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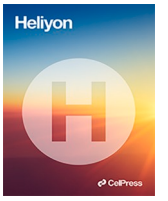

Review article

Target-based drug discovery: Applications of fluorescence techniques in high throughput and fragment-based screening

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ABSTRACT

Target-based discovery of first-in-class therapeutics demands an in-depth understanding of the molecular mechanisms underlying human diseases. Precise measurements of cellular and biochemical activities are critical to gain mechanistic knowledge of biomolecules and their altered function in disease conditions. Such measurements enable the development of intervention strategies for preventing or treating diseases by modulation of desired molecular processes. Fluorescence-based techniques are routinely employed for accurate and robust measurements of *in-vitro* activity of molecular targets and for discovering novel chemical molecules that modulate the activity of molecular targets. In the current review, the authors focus on the applications of fluorescence-based high throughput screening (HTS) and fragment-based ligand discovery (FBLD) techniques such as fluorescence polarization (FP), Förster resonance energy transfer (FRET), fluorescence thermal shift assay (FTSA) and microscale thermophoresis (MST) for the discovery of chemical probe to exploring target's role in disease biology and ultimately, serve as a foundation for drug discovery. Some recent advancements in these techniques for compound library screening against important classes of drug targets, such as G-protein-coupled receptors (GPCRs) and GTPases, as well as phosphorylation- and acetylation-mediated protein-protein interactions, are discussed. Overall, this review presents a landscape of how these techniques paved the way for the discovery of small-molecule modulators and biologics against these targets for therapeutic benefits.

1. Introduction

The primary step in a drug discovery campaign involves identifying new chemical entities that have the potential to become therapeutic agents. Target-based and phenotypic screening are the two approaches that have majorly contributed to discovering first-in-class drugs in the past few decades [1,2]. Phenotypic screening does not rely on knowing the identity of the specific molecular target or its role in the disease [3], instead active chemical compounds are identified with desired physiological responses or phenotypes, typically in animal models or cell-based assays [4]. Whereas target-based screening relies on the knowledge of the specific molecular target that has a vital role in the disease [5–7]. The ever-growing genetic, biochemical and structural insights into disease conditions

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have expanded the list of potential molecular targets amenable to therapeutic intervention [5,6,8]. Once the molecular target is identified and validated, a typical discovery process involves assay development, hit identification and lead optimization followed by preclinical and clinical development [9]. High-throughput screening (HTS) and fragment-based ligand discovery (FBLD) are currently

