Multiplexed experimental strategies for fragment library screening against challenging drug targets using SPR biosensors

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Principles for fragment-based drug discovery

Fragment-based drug discovery (FBDD) involves screening of specially designed libraries containing structurally diverse compounds with a molecular weight below ca. 300 Da. Fragment libraries typically comprise hundreds to thousands of compounds, rather than hundreds of thousands of compounds, as for high-throughput screening (HTS). The chemical diversity of fragments makes it possible to identify novel scaffolds, alternative binding sites and modes-of-action in the early stages of a discovery program. But their small size has the disadvantage of providing only a few possible intermolecular contact points, exhibited as typically weak and transient interactions with their targets. Fragments therefore have to be detected using very sensitive biophysical methods that must allow relatively high concentrations of fragments to be screened without running into experimental artifacts. Interactions are therefore ideally monitored directly without using reporter molecules (e.g. substrates) or coupled assays.

In order of popularity, the methods currently used include X-ray crystallography, nuclear magnetic resonance (NMR), surface plasmon resonance (SPR)-based biosensors, thermal shift and in silico methods, functional screening and isothermal calorimetry (ITC). They differ in their sensitivity, capability of providing kinetic or structural information, experimental flexibility and risk of experimental artifacts. Two complementary methods are ideally used in a project since screening hits should be confirmed by an orthogonal method. An advantage of FBDD is that the same methods used for the screening can also be used also in the later stages of FBDD. The first of these is to follow up hits by analysing commercially available analogues (aka “analogue-by-catalogue”) to confirm that closely related fragments also interact, and eventually to establish structure-interaction relationships using specifically designed structural analogues. Similarly, the methods can be adjusted for in-depth characterization of interactions required for evolution of hits into leads, guided using structure-based medicinal and computational chemistry approaches.

Principles for SPR biosensor-based systems for FBDD

SPR biosensor systems used for time-resolved analysis of interactions between small molecules and drug targets have a derivatized gold sensor surface to which the protein has been immobilised, embodying the sensor surface (SI 1, Fig. 1a). The biosensor terminology is confusing as the immobilised interaction partner is often referred to as the ligand (originating from the terminology for affinity chromatography). Here we reserve the term “ligand” for the low molecular weight analyte to avoid confusion.

The detection of molecular interactions in real time using SPR is due to the changes that take place at the surface by continuously monitoring the angle where the intensity of the reflected light has a minimum due to surface plasmon resonance. Signals depend on the refractive index of the medium close to the gold layer and are dominated by interactions between analytes and immobilised
molecules. Fragments are injected in a continuous flow of buffer via a microfluidic flow system that controls how the surface is addressed in time and space. State-of-the-art flow-based instruments vary in the number of channels in the microfluidic flow system and the number and relative position of sensor surfaces (SI 1, Fig. 1b). This influences the flexibility of the experimental design and the throughput of assays. Instruments that allow the use of multiple sensor surfaces in a single flow channel are advantageous for advanced referencing of data, e.g. using multiple target variants or off-targets, while screening efficiency can be higher with systems that have multiple flow channels. For fragment analysis, at least two surfaces are used to discriminate the typical weak binding to the properly folded and functional target from non-specific binding to unfolded or otherwise non-functional forms of the target, immobilised contaminants or the matrix. A reference surface can consist of the matrix alone, a variant of the target (mutant, chemically modified or denatured), or another protein.

Figure SI 1_1. Overview of SPR biosensor technology. a, Graphical representation of SPR biosensor flow system, sensor surface and detection principle. b, Illustration of alternative layouts of flow systems and sensor surfaces in instruments used in this work. c, Visualization of targets immobilized to sensor surfaces in random orientation via covalent amine coupling (top) and in fixed orientation via non-covalent SA-biotin conjugation (right).

Sensor surfaces

When adopting time-resolved SPR biosensor systems for analysis of fragments, there are some unique challenges. Due to the small size, fast kinetics and weak affinities of fragments, screening of fragment libraries requires very sensitive sensor surfaces that are stable for the entire screening experiment. In practice, assay development is a trial-and-error process where success relies on
practical considerations, such as the availability of high-quality target protein samples and an understanding of how to handle the protein and select conditions that allow the protein to be functional. It is often necessary to produce target proteins specifically constructed for SPR biosensor analysis, increasing stability or introducing residues or tags for improved immobilisation. It is important to check the structural integrity of new batches of protein and aliquoted samples subjected to storage and handling, and for identifying buffer conditions suitable for immobilisation and interaction experiments. Differential scanning fluorimetry based on intrinsic fluorescence (nanoDSF) is convenient for routine assessments, although it cannot be used for disordered proteins, or proteins lacking Trp and Tyr residues.

