dextran	High-affinity capture of biotinylated ligands	For oriented ligand presentation, DNA/RNA studies	(GE-Healthcare, 2005; Yang et al., 2007)
Protein A	Recombinant protein A immobilized on surface	Fc region capture of IgG	Ideal for oriented antibody capture	(Champion and Beck, 2013)
Protein G	Protein G on surface	Fc capture with broader species recognition	Better for antibodies from multiple species	(GE-Healthcare, 2005; Yang et al., 2007)
Biotin CAPture	Biotin-streptavidin based, regenerable	Hybridization with biotinylated capture probes	DNA and RNA analysis, regenerable format	(Cytiva, 2025f; Li et al., 2006)
C1	Flat surface with minimal dextran	Direct coupling without matrix interference	Small molecule analysis, membrane protein reconstitution	Cooper (Cooper, 2002)

2008). Innovations include polymer brush coatings that reduce nonspecific binding, site-specific immobilization techniques using click chemistry, and nanostructured surfaces that amplify SPR signals. Regenerable chips, such as the Biotin CAPture series, are particularly attractive in high-throughput settings where repeated use of the chip surface is economically and technically advantageous. These advancements are poised to play a significant role in expanding SPR applications, particularly in FBDD, where the detection of weak and transient interactions requires high-performance chip platforms (Abdiche et al., 2011; Homola 2008; Mauriz et al. 2007).

Buffers for SPR experiments: Composition, function, and optimization

The performance and reproducibility of SPR experiments heavily depend on the composition of the running and immobilization buffers used throughout the assay. Buffers play a critical role in maintaining protein stability, ensuring the correct refractive index, minimizing nonspecific interactions, and enhancing binding kinetics. Therefore, selecting and optimizing the appropriate buffer is essential to generate reliable and interpretable kinetic and affinity data (Karlsson and Falt 1997; Myszka 1999; Rich and Myszka 2000).

SPR buffers must meet several stringent criteria to be compatible with the optical and biochemical requirements of the technique. One fundamental requirement is the stability of the refractive index. Since SPR detects changes in refractive index upon analyte binding, any fluctuation in buffer composition can lead to signal drift and baseline noise. Equally important is the need to reduce nonspecific binding, which can occur either on the chip surface or on immobilized ligands, potentially masking true interactions. Buffers must be biologically compatible with the proteins or nucleic acids being studied, preserving their native conformation and functional activity. Finally, the buffer must be chemically compatible with the immobilization chemistry employed, whether that be amine coupling, thiol coupling, or capture strategies. In general, all SPR buffers should be filtered through a 0.22 µm membrane and thoroughly degassed to remove particulates and dissolved gases that may cause microbubbles and artifacts during sensorgram acquisition (Homola 2008; Schuck 1997). The buffer composition typically includes a buffering agent, salts to maintain ionic strength, and additives to suppress nonspecific binding. Among buffering agents, HEPES, phosphate-buffered saline (PBS), Tris, and acetate-based systems are commonly used, with selection guided by target stability and assay requirements. HEPES is frequently chosen because of its minimal temperature-dependent pKa shift and limited interaction with protein surfaces. While concentrations around 10 mM may be sufficient for stable protein systems, fragment screening at millimolar compound concentrations may require higher buffer strengths (e.g., 20–50 mM) to ensure adequate buffering capacity and maintain pH stability. Buffer concentration should therefore be adjusted based on ligand load, protein sensitivity, and instrument compatibility rather than fixed convention.

Tris buffers may be selected in systems sensitive to small pH deviations, particularly where enzymatic activity or conformational equilibria are involved. However, most protein–ligand interactions exhibit some degree of pH dependence, and buffer choice should be determined empirically based on stability and reproducibility under screening conditions. Sodium acetate buffers are commonly used during the immobilization phase due to their tunable pH range (4.0–5.5), which facilitates electrostatic preconcentration of analytes onto carboxymethyl dextran surfaces prior to covalent coupling.

Salts such as NaCl or KCl are incorporated to maintain ionic strength, typically around 150 mM. Maintaining consistent ionic strength is essential to minimize nonspecific electrostatic interactions and stabilize baseline responses, particularly in high-sensitivity SPR assays or when analyzing weak, low-affinity ligands (Homola 2008; Schuck 1997). Variations in ionic strength can significantly influence both bulk refractive index and surface charge interactions, and may therefore require adjustment depending on protein stability and analyte charge distribution (Myszka and Rich 2000).

Non-ionic surfactants such as Tween-20 are routinely included at low concentrations (typically 0.005%−0.05%) to reduce nonspecific adsorption of proteins to the sensor surface and internal microfluidic channels (Karlsson and Falt 1997; Myszka 1999). Their inclusion improves baseline stability and reproducibility by limiting hydrophobic interactions between analytes and the dextran matrix or fluidic tubing, thereby reducing signal drift and enhancing signal-to-noise ratio (Rich and Myszka 2000). The use of Tween-20 and related non-ionic detergents has been extensively validated in SPR methodology and is standardized in commonly employed running buffers such as HBS-EP in Biacore systems (Katsamba et al. 2006; Rich and Myszka 2000).

Additional additives may be included depending on the experimental setup. For example, EDTA is often incorporated to chelate divalent cations such as Ca^{2+} or Mg^{2+} that could cause aggregation or nonspecific bridging effects between proteins, especially in systems employing metal-chelating surfaces like NTA sensor chips (Abdiche et al., 2009; Sjolander and Urbaniczky, 1991; Stenberg et al., 1991). Conversely, divalent cations such as Ca^{2+} or Mg^{2+} are sometimes required in the buffer to maintain the functional conformation of certain ligand classes, such as integrins,

nucleotidyl transferases, or other metalloenzymes, where divalent ions act as essential cofactors (Homola 2008; Katsamba et al. 2006).

Proteins such as bovine serum albumin (BSA) or casein are occasionally added to running buffers at low concentrations (e.g., 0.1%) to reduce nonspecific adsorption and stabilize ligands prone to aggregation, particularly for serum-derived or 'sticky' analytes (Karlsson and Falt 1997; Lichtenberg et al., 2019). Selecting the appropriate buffer is often a case-specific decision, influenced by the target protein's biochemical properties and the nature of the intended molecular interaction. Optimization is typically achieved empirically, using pilot titration experiments, buffer scouting, and published protocols for similar ligand classes. During immobilization steps, a preliminary phase known as pH scouting is widely recommended. In this procedure, the ligand is diluted in a series of sodium acetate buffers with pH ranging from 4.0 to 5.5. The objective is to identify the pH that ensures strong electrostatic attraction to the negatively charged dextran matrix on the sensor surface without compromising ligand stability or solubility (Karlsson and Falt 1997; Schuck 1997).