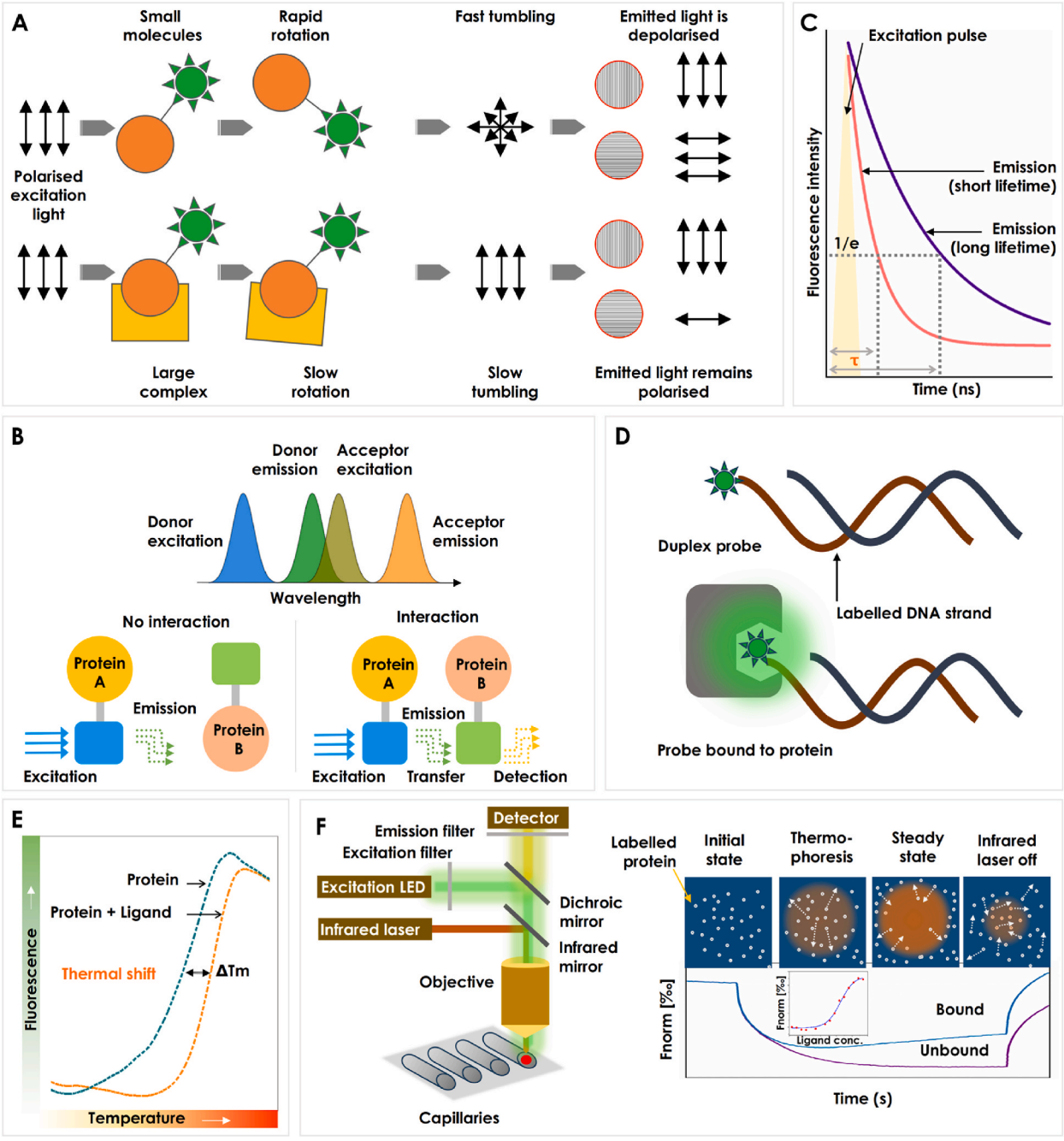

Fig. 1. A schematic representation of fluorescence-based assays (A). FP assay: the fluorophore-labeled substrate is excited by linearly polarised light, and the amount of polarised light emission determines the binding with the protein. (B). FRET assay: schematic illustration of the donor-acceptor spectral overlap region; distant donor and acceptor - no FRET, and close donor and acceptor - successful FRET detection. (C). FLT assay: The fluorescence intensity decay of a fluorophore is plotted on an intensity scale versus time. The changes in the lifetime of fluorophore measure the effect of protein binding. (D) PIFE assay: fluorescence enhancement when a fluorophore-tagged nucleic acid binds to a protein. (E) FTSA assay: The curve shows the protein unfolding with rising temperature, which alters upon ligand binding. (F) MST assay: schematic representation of an MST setup showing infrared laser directed at the protein-ligand solution in capillaries and excitation light to track the fluorescently labeled protein in the solution. An increase in temperature results in a binding-dependent decrease in fluorescence in the region followed by a steady state and back diffusion.

the two leading paradigms to identify chemical probes or "hits" against a molecular target [10,11]. In FBLD, a library of a few thousand very-small-molecular-weight compounds (MW ~100–250 Da) at high concentrations are screened using biophysical methods such as NMR, X-ray crystallography, SPR, MST and FTSA [12]. HTS involves screening large numbers of chemical compounds, typically a few hundred thousand with their molecular weights ranging between 200 and 600 Da, against the target using biochemical activity and binding-based methods [10,13–16]. HTS and FBLD have become standard tools within the pharmaceutical industry and academia, identifying the hits that affect the molecular targets in the desired way. The identified hits from the screening campaign provide a good starting point for lead optimization.

Screening cascades with suitable absorbance-, fluorescence- and luminescence-based high throughput biochemical and binding assays are developed for many important drug target classes [14,15]. However, developing robust, reliable and cost-effective assays while testing large libraries through them is quite challenging for the repertoire of novel molecular targets [17]. Towards this end, fluorescence-based assays are widely implicated in HTS and FBLD as they provide us with highly sensitive, rapid, homogeneous assays that are mix-and-read kind and compatible with automation. A wide diversity of available fluorescent dyes and technological advancements in readout modes have made them an ideal assay platform for most of the molecular target classes [18–21]. This review focuses on general concepts of fluorescence-based biochemical and binding assays routinely used in HTS and FBLD, namely fluorescence polarization (FP) and anisotropy (FA), Förster resonance energy transfer (FRET), time-resolved Förster resonance energy transfer (TR-FRET), fluorescence lifetime (FLT), protein-induced fluorescence enhancement (PIFE), fluorescence thermal shift assay (FTSA) and microscale thermophoresis (MST). In addition, the article also focuses on the rationale for the development of high-throughput fluorescence assays and their utilization in an array of screening activities for some therapeutic targets: G-protein coupled receptors (GPCRs), GTPases and phosphorylation- and acetylation-mediated protein-protein interactions.

2. Principles, advantages and limitations of fluorescence-based techniques

2.1. Fluorescence polarization (FP)/fluorescence anisotropy (FA)

FP/FA assay is based on the principle that the degree of polarization of a fluorescently labeled molecule, called a tracer, is inversely proportional to its rate of molecular rotation [22]. Molecular rotation, in turn, depends on molecular mass, with larger masses having slower rotation frequency. When a small tracer is excited by plane-polarized light, it emits depolarized light due to its rapid motion in solution. However, when the tracer binds to a much larger molecule, it rotates more slowly, and the emitted light remains largely polarized (Fig. 1A) [22]. Polarization is expressed as milli polarization units (mP). FP/FA technique is employed to study molecular interactions, i.e., association and dissociation between two molecules, e.g., protein-peptide, protein-nucleic acid, and protein-ligand [23,24]. The assay is sensitive, mix-and-read type and readily miniaturized for HTS. However, the FP assay works best for large protein-small ligand combinations rather than two proteins of similar molecular weight. Moreover, FP is amenable to interference from fluorescently active small molecules [24,25]. Some commonly used fluorophores in FP assay are listed in Table 1 and the chemical structures are shown in Fig. 2. FP assay readouts are measured using a range of multimode readers such as Victor Nivo and Envision (PerkinElmer), Spark (Tecan), Pherastar FSX (BMG Labtech), and Spectramax iD5 (Molecular Devices) etc. Optimization of parameters

Table 1
List of common fluorophores arranged in increasing order of their excitation spectra with their characterization and usages in different assays.