Target proteins are immobilised to sensor chips pre-coated with a carboxylated polymer, varying in density, degree of carboxylation and chain length using well-established chemical and biological approaches, such as covalent amine or thiol coupling, capture via His-tags or biotin groups proteins using chelating groups (nitrolotriacetic acid, NTA), antibodies or streptavidin covalently attached to the surface (SI 1, Fig. 1c). Coupling techniques that directly immobilize the target protein are preferred over the use of antibodies or other protein constructs as these additional proteins increase the density on the sensor surface and can lead to artifacts (e.g., interaction of fragments with non-target binding sites). When screening fragments against large proteins (>100 kDa), it can be advantageous to use surfaces with a denser matrix and a polymer with a higher degree of carboxylation. Targets can be immobilised in a random or defined orientation depending on the number of possible attachment points (SI 1, Fig. 1c, left and right, respectively) and further modification of surfaces are possible using biological and chemical modification methods, either before or after immobilisation of target proteins. The orientation is not critical for FBDD as small fragments are expected to easily be able to access all parts of the protein unless it has been immobilised to a very high density. However, potentially relevant binding sites should not be blocked. Multiple immobilisation strategies often have to be explored since it is critical to have a sensitive sensor surface with a fully functional target throughout the experiment.

Biosensor assay development involves exploration of different coupling techniques and experimental conditions. The quality of the generated sensor surfaces can be evaluated by analysis of interactions with compounds known to interact specifically with functional targets. However, such reference compounds are not always available at this early stage of a project.

**Data output**

The output from experiments is in the form of sensorgrams, representing response signals in resonance units (RU) and as a function of time (SI 1, Fig. 2). First, the system is equilibrated using running buffer and a baseline signal representing the surface with free target (P) is recorded. Binding events are observed as an increased signal upon the injection of a sample (L) over the derivatized
surface during the association phase. The signal represents the ligand target complex (PL) whose concentration is defined by the concentration of injected analyte and the association rate constant $k_{\text{on}}$ (units s$^{-1}$). The dissociation of bound molecules is observed during the dissociation phase, when only running buffer flows over the surface. The curvature is defined by the dissociation rate constant $k_{\text{off}}$ (units M$^{-1}$ s$^{-1}$). Rate constants and the equilibrium dissociation constant $K_D$ are quantified from sensorgrams recorded for a series of analyte concentration, using global non-linear regression analysis. $K_D$ values can also be estimated from steady state data for concentration series.

**Figure SI 1.2. Overview of SPR biosensor data output (sensorgram).** Depiction of the different steps in a typical experiment and idealized sensorgram.

**Assay sensitivity**

The detection limit depends on instrument features, how the assay is set up and the characteristics of the sensor surfaces used. The theoretical maximal response ($R_{\text{max}}$) that can be expected for an interaction is dependent on the analyte binding capacity of the surface. By assuming that the target is fully functional and that the interaction follows a reversible 1-step mechanism with a 1:1 stoichiometry, it can be estimated as:

$$R_{\text{max}} = \frac{\text{Immobilization level of target}}{\text{MW}_{\text{target}}} \times \text{MW}_{\text{analyte}}$$

(Equation 1)

$R_{\text{max}}$ can be determined experimentally by injecting a saturating concentration of a tool compound with a well-defined and reversible interaction with the target. Comparisons of theoretical and experimental $R_{\text{max}}$ values define the degree of surface functionality and are important sensor surface quality controls, critical when establishing new surfaces and ensuring that surfaces do not deteriorate or become blocked during a screen and can be used to predict if surfaces have adequate sensitivity for fragment studies. It is useful to normalize the signal with respect to $R_{\text{max}}$ and use this normalised signal ($R_{\text{norm}}$) to detect interactions that have a different stoichiometry than 1:1, as expected in the ideal case if there is a single binding site for the fragment on the protein.

To optimise the sensitivity of a surface, it is tempting to maximise the immobilization level of the target since it directly affects $R_{\text{max}}$ (Equation 1). But this may result in mass transport problems and suboptimal surface characteristics. It is more important to use surfaces with pure and fully functional protein which also avoids the risk of false positives resulting from interactions with unfolded,
denatured or aggregated protein. Selection of experimental conditions with respect to protein stability for the duration of experiments is particularly important for fragment screening where concentrations are high and specificity is low.