Careful buffer selection and validation are essential to maximize SPR performance and data quality. A well optimized buffer not only preserves ligand activity but also improves signal-to-noise ratio, minimizes baseline drift, and ensures reproducible binding responses. As such, buffer optimization should be considered an integral part of SPR assay development rather than a peripheral concern (Table 7).

Buffer selection in FBDD is particularly crucial due to the physicochemical nature of fragments. Fragments are typically small and weak binders, and even minor variations in buffer composition can substantially affect binding kinetics or lead to false positives or negatives. A well optimized buffer reduces the chance of nonspecific electrostatic interactions, stabilizes proteins that may otherwise be marginally stable in solution, and maintains a consistent refractive index across all injections (Karlsson and Falt 1997; Katsamba et al. 2006).

Another key factor is the prevention of analyte or ligand aggregation, which can distort kinetic profiles or mask true fragment binding. Buffer additives such as low concentrations of surfactants (e.g., Tween-20) or carrier proteins (e.g., BSA) are often employed to mitigate such effects. Moreover, the ionic strength and pH of the buffer must be finely tuned to support the folded, functional conformation of the target while avoiding destabilization of fragments or interference with their solubility (Homola 2008).

Because DMSO introduces concentration-dependent refractive index changes, precise solvent matching and solvent correction are essential for reliable SPR-based fragment screening. These practices are well established in SPR methodology and have been extensively described in foundational studies of biosensor experimental design and data interpretation (Karlsson and Falt 1997; Myszka 1999; Rich and Myszka 2000; Katsamba et al. 2006). Similar bulk refractive index effects have also been documented in broader SPR applications, including immunoassay formats, underscoring the general importance of solvent control in optical biosensing (Mauriz et al. 2007).

To mitigate these effects, a series of corrective strategies have been developed and standardized. One essential practice is to precisely match the DMSO concentration in the running buffer with that of the fragment solution. This

Table 7 Common SPR running and immobilization buffers: composition and applications

Buffer	Composition	Application	Notes	References
HBS-EP	10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% Tween-20, pH 7.4	General protein–protein and antibody binding assays	Gold standard Biacore buffer; suitable for most proteins	(Katsamba et al. 2006; Rich and Myszka 2000)
PBS-T	10 mM phosphate, 137 mM NaCl, 2.7 mM KCl, 0.05% Tween-20, pH 7.4	Antibody-antigen interactions	Suitable for antibody studies, especially IgG	(Abdiche et al., 2009; Karlsson and Falt 1997)
Tris Buffer	10 mM Tris, 150 mM NaCl, 0.005% Tween-20, pH 7.5	Enzyme–substrate binding	Ideal for pH-sensitive interactions	(Homola 2008)
Running buffer with DMSO	10 mM HEPES, 150 mM NaCl, 0.05% Tween-20 + 1–5% DMSO	Fragment screening	Requires refractive index correction	(Mauriz et al. 2007; Myszka 1999)
Acetate Buffer	10 mM sodium acetate, pH 4.0–5.5	Immobilization buffer	Used for pH scouting during amine coupling	(Karlsson and Falt 1997; Schuck 1997)
Biotin CAP Regeneration Buffer	10 mM glycine–HCl at pH 1.5	Regeneration of streptavidin surfaces	Harsh, use with caution	(GE-Healthcare, 2005)

eliminates differential bulk refractive index contributions, allowing the SPR signal to primarily reflect binding events. In addition, solvent correction curves are generated by injecting reference samples containing different concentrations of DMSO (typically spanning 0.1–1% above and below the working concentration). These curves allow interpolation and post-processing corrections for minor discrepancies in DMSO content and help isolate true binding signals from solvent-induced artifacts (Katsamba et al. 2006; Myszka 1999).

Instrument-specific calibration tools, such as Biacore's solvent correction wizard, further aid in managing DMSO variability. It is also advisable to run control injections using buffer plus DMSO alone to evaluate baseline stability and to exclude compound aggregation or nonspecific interactions. Moreover, fragment concentrations should be optimized to ensure that the observed responses remain within the linear response range of the instrument while avoiding artifacts like nonspecific sticking or surface saturation (Karlsson and Falt 1997).

Taken together, the careful selection and conditioning of SPR buffers, with a specific focus on DMSO calibration and refractive index correction, is fundamental to the successful execution of fragment-based screening campaigns. These practices enable high-throughput identification of genuine binders, allowing for the precise characterization of fragment-target interactions and facilitating the progression of hit-to-lead optimization in modern drug discovery.

Kinetic constants from SPR sensorgrams: principles, equations, and experimental considerations

We have already established that by monitoring real-time changes in refractive index at the sensor surface, SPR generates sensorgrams that describe the association, steady-state, and dissociation phases of an interaction (Fig. 2). From these curves, kinetic constants such as the association rate constant (k_{on}), dissociation rate constant (k_{off}), and the equilibrium dissociation constant (K_D) can be extracted. These constants offer insight into both how rapidly a complex forms, and how stable it is once formed (Schuck 1997).

An important limitation in the application of SPR to FBDD concerns the interpretation of kinetic parameters for weakly binding fragments. Fragment-protein interactions are typically characterized by low affinities and rapid dissociation rates, with k_{off} values that are frequently too fast to be reliably resolved by current SPR instrumentation under routine experimental conditions (Navratilova and Hopkins 2011; Rich and Myszka 2000; Schuck 1997). As a result, extraction of well-defined association and dissociation rate constants is often impractical at the fragment stage, even when binding can be robustly detected. In these cases, steady-state or equilibrium-based analyses provide a more appropriate and reliable description of fragment binding behavior and are widely used in fragment screening campaigns (Giannetti 2011; Navratilova and Hopkins 2011). Kinetic characterization becomes increasingly informative as fragment hits are elaborated into higher-affinity, lead-like compounds, where dissociation rates slow sufficiently to allow meaningful resolution of k_{on} and k_{off} values (Navratilova and Hopkins 2010, 2011). Accordingly, the strength of SPR in FBDD lies not in routine kinetic dissection of weak fragment interactions, but in its sensitivity, reproducibility, and flexibility to support both equilibrium and kinetic analyses across different stages of the discovery pipeline, when applied with appropriate experimental and interpretative caution (Giannetti 2011).