Fluorophore	Ex/Em (nm)[a]	Lifetime (ns)[b]	Assay
Europium cryptate[d1]	320-340/615	>1000	TR-FRET
Europium TMT chelate (donor)[d2], isothiocyanato chelate (donor)[d2]	320-340/618	>1000	TR-FRET
Terbium chelate[d3], Terbium cryptate[d3]	320-340/490, 545	>1000	TR-FRET
8-Anilino-1-naphthalenesulfonic acid	366/480	0.24	FTSA
9-Aminoacridine	405/450	17	FLT
PT14	405/450	14	FLT
SYPRO orange	470/570	<1	FTSA
PT22	473/556	22	FLT, FP
Fluorescein[a3]	475/520	4	FP, MST, TR-FRET
Alexa 488[a3]	490/525	4.1	FP, MST, TR-FRET
Rhodamine 110[a3]	496/520	4	FLT, FP, TR-FRET
BODIPY-FL[a3]	503/512	5.7	FLT, TR-FRET
TAMRA[a3]	550/580	1.0	FP, MST, FRET
Cy3[a3]	555/569	0.3	FP, PIFE, MST, FRET
Dylight DY547	558/574	0.18	PIFE
Rhodamine B	562/583	1.68	FLT, FP
ROX	580/621	4.8	FTSA
XL665[a1,a2]	625/665	3.0	TR-FRET
D2[a1,a2]	625/665	NA	TR-FRET
Alexa Fluor 647[a1,a2]	650/671	1.0	FP, MST, TR-FRET
Cy5[a1,a2]	651/670	1.0	FP, PIFE, MST, FRET, TR-FRET

d – donor in FRET pair, a – acceptor in FRET pair; respective donor-acceptor pairs have been numbered d1-a1, d2-a2 and d3-a3.
[a] Approximate excitation and emission maxima.
[b] Approximate fluorescence lifetime.

such as Z-height, G-factor, and number of flashes per second improves the assay performance. These readers are also used for the fluorescence-based techniques described in later sections, such as FRET, TR-FRET, FLT, PIFE and fluorophore substrate-based enzymatic assays.

2.2. *Förster resonance energy transfer (FRET)*

FRET measures the efficiency of non-radiative energy transfer between donor and acceptor fluorophores (FRET pairs) when they are in close proximity [26]. FRET efficiency varies inversely to the sixth power of the distance between the FRET pairs, indicating that small changes in distance result in a significant FRET signal (Fig. 1B) [27]. This advantage suites FRET to study intermolecular interaction. In general, the donor's quantum yield, the acceptor's extinction coefficient, spectral overlap (donor emission spectra should match acceptor absorption spectra), and inter-fluorophore distance (10–100 Å) and their angular orientation are the elements that must be considered to maximise the FRET signal. Time-resolved *Förster* energy transfer (TR-FRET) relies on resonance energy transfer between the long-lived donor fluorophore and short-lived acceptor fluorophore [28]. TR-FRET significantly reduces the short-lived fluorescence interference in the assay condition. The FRET-based assay measures biochemical activities, biomolecular interactions, and protein conformational dynamics [29]. FRET-based assays are highly sensitive, rapidly measurable, and readily adaptable to the HTS format. It can be used for both *in vitro* and *in vivo* assay systems. Also, there is no limitation on the molecular