The signal expected for fragment screening hits is lower than $R_{\text{max}}$ since they typically have low affinities and do not saturate the target, even when screening is performed at the highest practical concentration. Instead, for fragments with rapid interactions, the measured response corresponds to the equilibrium response ($R_{\text{eq}}$) which depends on its affinity for the target (expressed as the equilibrium dissociation constant $K_D$) and the concentration injected (L):

$$R_{\text{eq}} = \frac{R_{\text{max}} [L]}{[L] + K_D}$$

(Equation 2)

$K_D$ can be defined either as the ratio of the concentrations of free target (P), free ligand (L) and the target-ligand complex (PL) at equilibrium, or as the ratio of the association and dissociation rate constants ($k_{\text{on}}$ and $k_{\text{off}}$, respectively):

$$K_D = \frac{[P][L]}{[PL]} = \frac{k_{\text{off}}}{k_{\text{on}}}$$

(Equation 3)

For successful fragment library screening, $R_{\text{eq}}$ should be much higher than the detection limit for the system to be used. Due to the low molecular weight of fragments (affecting $R_{\text{max}}$) and their weak and transient interactions with the target (affecting $K_D$), signals are very low. The possibility to maximise $R_{\text{eq}}$ by using high fragment concentrations ([L]) may in practice be limited by fragment availability, but other issues are common. Firstly, the relative contribution of secondary (non-specific) interaction with the target or sensor surface to the signal increases with concentration, making it essential to use as low concentrations of the fragments as possible. Secondly, inadequate fragment solubility in the assay buffer can result both in a lower effective concentration and aggregation on the surface thus potentially blocking the binding of subsequently injected fragments and obscuring the detection of hits. Increasing the solubility by increasing the solvent (DMSO) concentrations is not easily done since DMSO affects signals by changes to the refractive index as well as the functionality of targets. It is consequently essential to be accurate when pipetting samples in DMSO and to optimise and match running and sample buffers with respect to DMSO concentrations. Multiple controls and data correction routines are critical to detect and avoid solubility problems.

**Data analysis**

For many biomolecular interactions, sensorgrams have a clear curvature in both the association and dissociation phases (SI 1, Fig. 2). For fragments, the interactions are typically very rapid and sensorgrams are essentially “square” with no discernible curvature in the association or dissociation phases (SI 1, Fig. 3). However, data often show complexities due to weak interactions with multiple sites (>1:1 stoichiometry). Secondary/non-specific binding due to promiscuity or poor compound solubility can slow down association, while non-specifically interacting “sticky compounds” and
reversibly covalently interacting compounds can slow down dissociation and sometimes causing a base line shift. Such behaviour is typical of pan-assay interference compounds (PAINS) which should preferably be excluded from the library before screening. In addition, it is possible to detect ligand binding induced effects on the target structure resulting in curvature once a complex has been formed.

![Figure SI 1.3. Schematic of typical sensorgram shapes for fragments.](image)

Since fragments are expected to interact only very weakly and transiently with targets, square sensorgrams with signals that do not exceed the theoretical $R_{\text{max}}$ are often considered ideal (SI 1, Fig. 3, left). Depending on the target and goals of experiments, non-ideal kinetic profiles can be more or less problematic. For example, fragments showing curvature in the association or dissociation phases are often excluded when selecting hits, but relevant fragments may thereby be overlooked. Similarly, slow association (blue) is although fragments interacting rapidly with single binding sites at low concentration and secondary sites at high concentrations will be excluded.

**Fragment library screening**

Screening is efficiently done by injecting fragments at a single concentration and selecting hits based simply on the basis of signal levels at a defined time after injection, with controls taken before injection of samples and after a certain dissociation time (double base line controls). For SPR biosensor-driven screening it is essential that fragments have a high solubility under the experimental conditions and do not block the surface or result in carry over between injections. A pre-screening routine to identify compounds that may give rise to artifacts under the selected screening conditions and to confirm that the assay is sensitive and robust, is recommended. An option is to run the actual screen in forward and reverse order, and look for differences in signals, thus identifying problematic fragments and a deteriorating surface.5

To reliably identify fragments, screening should be done with sensitive assays that can discriminate compounds that have low $R_{\text{norm}}$ values from baseline and at concentrations higher than the expected $K_D$ values, thus maximising $R_{\text{norm}}$. Still, not much higher, due to the risk of signals arising from non-specific interactions and super-stoichiometric binding. Fragments giving rise to square-shaped sensorgrams are prioritized, reflecting the typical fast on/fast off kinetics of fragment
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SPR biosensor basics for FBDD

interactions with target proteins (SI 1, Fig. 3). However, fragments with deviations from rapid 1:1 interaction resulting in slow association, slow dissociation, or \( R_{eq} > R_{\text{max}} \) (illustrated in SI 1, Fig. 3), or other secondary effects should not immediately be rejected as they may represent mechanistically interesting interactions. The number of expected hits depends on the size and quality and relevance of the library screened. It is often practical to select the 10% “best hits” based on signal levels and sensorgram shapes.