Association phase

The association phase represents the time during which the analyte is actively injected over the immobilized ligand surface (Fig. 2A). During this phase, the rate of complex formation increases as analyte molecules bind to the available ligand. The interaction is governed by second-order kinetics and can be modeled by:

$$A+L \rightleftharpoons AL \tag{1}$$

The rate of complex formation is described by:

$$\frac{dR}{dt}=k_{on} \times C \times \left(R_{max} - R_t\right) - k_{off} \times R_t \tag{2}$$

where R_t is the SPR response at time t, R_{max} is the maximum response when all ligand sites are occupied, and C is the analyte concentration in mol/L.

The increase in R is directly proportional to the refractive index change (Δn), which is linearly related to mass accumulation:

$$\Delta n \propto R_t \propto \Gamma_t \tag{3}$$

where Γ_t is the surface concentration of bound analyte in ng/mm^2 (Homola 2008).

Steady-state phase

In some binding events, especially under high analyte concentrations or with rapid kinetics, the interaction may reach equilibrium during the association phase. At this point, the rate of complex formation (association) equals the rate of complex dissociation, resulting in a stable plateau in the sensorgram (Fig. 2). This plateau represents the steady-state binding response (R_{eq}).

The steady-state approach involves injecting increasing concentrations of analyte and recording the equilibrium

response values. By plotting R_{eq} against the analyte concentration C, a binding isotherm is constructed that follows a Langmuir model:

$$R_{eq}=\frac{(R_{max} \times C)}{K_D+C} \tag{4}$$

where: R_{max} is the theoretical maximum response at saturation; K_D is the equilibrium dissociation constant (in mol/L or M) and C is the analyte concentration (in mol/L or M).

The experimental data points are then fitted to this equation using nonlinear regression, and the K_D value is extracted from the best-fit curve. This method is particularly advantageous when rapid on- and off-rates make it difficult to resolve kinetic parameters accurately. It is also highly useful in fragment screening campaigns, where low-affinity interactions generate relatively flat association curves but can still reach detectable equilibrium levels (Karlsson and Falt 1997; Myszka 1999; Navratilova and Hopkins 2011; Rich and Myszka 2000).

Important considerations include ensuring that equilibrium is indeed reached within the injection time for each concentration and that the analyte concentration range spans both below and above the anticipated K_D to generate a reliable isotherm curve. Additionally, the use of control surfaces and solvent correction is essential to eliminate nonspecific binding and refractive index mismatches, respectively.

Dissociation phase

The dissociation phase begins when the analyte flow is stopped and replaced by buffer, causing the complex to decay as analyte molecules dissociate from the ligand. The response decreases over time following first-order kinetics:

$$R_t=R_0 \times e^{-k_{off} \times t} \tag{5}$$

Here, R_0 is the response at the start of dissociation. The slope of the exponential decay curve is directly used to extract k_{off}, which defines the stability of the complex.

Integrated rate equation and global fitting

Advanced analysis uses integrated rate equations across time and concentrations to extract parameters globally. A widely applied expression in pseudo-first-order conditions is:

$$R_t=\frac{(k_{on} \times C \times R_{max})}{(k_{on} \times C+k_{off}) \times [1-e^{-(k_{on} \times C+k_{off})} \times t]} \tag{6}$$

This enables simultaneous fitting of multiple sensorgrams for a more robust determination of kinetic parameters (Myszka and Morton, 1998).

Relation to refractive index and surface mass density

SPR response (R) is proportional to the change in refractive index (Δn), which itself is related to the surface mass density (Γ) via:

$$\Delta n=(\frac{dn}{dC}) \times \Gamma \tag{7}$$

where: dn/dc is the refractive index increment (typically ~ 0.185 mL/g for proteins); Γ is in units of mass per surface area (ng/mm^2).

Thus, binding curves not only inform kinetic parameters but also offer quantitative insights into the amount of biomolecule captured or bound on the surface (Homola 2008; Schuck 1997).

Practical examples from fragment-based screening

Extraction of reliable SPR kinetic parameters requires high-quality experimental design.

Table 8 summarizes key considerations, the potential issues they may cause, strategies to mitigate them, and supporting information. These considerations are essential to ensure reproducibility and accuracy in SPR kinetic studies, particularly in challenging applications such as FBDD.

Example applications in FBDD

As an example of SPR resolving weak, fast on/off interactions, Myszka (1999) discusses how appropriate experimental design and analysis enable meaningful kinetic and affinity estimates even when binding responses are small and kinetics are rapid (Myszka 1999).

As an illustrative example, fragment libraries have been screened by SPR against HIV-1 protease as part of fragment library validation workflows, demonstrating that weak binders can be detected reproducibly at low response levels and triaged for follow-up analysis (Elinder et al., 2011).

Steady-state affinity analysis is frequently used for weak fragment interactions when rapid kinetics or low signal-to-noise complicate reliable kinetic fitting (Giannetti 2011; Navratilova and Hopkins 2011; Rich and Myszka 2000).