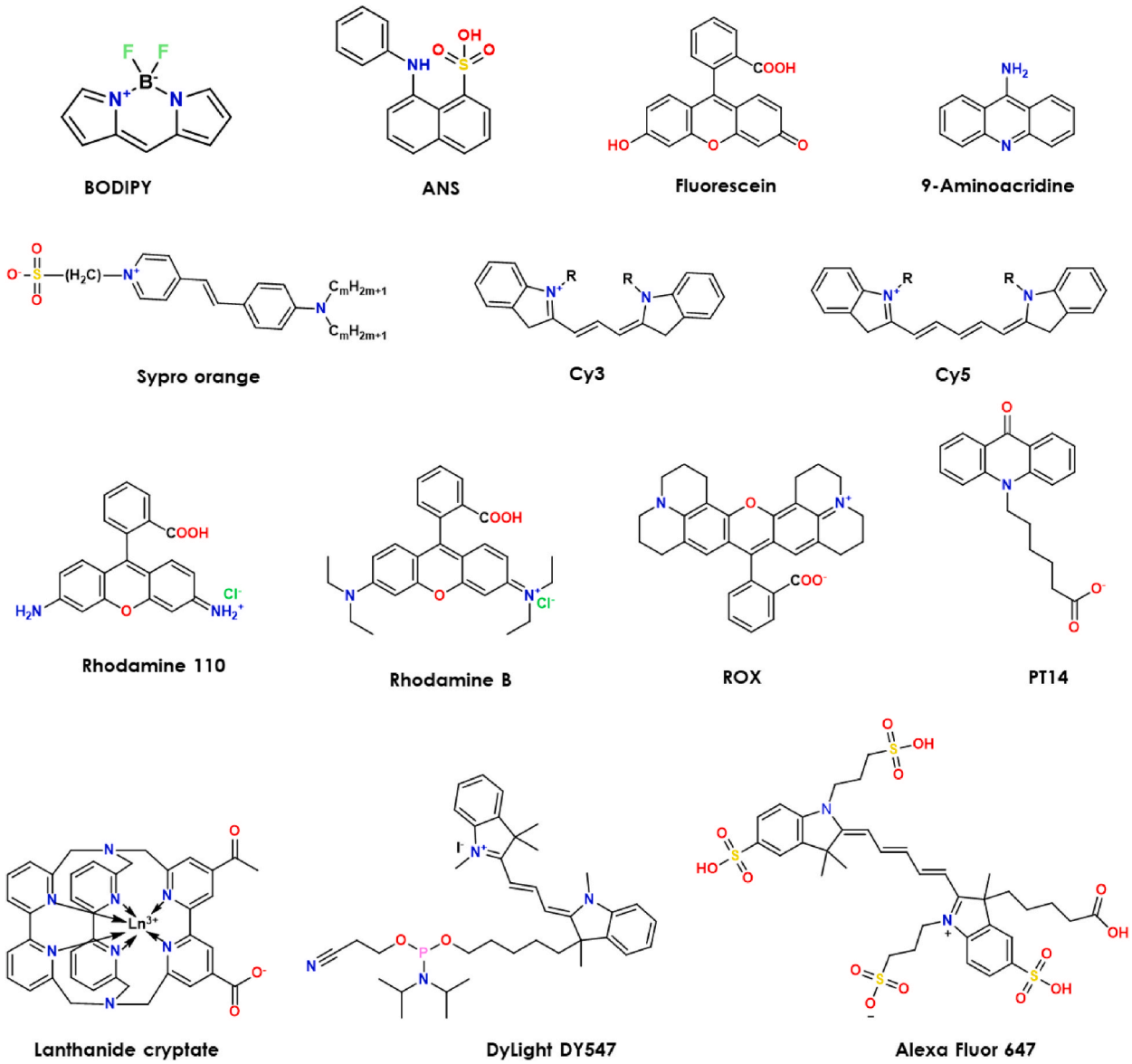

Fig. 2. Chemical structures of some commonly used dyes.

weight of biomolecules to be studied. However, both the interacting partners need to be labeled with suitable fluorophore pairs (refer to Table 1 and Fig. 2 for commonly used FRET pairs), and fluorophores should lie between 10 and 100 Å with correct orientation and the possibility of crosstalk between donor and acceptor fluorophores [30,31].

2.3. Fluorescence lifetime (FLT)

Fluorescence lifetime is an intrinsic property of a fluorophore defined as the average time a fluorophore takes in the excited state before returning to the ground state [32]. The lifetime of a fluorophore (τ) is dependent on the physicochemical molecular environment, so changes in the environment influence the changes in the FLT measurement [33]. The intensity decay of a fluorophore is plotted on an intensity scale versus time, and the fluorescence lifetime is calculated based on the fluorescence intensity decays to 1/e of the initial intensity (Fig. 1C). FLT measurement is independent of fluorescence intensity or fluorophore concentration [34]. Most commonly used fluorophores and compound interference typically have fluorescent lifetimes ranging from 100 ps to 5 ns [35]. The FLT fluorophores such as 9AA (Almac Group, FLEXYTE™), PT14 and PT22 (GE Healthcare) have fluorescence lifetimes of 14, 17 and 22 ns, respectively (Table 1 and Fig. 2). Principles of FLT can be used in different formats such as FLT assay, FLT sensing and fluorescence lifetime imaging [36]. FLT assays are successfully applied for enzyme activity assays for proteases, kinases and phosphatases for inhibitor screening [37,38]. Whereas FLT sensing is used for sensing pH, Ca^{2+}, Mg^{2+}, K^{+}, glucose, antigen, and other metabolites *in vitro* and FLT imaging applications involve studying molecular environments of the probes in cell [36]. With improved plate readers and a new range of long-lifetime dyes, the application of FLT is being extended to molecular targets other than enzymatic peptide modifications [33].

2.4. Protein-induced fluorescence enhancement (PIFE)

PIFE is based on the photophysical phenomenon whereby the intensity of fluorophore increases upon proximal binding of a protein (Fig. 1D) [39]. The technique is routinely used to probe the binding affinity and kinetics of protein and DNA/RNA interactions, wherein the nucleic acid is labeled with a single dye and binding to unlabeled protein causes a change in the fluorescence signal [40,41]. Since the technique bypasses protein labeling, it mitigates the risk of labeling-induced structural changes. Also, PIFE is sensitive within short distances (0–3 nm) [40]. So far, the technique is limited to studying protein-nucleic acid interactions and has not been routinely used in HTS format. This phenomenon is limited to the dyes exhibiting *cis*-trans photoisomerization [42,43]. A class of cyanine dyes (Cy3 and Cy5), Dylight DY547 and Alexa dyes (Thermo Fisher Scientific) are commonly used (Table 1 and Fig. 2).

2.5. Fluorescence thermal shift assay (FTSA)

FTSA exploits the phenomenon whereby a ligand binding to a target protein enhances the target protein's thermal stability [44]. Specific binding of a ligand to a defined site of the protein will enhance or decrease the structural stability of the protein and hence change the melting temperature (Tm), the temperature at which there is 50 % denaturation (Fig. 1E) [44]. The Tm of a protein can be measured either under native and label-free conditions by detecting temperature-dependent changes in the intrinsic fluorescence of tryptophan residues in proteins as the protein unfolds or by monitoring the fluorescence signal of a fluorescent dye that binds preferentially to the denatured state of a protein as a function of temperature [44]. A variant of FTSA is cellular thermal shift assay (CETSA), which is used for testing ligand binding to the target protein inside cells [45]. Since FTSA provides information on protein stability, it is helpful for the optimization of buffer conditions needed in protein purification, storage, and crystallization [46,47] and protein-ligand binding [48]. Due to the ease of optimization and miniaturization, it has been used for the screening of small molecules (*e.g.,* fragments) and compounds with high affinities [49–51]. FTSA does not require special plate readers, as real-time PCR instruments such as Applied Biosystems, Qiagen, Biorad etc., can be used for measuring Tm. Although FTSA is often used for qualitative measurement of the binding event, it can be leveraged for quantitative measurement of binding constant at a constant temperature [52]. Another caveat is that intrinsically disordered proteins are unsuitable for FTSA [53]. SYPRO Orange (Sigma-Aldrich), ROX and ANS (Thermo Fisher Scientific) are routinely used fluorophores for FTSA (Table 1 and Fig. 2). Although beyond the scope of FTSA, it is worth mentioning two luminescent methods: (i) a highly sensitive protein-probe method uses Eu^{3+} chelate conjugated to a peptide probe that preferably binds to an unfolded state of a protein, helps measure protein denaturation and ligand binding [54–56]. (ii) Aggregation-induced emission (AIE), based on the phenomenon, in which luminescent molecules emit more efficiently in the aggregation state than in the solution state [57]. Though AIE has potential applications in antibiotic screening, biosensing and biomedical sciences, their use in target-based compound library screening is yet to be explored [58–60].