**Hit validation**

Hits from a single concentration screen are validated in a follow-up experiment where hits are injected in a concentration series. Sensorgram shape and concentration dependence are evaluated. Signals for report points taken at steady state (i.e. corresponding to \( R_{eq} \)) and different concentrations should ideally result in a hyperbolic saturation curve and be below \( R_{\text{max}} \), as described by Equation 2 (see SI 1, Fig. 4a). To establish the interaction mechanism and estimate a \( K_D \)-value, it is essential to use an analyte concentration range that is \( >> K_D \). For fragments, the affinity is often too weak for a practically useful concentration to be used (see SI 1, Fig. 4b).

**Figure SI 1_4. Quantification of interaction data.**

**a**, Steady state data vs. ligand concentration for interactions with different characteristics. Levels of, representing \( R_{\text{max}} \). A simple reversible 1:1 interaction described by the Langmuir equation is hyperbolic (red dotted line) and . For higher order interactions, i.e. interactions with multiple sites (2:1, 3:1 etc.) or non-specific interactions (\( >> 1:1 \)), signals higher than 1 will be seen and the curve does not reach saturation within the concentration range used (green line). The binding efficiency (BE) represents the initial slope of the graph, i.e. the total binding to the target at low concentration. **b**, Relationship between fractional occupancy at different screening concentrations and \( K_D \) values.

Although it is not possible to quantify \( K_D \) values for interactions that do not reach saturation (SI 1, Fig. 4a, dashed curve), approximate \( K_D \) values (\( K_D^{\text{app}} \)) can be estimated by non-linear regression analysis using a 1:1 interaction model if the \( K_D \) is not much higher than the highest concentration used for screening (typically \( \mu \)M range), providing that \( R_{\text{max}} \) is not exceeded. When screening the library at 250-500 \( \mu \)M (suitable from the perspective of compound solubility), only fragments with \( K_D < 1 \) mM can be expected to result in > 50% fractional occupancy (SI 1, Fig. 4b).
When secondary/non-specific interactions occur, signals can be higher than $R_{\text{max}}$ and a more complex model is required for analysis (SI 1, Fig. 4a, solid curve). For very weak interactions, the relationship between signal and analyte concentration can be essentially linear, with only a slight curvature. It is then useful to estimate the Binding Efficiency (BE), which provides a measure of the ability of the fragment to bind to the target, without assuming an interaction model or stoichiometry. BE is determined from the initial slope of the signal vs. concentration graphs, i.e. at very low ligand concentrations where the amount of complex ($R_{\text{eq}}$) is linearly dependent on BE, at different compound concentrations ($L$) (SI 1, Fig. 4a, tangent).

For FBDD, it is common to use ligand efficiency (LE) to prioritize hits. It is essentially a normalization of affinities with respect to the size of the molecule, giving small molecules the chance to be selected despite their weak affinities. The LEs that can be expected for hits can be estimated on the basis of $K_D$ values and heavy atom counts (HAC). For FL1056 (see main text), the LEs of potential hits is shown in Table 1.

### Table SI 1. Analysis of potential ligand efficiencies (LE) for fragments in FL1056.

| HAC | LE calculations account for size (defined as Heavy Atom Count, HAC) and potential $K_D$ values at 25 °C.
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**Multiplexed experimental designs for single- and multi-channel systems**

By using multiple target variants immobilised to different surfaces and several experimental conditions it is possible to overcome problematic target characteristics or lack of reference compounds. Such multiplexed experimental approaches for screening of FL90 and FL1056 are
illustrated using two biosensor systems that differ with respect to the possible/required experimental design and throughput (SI 1, Fig. 1b).

A single flow channel system (SI 1, Fig. 1b, top) can readily be used for screening of a small library. The screening of FL90 simultaneously against three target variants is shown. It allowed a direct specificity analysis where the fourth sensor surface served as a reference. A multi-channel system is more suitable for screening of larger libraries since it enables screening with a higher throughput (SI 1, Fig. 1b, bottom). The rapid addressing of 16 biosensor surfaces via parallel injection of analyte across 8 channels is illustrated for a screen of FL1056. For comparison, the screening of FL90 against the three FPPS variants using the multichannel system is also shown. In this set-up, each variant is immobilized in a separate channel and referenced by a blank surface for each surface. Hits were identified for all targets using these multiplexed experimental designs (Main manuscript, Table 1 and Fig. 4).

References