These examples highlight how careful experimental design combined with rigorous kinetic or equilibrium

Table 8 Key experimental challenges and mitigation strategies in SPR-Based kinetic analysis

Consideration	Potential Problem	Recommended Solution	References
Analyte concentration range	Poor definition of binding kinetics and inaccurate K_D estimation	Use at least five analyte concentrations covering a tenfold range above and below K_D	(Karlsson and Falt 1997; Myszka 1999)
Flow rate	Mass transport limitations leading to distorted kinetics	Use high flow rates (> 30 µL/min) to ensure analyte supply exceeds binding rate	(Belcher et al., 2024; Schuck and Zhao, 2010)
Surface ligand density	Rebinding or steric hindrance; non-ideal kinetics	Optimize immobilization to maintain low-to-moderate surface density (100–500 RU)	(Myszka and Rich 2000)
Reference subtraction	Signal distortion due to nonspecific binding or bulk refractive index effects	Include blank/reference channels and subtract baseline artifacts from raw data	(Homola 2008; Myszka and Morton, 1998)
Signal stability & replicate analysis	Data variability and unreliable kinetic fitting	Run at least three technical replicates and assess baseline drift	(Abdiche et al., 2009; Myszka 1999)
DMSO and solvent effects	Bulk refractive index shift and artifacts in fragment binding studies	Match DMSO concentration in sample and buffer; apply solvent correction curves	(Karlsson and Falt 1997; Mauriz et al. 2007)

analysis can yield meaningful data even for weak, transient fragment interactions. Some key considerations include: *i)* analyte concentrations: should span at least tenfold above and below K_D; *ii)* flow rate: should minimize mass transport effects (typically > 30 µL/min); *iii)* surface density: must be optimized to avoid rebinding and secondary effects; *iv)* reference subtraction: use of control surfaces or blank injections to remove nonspecific binding; *v)* replicates: multiple replicates improve robustness of fit and detection of outliers for fragment-based screening, where interactions are weak and transient, noise reduction and accurate solvent correction (e.g., for DMSO) are essential (Abdiche et al., 2009).

The role of clean-screening in SPR-based fragment screening

In the context of fragment-based screening using SPR, the clean screen step plays a fundamental role in the early triage of compounds that might otherwise produce misleading or artifactual binding data. In FBDD led by SPR assays, it is crucial to ensure that observed interactions are genuine and specific. Fragments are prone to nonspecific binding, aggregation, and refractive index artifacts, which can complicate interpretation of kinetic data (Elinder et al., 2011; Geschwindner et al., 2012; Giannetti 2011; Myszka 1999; Navratilova and Hopkins 2011; Shuker et al. 1996).

A clean screen is designed to identify problematic fragments before the main screening process begins, thereby preventing false positives and protecting the integrity of the biosensor surface. In practice, fragments are tested against a reference surface or a blank chip to detect nonspecific adsorption, detergent sensitivity, or baseline instability. Compounds that produce high background responses, fail to dissociate cleanly, or display bulk refractive index shifts are flagged and excluded from subsequent kinetic analysis (Geschwindner et al., 2012; Giannetti 2011).

Additionally, the clean screen step often involves parallel injection of fragments over both the target surface and a control surface to verify differential binding. This helps distinguish genuine target interactions from artifacts arising from compound properties such as stickiness, aggregation, or DMSO incompatibility (Giannetti 2011; Navratilova and Hopkins 2010; Shepherd et al., 2014).

By removing these confounding factors early, clean screening enhances the reliability of SPR-based FBDD campaigns and contributes to more efficient identification of high-quality starting points for drug discovery. During a clean screen, each fragment compound from the library is injected over a reference surface to identify nonspecific binders and surface artifacts. In fragment-based workflows, a blank reference channel containing activated and blocked dextran without immobilized target is generally preferred as the primary control, as it isolates bulk refractive index contributions and matrix-binding artifacts without introducing additional protein–protein interaction variables (Giannetti 2011; Rich and Myszka 2000). In some implementations, an unrelated, inert protein may be used as a secondary control to identify promiscuous binders with affinity for protein surfaces more broadly.

This enables the detection of fragments that nonspecifically bind to the chip surface matrix, interact promiscuously with protein surfaces, or display abnormal sensorgram characteristics such as excessive binding, refractive index artifacts, long dissociation tails, or aggregation behavior. Fragments producing responses above a set threshold in these

control channels are flagged and excluded from further assays. Additionally, fragments that alter baseline stability or are sensitive to DMSO content shifts are also removed. The proportion of excluded compounds varies with library quality but typically ranges between 1–5% in well curated sets. Implementing this step reduces downstream noise and improves hit-to-lead efficiency. Publications by Congreve et al. (Congreve et al. 2003) and by Navratilova and Hopkins (Navratilova and Hopkins 2011) emphasize the importance of pre-screening in the biophysical assessment of fragment libraries, particularly for high-throughput formats.

FBDD workflow using SPR: direct fragment binding

As described in Fig. 2B in FBDD by SPR, fragments are ranked by RU *versus* fragment ID. In this kind of screening, the hits exceeding the established RU cutoff are selected for further analysis. The fragments are re-tested by SPR (single point), and the new sensorgrams are analyzed for hit identification. The fragments that still show reasonable binding profiles are subjected to hit validation. In a hit validation assay by SPR, several concentrations of the identified hit (fragment) are analyzed in a dose response manner to confirm reproducibility, and to estimate binding kinetics and K_D.

A structured workflow is essential when SPR is employed as the primary platform in a fragment-based drug discovery campaign. The process begins with the preparation of the target protein, which must be biochemically pure, monodisperse, and correctly folded, as confirmed by methods such as SDS-PAGE, dynamic light scattering (DLS), analytical size exclusion chromatography, and circular dichroism (CD) (Batista et al., 2015). Moreover, if possible, its functional activity should be validated using a known ligand or cofactor to ensure that the immobilized form of the protein retains native-like behavior.

Once the protein is confirmed as suitable, it is immobilized onto a sensor surface using methods described before. Immobilization levels should be optimized to produce reliable response signals while minimizing mass transport limitations and rebinding effects. After immobilization, a DMSO calibration series is typically run to correct for refractive index differences introduced by the solvent carrier in which fragments are dissolved.

The clean screen procedure is then applied, as described above, to eliminate fragments that exhibit undesirable physicochemical properties. Following this, primary screening is conducted by injecting each fragment individually at a fixed concentration, typically in the 200–1000 µM range. Sensorgrams are analyzed for signs of specific, reproducible binding, typically defined by response thresholds exceeding three times the baseline noise and showing characteristic association/dissociation phases. Fragments with square shaped sensorgrams and no drift or bulk effects are selected as preliminary hits (Fig. 2B).

Secondary screening (hit validation) typically involves a multi-concentration series for each confirmed fragment hit to quantify binding strength. At the fragment stage, interactions often exhibit rapid association and dissociation kinetics that preclude reliable extraction of k_{on} and k_{off} values under standard experimental conditions. Accordingly, steady-state binding analysis is generally preferred for early-stage fragments, with equilibrium response levels fitted to appropriate binding models (Giannetti et al. 2011; Myszka and Rich 2000). Kinetic parameterization becomes increasingly informative as fragments are elaborated into higher-affinity compounds with slower dissociation rates (Navratilova and Hopkins 2011).