2.6. Microscale thermophoresis (MST)

MST measures the affinity between two molecules based on temperature-related intensity change in fluorescence signal plus the movement of molecules along the temperature gradient induced inside a thin glass capillary using an IR-laser [61] (Fig. 1F). The thermophoretic movement of the biomolecules is tracked either by using the intrinsic fluorescence of tryptophan in proteins or an extrinsic fluorophore coupled to any one interaction partner [62]. MST can measure the binding affinity of molecular interactions. The technique has low sample consumption, short analysis time, no limitation on the molecular weight of interacting molecules and requires no immobilization of probes. MST has mostly been used as an orthogonal assay to validate hits found from primary screening [63,64]. The technique has capillary-based as well as plate-based formats available. Commercial kits for labeling proteins are available

from NanoTemper [65], which provides dyes that can label amino acid residues lysine (RED-NHS 2nd Generation), cysteine (RED--MALEIMIDE 2nd Generation) and histidine (RED-tris-NTA 2nd Generation). The availability of these three labeling residues on the protein's surface and their role in the binding event under study must be considered before the dye selection. The degree of labeling, i.e., the number of residues labeled per molecule, is a critical factor in assay quality and affects the reproducibility of measurements. To avoid such variations, proteins or peptides synthesized with tags such as GFP, FITC and Alexa-488 can be used [66]. For MST readouts, NanoTemper provides capillary (Monolith) and plate-based (Dianthus) readers. The availability of residues amenable for covalent labeling without altering the interaction or stability of a protein is a limitation [67,68]. Since the thermophoretic ability of a biomolecule depends upon its charge along with size and solvation shell, a better response is observed with charged biomolecules such as DNA and RNA [62,69,70]. Further, the capillary-based technique is incompatible with high-throughput screening and the plate-based approach is relatively new.

3. Development of HTS assays for therapeutic targets

3.1. G protein-coupled receptors (GPCRs)

GPCRs are the largest membrane receptor superfamilies regulating diverse physiological processes and are the most sought-after drug targets, with ~35% of approved drugs targeting these receptors [71–74]. In general, upon the ligand binding, GPCRs activate downstream heterotrimeric G-proteins consisting of Gα, Gβ and Gγ subunits (Fig. 3) [71]. Due to the challenges involved in the purification of stable GPCRs, considerable efforts have been made using mutagenesis [75,76], protein microarrays [77,78], reconstituted lipid nanodisc [79,80] and viral envelope [81] to obtain the active GPCRs for biochemical and biophysical studies. So far, HTS assays on GPCRs primarily rely on changes in the intracellular concentration of Gα-protein mediated secondary messengers such as cyclic adenosine monophosphate (cAMP), calcium, diacylglycerol (DAG), inositol 1,4,5-trisphosphate (IP_3) and RhoA activation (Fig. 3) [82]. G-protein independent β-arrestin signaling can be detected either by β-arrestin recruitment or β-arrestin mediated internalization