Fragments with sub-millimolar affinity and acceptable ligand efficiency (LE $\geq$ 0.30 kcal/mol per heavy atom) are prioritized. Orthogonal assays, such as DSF, HDX-MS, DEPC-MS, NMR, X-ray crystallography or cryo-EM are then used to confirm binding and elucidate binding modes.

In some cases, SPR competition experiments are conducted using a known ligand to determine whether the fragment binds to the orthosteric or an allosteric site. Additionally, truncated domain constructs or point-mutant versions of the protein can be immobilized to further localize the binding epitope. Confirmed fragment hits may then enter hit-to-lead programs that include structure-based optimization, which usually involves iterative cycles of co-structure determination and medicinal chemistry design. The described approach has been widely validated in both industrial and academic settings and remains a gold standard for biophysical fragment screening, as exemplified in studies by Giannetti et al. (Giannetti et al., 2008), Shuker et al. (Shuker et al. 1996), and Navratilova et al. (Navratilova and Hopkins 2011).

Functional SPR-Based Screening for Protein–Protein Interaction (PPI) modulators

Functional fragment screening using SPR is particularly powerful for identifying compounds that modulate PPIs. These interactions often involve shallow or dynamic interfaces that are poorly suited for traditional high-throughput assays, making biophysical techniques like SPR invaluable. In functional PPI-based screening, the SPR assay is designed to recapitulate the biological interaction between two proteins (typically one immobilized on the chip surface and the other injected in solution) while monitoring how fragments influence the association in real time (Fig. 2C).

To perform a functional PPI SPR screen, the first protein (Protein X-ligand) is immobilized onto the sensor chip using

standard surface chemistries, ensuring that its binding site for the partner protein (Protein Y-analyte) remains accessible. The baseline interaction between ligand and analyte is first characterized by injecting Protein Y at a fixed concentration to determine the strength and kinetics of the interaction. Subsequently, fragments are pre-incubated with Protein Y or co-injected with it, and the assay monitors whether the presence of the fragment alters the binding profile compared to the baseline signal.

In functional SPR assays designed to monitor PPIs, one interaction partner is immobilized while the second binding partner is injected in the presence or absence of fragment compounds. Fragments that disrupt the interaction typically produce a reduction in response units (RU) relative to the control injection, whereas fragments that stabilize or enhance the interaction may increase or prolong the RU signal. These formats enable detection of orthosteric inhibitors as well as allosteric modulators that alter complex stability. Careful interpretation is required to distinguish genuine modulation from nonspecific surface effects or mass transport artifacts (Giannetti 2011; Rich and Myszka 2000).

Importantly, functional SPR screening for PPIs must include stringent controls, such as testing fragments against each component separately to exclude nonspecific binding or aggregation. Sensorgrams should be carefully analyzed for reproducibility and stoichiometry of inhibition or enhancement. Fragments validated through this approach are strong candidates for development into PPI-targeting leads, a class of therapeutics with growing clinical relevance across oncology, virology, and inflammation.

Besides SPR, other immobilization-based biophysical technologies can be applied to FBDD campaigns. Bio-Layer Interferometry (BLI), Quartz Crystal Microbalance (QCM), and Grating-Coupled Interferometry (GCI) are widely used to monitor real-time biomolecular interactions under label-free conditions, offering complementary kinetic and affinity information depending on platform sensitivity and experimental configuration. These techniques differ in surface chemistry, detection physics, and dynamic range, but share the advantage of enabling direct observation of fragment–target interactions in a controlled format (Giannetti 2011; Rich and Myszka 2000).

Second Harmonic Generation (SHG) has also emerged as a surface-based, immobilization-dependent technology capable of detecting ligand-induced conformational changes and binding events at interfaces through nonlinear optical responses from oriented molecules. Unlike conventional mass-sensitive biosensors, SHG is particularly sensitive to changes in molecular orientation and conformational dynamics, enabling detection of binding-induced structural perturbations even in cases where mass changes are minimal. SHG has been successfully integrated into fragment screening and optimization workflows targeting challenging oncogenic drivers, including KRAS. The translational relevance of this approach has been highlighted by the clinical advancement of Quanta Therapeutics' KRAS inhibitor program, in which SHG-supported screening contributed to fragment evolution and structure-guided optimization (QuantaTherapeutics 2023). This example underscores the expanding role of complementary surface-based technologies within modern FBDD campaigns.

Label-free biophysical techniques involving protein immobilization in FBDD: principles, performance, and comparative evaluation to SPR

Bio layer interferometry in FBDD

Bio Layer Interferometry (BLI) is a label-free optical analytical technique that enables real time monitoring of biomolecular interactions. It operates by measuring changes in the interference pattern of white light reflected from two surfaces: a reference layer and a biolayer where the target biomolecule is immobilized. When an analyte binds to the immobilized target, it alters the optical thickness of the biolayer, producing a shift in the interference pattern that is detected and quantified. This shift is proportional to the amount of bound material, allowing for the real-time analysis of association and dissociation events (Abdiche et al., 2009).

BLI offers several advantages in drug discovery applications, particularly due to its simplicity, robustness, and suitability for high-throughput screening. The technology is amenable to automation and does not require microfluidics or complex instrumentation, which makes it attractive for screening large libraries in a cost-effective manner (Concepcion et al., 2009).

In the context of FBDD, biolayer interferometry (BLI) has been explored as a complementary label-free method to SPR. Although BLI generally exhibits lower sensitivity, increasing the density of immobilized target proteins and optimizing buffer conditions can improve the signal-to-noise ratio and enable detection of fragment binding events. Accordingly, BLI has been applied to fragment screening and hit validation in FBDD campaigns, including studies targeting interaction-relevant or conformationally sensitive binding sites (Bothe et al., 2022; Wartchow et al., 2011).

Nevertheless, due to its inherent limitations in sensitivity compared to SPR and NMR, BLI is more often employed as a secondary tool in FBDD workflows, typically for orthogonal validation rather than primary screening. Researchers must carefully design their experiments, considering target immobilization strategy, surface regeneration conditions, and analyte concentration, to obtain reliable data from BLI platforms like the ForteBio Octet system (Apiyo, 2022).