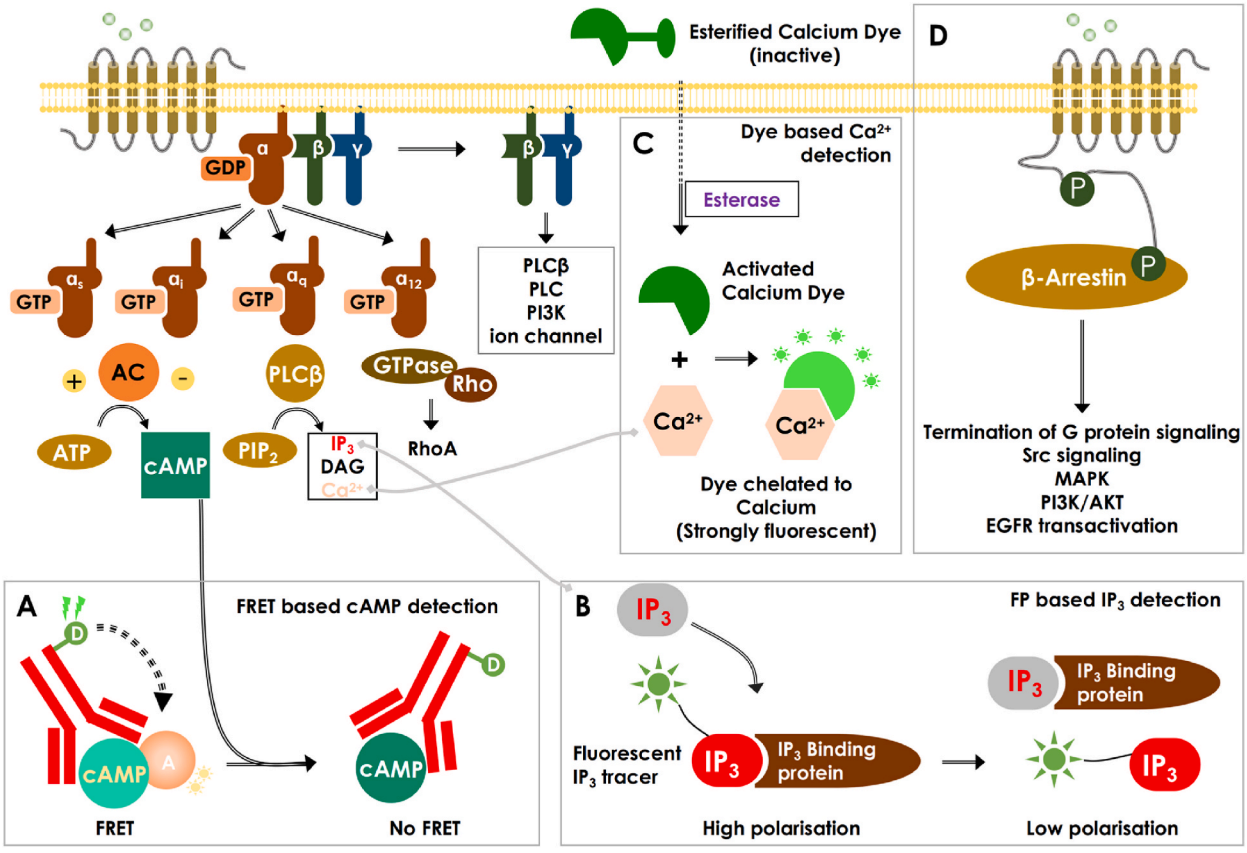

Fig. 3. An illustration of the G-protein-coupled receptor in action and β-arrestin signaling. The G-protein alpha subunit separates from the beta-gamma subunit in response to external stimulation, and GDP is exchanged for GTP, activating additional molecules in the cell. The three assays to quantify this, namely (A) cAMP detection by FRET-based assay, (B) IP_3 detection by FP-based assay, and (C) calcium detection by the dye-based method are shown as insets. (D) The recruitment of β-arrestin to the phosphorylated GPCR terminates the G-protein mediate signaling and initiates G-protein-independent signaling.

of GPCRs (Fig. 3) [83].

3.1.1. Fluorescence-based secondary messenger GPCRs assays

The GPCRs-dependent activation of Gαs and Gαi subunits stimulate and inhibit adenylate cyclase, respectively, which in turn increases and decreases the cellular cAMP levels [71]. Activation of Gαq results in the accumulation of inositol phosphates (IP_1/IP_3) and calcium (Ca^{2+}) levels (Fig. 3) [84]. FRET, TR-FRET and FP-based robust and sensitive assays are best suited to quantitatively measure cAMP, IP_1/IP_3 and DAG modulation in response to Gα-proteins [85]. In TR-FRET, the exogenous cAMP, IP_1 and IP_3 molecules labeled with an acceptor fluorophore compete with GPCR-coupled endogenously produced cAMP, IP_1 and IP_3 molecules, respectively, for binding to their donor-labeled monoclonal antibodies [85–87] (Fig. 3A). In FP assay, the cellular cAMP and IP_1/IP_3 displace a fluorescent derivative of counterparts from an unlabeled specific binding protein (Fig. 3B) [82,87]. Some of the commercially available HTS assay formats of GPCR modulators are cAMP-Gi kit (Cisbio), LANCE cAMP kit (PerkinElmer), HTRF cAMP (Cisbio), cAMP Gs dynamic kit (Cisbio), cADDis (Montana Molecular), IP-One Gq kit HTRF (Cisbio), HitHunter IP_3 assay (DiscoveRx), HTRF cAMP/IP-One HTplex (Cisbio) etc. FRET and FP assays are widely used to identify small-molecule and peptide agonists and antagonists as well as to study monoclonal antibodies [88–93].

3.1.2. Calcium assay

As mentioned earlier, calcium is another secondary messenger for GPCR signaling. Gαq activation results in the accumulation of inositol phosphates (IP_3), which increases the intracellular Ca^{2+} levels by opening the endoplasmic IP_3-gated calcium channel [94]. Commercial availability of Ca^{2+}-sensitive fluorescence dyes (Fluo, Fura, Indo, Quin etc.) [95,96] and "no-wash" assays such as Fluo-4 Direct Calcium assay kit (Life Technologies), FLIPR Calcium 4 assay kit (Molecular Devices), Calcium No WashPLUS (DiscoverX) and FLUOFORTE Calcium assay kit (Enzo Life Sciences) has enabled the measurement of Ca^{2+} levels in HTS format. The assay is based on the principle that the inactive esterified dye penetrates the cell membrane and becomes active once inside the cell upon binding to Ca^{2+}. Esterification is an effective way to increase the lipophilicity of the dye to improve cellular penetration of chemical entities. Intracellular esterases hydrolyse the dye once inside the cell, generating the negatively charged form of impermeable dye through the cell membrane. The calcium-binding moiety portion of the probe quenches the fluorescence signal in the absence of calcium through photo-induced electron transfer. In the presence of calcium, it relieves quenching and increases the fluorescence emission intensity in several order magnitudes, with no shift in wavelength, thus characterizing GPCRs (Fig. 3C) [97–100].