Quartz crystal microbalance in FBDD

Quartz Crystal Microbalance (QCM) is a label free, real time analytical technique that exploits the piezoelectric properties of quartz crystals to detect minute changes in mass on a sensor surface. When an alternating voltage is applied to the quartz crystal, it oscillates at a specific resonant frequency. Binding of analytes to the immobilized target on the crystal surface adds mass, which in turn decreases the oscillation frequency proportionally. This mass sensitivity enables the detection of binding events down to the nanogram scale (Janshoff et al., 2000; Marx, 2003).

In FBDD, QCM has gained interest for its ability to monitor low affinity interactions without the need for labeling. Although its adoption has lagged behind techniques like SPR and BLI, QCM presents several advantages, such as the capability to study binding events in complex or non-optically accessible environments. Moreover, QCM can detect both specific and non-specific interactions, making it useful during the early triage of fragment hits (Cooper, 2002; Dixon, 2008; Johannsmann et al., 2021).

One of the unique applications of QCM in FBDD is its sensitivity to conformational changes and hydration layer alterations induced by ligand binding. Unlike purely optical methods, QCM detects changes in viscoelastic properties and solvent coupling, which can provide valuable insights into the binding mechanism and target dynamics. For example, QCM has been employed to investigate the conformational effects of fragment binding to intrinsically disordered proteins and membrane associated targets (Dixon, 2008; Nielsen and Otzen, 2013; Shur et al., 2011).

QCM has also been integrated into hybrid biophysical platforms for fragment screening. When used in conjunction with techniques such as NMR or X-ray crystallography, QCM provides orthogonal confirmation of binding and kinetic profiles. Recent studies have demonstrated the use of QCM-D (QCM with dissipation monitoring) to extract both mass and viscoelastic data, enhancing the interpretation of fragment induced effects on the protein surface and local microenvironment (Höök et al., 1998; Peh et al., 2007; Rodahl and Kasemo, 1996).

Although limitations such as lower throughput and complex data interpretation persist, continued advances in acoustic biosensor instrumentation and methodology are contributing to the growing utility of QCM in early-stage drug discovery. As FBDD continues to diversify its "*toolkit*", QCM and QCM-D offer complementary approaches for interrogating fragment binding, particularly for targets with challenging structural or dynamic properties (Renaud et al. 2016; St John-Campbell and Bhalay, 2025; Cooper and Singleton, 2007; Dixon, 2008).

Grating coupled interferometry in FBDD

Grating Coupled Interferometry (GCI) is a highly sensitive, label free optical technique that integrates waveguide interferometry with diffraction grating coupling. In GCI, coherent light is introduced into a waveguide via a diffraction grating, where it interacts with the evanescent field near the sensor surface. When an analyte binds to an immobilized target on the waveguide, it induces a change in the local refractive index, which shifts the phase of the light signal. This phase shift is detected with high precision, allowing the quantification of molecular interactions in real time (Kozma et al., 2011; Patko et al., 2012).

GCI offers several advantages over traditional label-free technologies. It provides exceptional baseline stability and low drift, making it ideal for detecting weak interactions within the affinity range typically encountered in fragment-based workflows. This sensitivity is particularly valuable in FBDD, where initial binding affinities are often in the high μM to mM range. Instruments like the Creoptix WAVE have demonstrated the ability to detect weak binding events with high accuracy, even under challenging buffer conditions or in the presence of complex matrices (Jankovics et al., 2020; Kartal et al., 2021; MalvernPanalytical, 2026).

A key benefit of GCI in FBDD is its capability to perform precise kinetic analyses. This method allows for the determination of association and dissociation rates (k_{on} and k_{off}), which are essential for characterizing fragment binding beyond simple affinity measurements. This kinetic resolution supports informed decisions during hit validation and lead optimization. Furthermore, GCI is compatible with standard surface-based immobilization approaches used in optical biosensing, supporting adaptable assay formats across diverse target classes (Jankovics et al., 2020; Kartal et al., 2021).

GCI is also well suited for analyzing membrane proteins and PPIs. Recent work has demonstrated GCI's utility for benchmarking, sensitivity assessment, and kinetic characterization of weak interactions relevant to fragment-based discovery on challenging targets, supported by high-quality kinetic readouts even at low immobilization levels (Jankovics et al., 2020; Kartal et al., 2021). Its ability to function effectively with crude extracts and serum containing buffers broadens its applicability in physiologically relevant conditions.

Overall, GCI represents a powerful and versatile addition to the biophysical arsenal for FBDD. As the technology matures, its adoption is expected to increase, particularly in applications demanding ultra-sensitive kinetic measurements and low noise detection of weakly binding molecules (Jankovics et al., 2020; Kartal et al., 2021; MalvernPanalytical, 2025).

Comparative performance in FBDD context

Label free biosensor technologies are essential components of FBDD due to their capacity to detect weak interactions in real time without the need for molecular labeling. Each technique that we present in this review (SPR, BLI, QCM and GCI) offers unique advantages and limitations based on factors such as detection principles, sensitivity, throughput, and applicability to different molecular targets.

SPR is sensitive enough to detect low-response fragment binding and can quantify affinities from high mM down to tight-binding ligands (as assay design permits), while providing kinetic resolution where kinetics are experimentally separable. It enables detailed kinetic dissection of molecular interactions through real-time monitoring of association and dissociation phases. In FBDD, it is widely used for hit validation, affinity ranking, and SAR expansion (Giannetti 2011; Rich and Myszka, 2007). Its compatibility with miniaturized sample volumes and advanced chip surfaces further enhances its utility in complex assay formats.

BLI offers a more accessible and high-throughput option, particularly for early-stage screening. It uses fiber-optic biosensor tips, enabling simultaneous analysis of multiple samples with minimal fluidics. While its sensitivity is lower than SPR, it is suitable for detecting moderate-to-strong fragment binders and has proven effective in epitope binning and antibody selection studies (Abdiche et al., 2009; Frenzel et al., 2016). BLI is also valued in FBDD for its ease of use and lower operational costs (Concepcion et al., 2009).

QCM stands out for its ability to detect mass and viscoelastic changes on the sensor surface. Unlike optical techniques, QCM provides insights into hydration layer dynamics and conformational alterations during fragment binding. It is particularly effective for studying intrinsically disordered proteins, membrane-associated systems, and ligand-induced structural transitions (Dixon, 2008; Marx, 2003). QCM-D (with dissipation monitoring) adds further granularity by revealing energy dissipation changes, which correlate with molecular rearrangements.

GCI, while relatively newer, combines optical waveguide technology and interferometry to deliver high-resolution binding data with low baseline drift. It provides sensitivity comparable to SPR and is well suited for analyzing weak binders under challenging buffer conditions. GCI enables kinetic ranking of fragment binders based on association and dissociation rate constants and has been successfully applied in drug-discovery studies targeting challenging proteins, including weakly binding and conformationally sensitive systems (Jankovics et al., 2020; Kartal et al., 2021).

Choosing the appropriate biosensor platform requires careful consideration of experimental goals, target characteristics, molecular weight ranges, and resource availability. In many cases, combining orthogonal techniques (such as using SPR for kinetic profiling, BLI for primary screening, and QCM for conformational readouts) provides a more comprehensive understanding of fragment target interactions.

Table 9 summarizes the performance characteristics of SPR, BLI, QCM, and GCI. It highlights differences in sensitivity, analyte molecular weight range, throughput, sample consumption, cost, chip reuse, and robustness. The accompanying references offer primary literature support for each evaluation criterion and are useful for guiding platform selection in FBDD workflows.

Applications and comparative insights

SPR has consistently proven its value in high-sensitivity kinetic screening and fragment validation. For example, SPR-based fragment screening and kinetic analysis were used to characterize weak binders targeting Bcl-2 family proteins such as Bcl-xL, enabling affinity ranking and extraction of association and dissociation rate constants to guide early optimization (Navratilova and Hopkins 2010). In addition, industrial groups including Roche and Astex Pharmaceuticals have reported extensive use of SPR in fragment screening and validation campaigns against kinases and bromodomains, highlighting its central role in modern FBDD workflows (Erlanson et al. 2016).

BLI has been effectively used in FBDD campaigns for hit triage and follow-up characterization, leveraging its throughput and ability to provide kinetic information. BLI-based fragment screening has been demonstrated in multiple drug-discovery settings (Bothe et al., 2022; Wartchow et al., 2011). In addition, BLI has been used to characterize binding kinetics of small-molecule hits against challenging targets including Hsp90 (Wang et al., 2019).

QCM has been used to probe conformational changes in intrinsically disordered systems, where hydration-layer coupling and viscoelastic effects can be critical to interpretation (Shur et al., 2011). In addition, QCM-D platforms based on supported lipid layers and tethered vesicle assemblies enable quantitative, label-free binding studies at membrane-like interfaces, including analysis of bound water contributions that can be central in soft, hydrated films (Patel and Frank, 2006; Patel et al., 2009).

GCI, with its phase-based detection and high baseline stability, has demonstrated strong performance in benchmarking and early drug-discovery applications. GCI-based assays enable the detection and kinetic ranking of weak-binding fragments, including interactions in the high-micromolar range, by extracting reliable association and dissociation rate constants (Jankovics et al., 2020; Kartal et al., 2021). In addition, the ability to operate at low surface densities and under challenging assay conditions supports the use of GCI for screening and characterization of difficult targets, including weakly binding and membrane-associated systems.

Table 9 Comparative performance metrics of label free biosensor technologies in FBDD

Feature	SPR (Biacore 8 K series)	BLI	QCM	GCI	Key References
Sensitivity	Very high (pM-mM)[&]	Medium (high nM-low µM)	Medium (nM-µM)	Very high (pM-mM)[&]	(Abdiche et al., 2009; Marx, 2003; Rich and Myszka, 2007)
Analyte MW range	~100–100,000 Da	> 5,000 Da	> 1,000 Da	~150–150,000 Da	(Frenzel et al., 2016; Giannetti 2011; Marx, 2003)
Throughput	High (up to 768/day)	High	Low	Moderate	(Abdiche et al., 2009; Giannetti 2011; Jankovics et al., 2020; Kartal et al., 2021; Marx, 2003)
Sample consumption	Very Low (nL to µL)	Moderate	Low-Moderate	Low	(Abdiche et al., 2009; Kartal et al., 2021; Marx, 2003; Rich and Myszka, 2007)
Cost per assay	Medium–High	Low-Medium	Low	High	(Concepcion et al., 2009; Giannetti 2011; Kartal et al., 2021; Marx, 2003)
Chip/sensor reuse	High	High	Moderate	Moderate	(Giannetti 2011; Jankovics et al., 2020; Jug et al., 2024; Marx, 2003)
Robustness	Very High	Moderate	Moderate	High	(Johannsmann et al., 2021; Jug et al., 2024; Kartal et al., 2021; Katsamba et al. 2006)

[&]This is the instrument range of sensibility. For fragments this range should be read as (high nM to mM)

Strategic advantages of SPR for fragment screening

SPR is well suited for detecting fragment interactions in the low-affinity range ($K_D \sim 10^{-3}$ to 10^{-6} M), particularly in cases where traditional biochemical assays lack sufficient sensitivity or dynamic range. However, this affinity range is also accessible to several complementary biophysical techniques, including NMR, MST, ITC, and MS. The advantage of SPR in this context lies not in exclusive detection capability, but in its real-time, label-free format, experimental flexibility, and compatibility with high-throughput workflows (Giannetti 2011; Rich and Myszka 2000).

Advanced SPR systems like the Biacore 8 K/K$^+$ support parallel channel architecture and rapid injection cycles, enabling high-throughput screening with accurate kinetic profiling. This facilitates ranking of fragment hits based on both affinity and kinetic behavior, which is invaluable for prioritizing follow-up compounds. SPR data can reveal structure-kinetic relationships (SKR), aiding in the selection of fragments with favorable residence times and binding mechanisms (a key factor in modern lead optimization).

SPR's chip-based system allows for diverse immobilization as shown in this review, preserving target protein activity and providing flexibility for screening challenging targets, including membrane proteins and PPI interfaces. Moreover, its high reproducibility and integration with kinetic modeling software allow users to derive mechanistic insights that go beyond simple binding affinity.