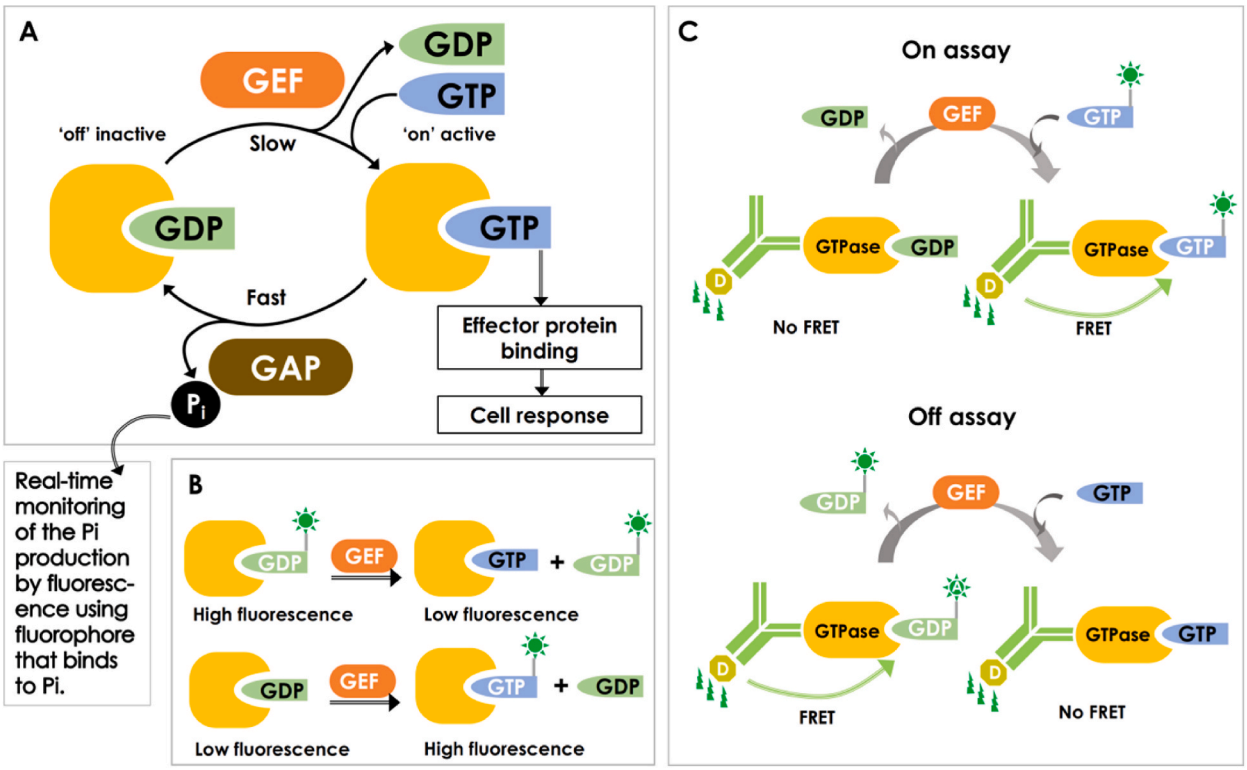

Fig. 4. A. An illustration of the GTPase activity cycle shows the interconversion between GDP-bound inactive and GTP-bound active forms, which are catalysed by GEFs and GAPs, respectively. B. Fluorescence-based and C. FRET-based nucleotide exchange assays measure the loading or unloading of labeling GDP and GTP, respectively. The antibody conjugated with donor fluorophore is mentioned as 'D' in Figure C.

3.1.3. β-arrestins recruitment assays

β-arrestins (1 and 2) are essential regulators of GPCRs. GPCR kinase-mediated phosphorylation of GPCR recruits β-arrestins, which eventually terminates G-protein signaling and leads to desensitization, inactivation, and internalization of GPCRs (Fig. 3D) [101]. There are chemiluminescence-based assay technologies such as PathHunter β-arrestin assay (DiscoverX) [102] and GPCR/β-arrestin Signaling Pathway LinkLight assay (BioInvenu) [103] available that measure β-arrestin recruitments. However, the Tango GPCR enabling model (Thermo Fisher Scientific) reporter gene assay is a fluorescence-based system that employs the release of non-native transcription factor (TF) linked to GPCR via a protease cleavage site. Recruitment of protease-tagged β-arrestin irreversibly cleaves and releases the TF to enter the nucleus for transcription. The TF directly regulates the transcription of a β-lactamase reporter construct, which catalyzes the turnover of an exogenous fluorescent substrate containing two fluoroprobes – coumarin and fluorescein. An HTRF-based total β-arrestin cellular kit (PerkinElmer) quantifies the expression level of β-arrestins [104]. Also, another HTRF-based β-arr2 recruitment kit (PerkinElmer) helps to characterize the β-arrestin recruitment by monitoring the interaction between the endogenous β-arrestin and adaptor protein AP-2, which plays a critical role in clathrin-mediated endocytosis [105].

3.1.4. Fluorescent ligand-based assay

Fluorescent-labeled ligands such as GPCR agonists or antagonists linked to fluorophore are employed in a range of fluorescence-based applications, including FP/FA and FRET, to characterize GPCR biology successfully, in particular, GPCR-ligand interactions [106–110]. GPCR-fluorescent ligands are advantageous over classical GPCR radioligands in HTS approaches for the development of novel therapeutics. However, care must be taken in designing the fluorescence ligand so that the attachment of fluorophore through a suitable linker should not affect the binding affinity and selectivity of the parent ligand [111]. Fluorescent dyes such as Cy5, ALEXA, BODIPY, TAMRA, Texas Red and Fluorescein (Thermo Fisher Scientific) linked to GPCR-binding peptides and small molecules are often used as fluorescent ligands in competitive binding experiments to identify novel small molecules [112–116]. More detailed applications of GPCR fluorescent ligands can be found in the published literature [117–119].