The synergistic role of biophysical and structural biology techniques in validating SPR-derived hits for drug discovery

SPR derived hits require rigorous validation to ensure specificity, relevance, and draggability. Biophysical and structural biology techniques provide critical, orthogonal insights that transform initial hits into viable leads. Their integration forms the backbone of modern FBDD campaigns, enabling the rational and efficient advancement of therapeutics from bench to bedside. These complementary approaches provide a multi-dimensional understanding of molecular interactions, aiding in hit validation, hit-to-lead, and ultimately, lead optimization (Myszka, 2004).

Biophysical techniques such as DSF, ITC, BLI and MST provide orthogonal validation of SPR hits by confirming binding through independent physical principles. For example, DSF can detect ligand induced thermal stabilization of a protein, providing evidence of direct interaction (Pantoliano et al., 2001). ITC, though low throughput, yields thermodynamic parameters that are invaluable for understanding binding mechanisms (Zhou and Gilson, 2009). MST complements SPR by confirming interactions in solution without immobilization artifacts (Jerabek-Willemsen et al. 2011).

With their capacity to reveal atomic level interactions, X-ray crystallography, cryo-EM, and NMR spectroscopy are essential for validating SPR hits by visualizing the exact binding mode of fragments. Structural confirmation ensures that binding occurs at the desired target site and informs

structure–activity relationship (SAR) development (Beck and Baumeister, 2016; Murray and Rees 2009).

Integration across the hit-to-lead pipeline Orthogonal biophysical and structural approaches integrate at every stage of the drug discovery pipeline. During hit validation, SPR hits are subjected to DSF or MST to eliminate false positives. Structural methods then map fragment binding and suggest vectors for chemical elaboration. In hit-to-lead development, ITC and SPR assess binding affinity improvements, while co-crystal structures guide medicinal chemistry. Lead optimization benefits from high-resolution structures that explain potency enhancements and pharmacophore tuning (Erlanson et al. 2016).

Conclusion

FBDD represents a conceptual shift in how molecular interactions are explored for therapeutic innovation. Rather than prioritizing highly optimized compounds at the outset, FBDD embraces weak and transient binding events as informative starting points, provided they are interrogated with sufficient sensitivity, rigor, and contextual understanding. As examined throughout this review, the deliberate integration of biophysical and structural biology techniques provides the framework through which such subtle molecular signals can be translated into interpretable and actionable biological insight.

Within this integrated framework, SPR has emerged as a widely adopted and highly informative technique that contributes sensitivity, quantitative resolution, and experimental flexibility across multiple stages of fragment-driven discovery. SPR enables affinity estimation, reproducibility assessment, and mechanistic interrogation in both direct-binding and functional assay formats. In addition, SPR requires relatively low sample consumption, both in protein mass and injection volume, making it particularly advantageous for studying complex or material-limited systems while reducing operational costs before transitioning to more sample-intensive orthogonal methods. Importantly, while kinetic analysis becomes increasingly informative as fragment hits are elaborated into higher-affinity, lead-like compounds, steady-state and equilibrium-based analyses often provide the most reliable description of fragment binding behavior at the earliest stages. In this context, the value of SPR lies not in the routine extraction of kinetic parameters for weak interactions, but in its robustness, adaptability, and suitability for stage-appropriate experimental strategies.

The impact of SPR is maximized when it operates as part of an orthogonal biophysical and structural biology ensemble. Complementary techniques such as DSF, ITC, MST, HDX-MS, and covalent labeling MS validate binding through independent physical principles and reveal thermodynamic, stoichiometric, and conformational features that SPR alone cannot resolve. Structural biology approaches, including X-ray crystallography, Cryo-EM, and NMR spectroscopy, provide the spatial context necessary to localize binding sites and guide rational optimization. Together, this convergence of evidence underpins confident hit validation and informed progression through the FBDD pipeline.

From practical experience, it is often the agreement between independent methods, rather than the apparent strength of any single dataset, that determines whether a fragment "hit" matures into a credible starting point or is efficiently triaged. This convergence reduces false positives, improves decision quality, and limits downstream attrition, reinforcing the central role of integration rather than methodological dominance in fragment-based workflows.

The success of FBDD is further grounded in the deliberate design of fragment libraries that emphasize simplicity, diversity, and physicochemical tractability. The reduced molecular complexity of fragments aligns naturally with the sensitivity of modern biophysical techniques, enabling reliable detection and mechanistic interrogation of weak binding events while facilitating efficient structure-guided optimization. In this sense, fragment libraries are not merely screening resources, but strategic extensions of the FBDD philosophy itself: minimal starting points refined through iterative, evidence-driven exploration.

At the same time, FBDD and its biophysical core face enduring challenges. The reliance on purified proteins, defined buffers, and controlled experimental systems inevitably simplifies biological complexity. Bridging the gap between reductionist screening and physiological relevance requires continued innovation in assay design, data interpretation, and integration with functional readouts. For SPR in particular, managing artifacts such as nonspecific binding, mass-transport effects, and solvent mismatches remains an essential component of experimental practice rather than a peripheral consideration.

As FBDD expands toward intrinsically disordered proteins, membrane-associated targets, and multi-component assemblies, SPR must continue to evolve to accommodate dynamic and heterogeneous interaction landscapes. Advances in recombinant formats, including nanodisc-based systems and functional screening configurations that preserve native-like assemblies, offer promising avenues for extending SPR into increasingly complex biological contexts.

Ultimately, the tension between reduction and emergence remains central to FBDD: how to reconcile the clarity of isolated binding events with the ambiguity of biological response *in-vivo*. Addressing this challenge requires judgment, experimental iteration, and interdisciplinary dialogue. When integrated thoughtfully with orthogonal biophysics,

structural biology, and medicinal chemistry, SPR remains well positioned to contribute meaningfully to this process, not as a singular solution, but as a critical component of an evidence-driven discovery ecosystem.

Acknowledgements This work was supported in part by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) under grant numbers 2017/26131-5 and 2024/02293-0, and by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) under grant numbers 310927/2021-8 and 303486/2025-2.

Author contributions All authors contributed to the conception, writing, and critical revision of the manuscript. All authors approved the final version.

Funding This work was supported in part by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) under grant numbers 2017/26131–5 and 2024/02293-0, and by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) under grant numbers 310927/2021–8 and 303486/2025–2.

Data availability No datasets were generated or analysed during the current study.

Declarations No additional external funding was received for the preparation of this review.

Competing interests The authors declare no competing interests.

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