3.2. GTPase activity assay

Activated GPCRs act as an exchange factor to enhance the release of GDP and subsequent binding of GTP to G-proteins to initiate unique intracellular signaling responses [120]. Moreover, $G\alpha_{12/13,}$ $G\alpha_q$ and $G\beta\gamma$ subunits are known to directly interact with guanine nucleotide exchange factor (GEF) RhoGEF through which GPCRs activate the small GTPase Rho (Fig. 4) [121–123]. Small GTPases constitute a superfamily of enzymes that function as molecular switches and are grouped into five subfamilies, i.e., Ras, Rho, Rab, Ran and Arf, based on sequence homologies [124,125]. The interplay between GTP-bound 'on' and GDP-bound 'off' states of GTPases is regulated by GEFs and GTPase activating proteins (GAPs), respectively (Fig. 4A) [126,127]. Usually, the interaction between the GTP-bound GTPases with the downstream effector proteins regulates the cellular responses [128,129]. Activating mutations in Ras GTPases are found in 30%–60% of human malignancies and hence are one of the most sought-after targets for drug discovery [130–132]. Most commonly, the fluorescence-labeled GTP, GDP or their inactive analogues are used in HTS assay formats such as FP, FI, FRET and TR-FRET and are demonstrated to be valuable tools to measure the activity and discovery of bioactive molecules for modulating GTPases, GEFs, GAPs and downstream effectors [133–138]. MANT (n-methyl-3′-o-anthranoyl) and BODIPY are the widely used dyes in these assays. Still, there are other commercially available fluorophores as well (e.g., Jena Biosciences provide a wide range of fluorophore-labeled nucleotides). Interestingly, fluorescently labeled γ-phosphate-linked GTP analogues were shown to readily displace the native bound GDP with high intrinsic exchange rates in the presence of Mg^{2+}, accompanied by substantial fluorescence enhancements and thereby acting as a synthetic low-molecular-weight nucleotide exchange factor [139]. GEF-mediated GTPases activity is measured in two complementary fluorescence intensity-based assays: (i) measuring the loss of fluorescence intensity due to GEF-mediated deloading of fluorescently labeled GDP and (ii) measuring an increase in fluorescence due to GEF-mediated loading of labeled GTP into a binding pocket occupied by unlabeled GDP (Fig. 4B). Bell Brook Labs have developed Transcreener GDP assay kits that selectively immunodetect the GDP produced by GTPases during GTP hydrolysis (www.bellbrooklabs.com). The kit contains a far-red fluorescent labeled GDP as a tracer and an antibody selective to GDP. The GDP produced in the reaction competes with the tracer, changing the fluorescent properties and providing fluorescence readout. This assay is available in HTS-compatible FI, FP and TR-FRET formats and provides a platform to screen compound libraries [140–146]. Also, KRAS WT GTP binding kit (Cisbio) helps test the compounds competing with GTP-red reagent as a tracer binds to 6-His tagged human GTPase protein and an anti-6His cryptate-labeled antibody. Recently, a TR-FRET based assay was developed to find inhibitors targeting Ras GTPase activity [147]. The assay measures the loading of fluorescently labeled GTP analogue (FRET-acceptor) onto GST-tagged GTPase that binds anti-GST-terbium (FRET-donor), which results in an increased FRET signal (On-assay). Similarly, a secondary assay was developed to measure the unloading of labeling GDP from GST-tagged GTPase that binds anti-GST-terbium, which results in decreased FRET signal (off-assay) (Fig. 4C). These on- and off-assays complement each other to pick GTP-competitive hits and eliminate false positives at the same time. Some fluorescence-based methods permit real-time monitoring of GTPase activity by measuring of inorganic phosphates (Pi) released during GTP hydrolysis (Fig. 4A). These assays measure the increase in fluorescence intensity when Pi binds to either phosphate-binding protein modified with a fluorophore (e.g., MDCC-tagged protein from Thermo Fisher Scientific) or coupled with other enzymes resulting in the formation of a fluorescent product (e.g., resorufin in case of P_iPer™ Phosphate assay kit from Invitrogen). Interestingly, a new dual-parametric FRET-based technique enables the GEF-mediated loading of Eu^{3+}-labeled GTP monitored by emission at 650 nm and subsequent Eu^{3+}-labeled GTP-loaded RAS interaction with effector protein labeling with Alex680 monitored at 730 nm. This homogeneous dual-parametric assay enables separable detection of nucleotide exchange and GTPase-effector interaction inhibitors [148].

3.3. Protein-protein interactions (PPIs)

3.3.1. Phosphorylation-mediated interaction

In almost all cellular pathways, PPIs have been recognized as crucial regulators [149,150]. Targeted modulation of PPIs has enormous potential as therapeutic targets in drug development as that would enable selective interference with specific signaling pathways. However, probing PPIs with small-molecule inhibitors or stabilizers is often difficult due to large and shallow binding interfaces, because of which PPIs are classically recognized as "undruggable" [151]. Notably, despite the inherent difficulty in targeting PPIs, there have been efforts leading to the successful discovery of novel PPI modulators [152]. Posttranslational modifications (PTMs) are reversible processes that change the functional diversity of proteins via the covalent addition of functional groups such as phosphorylation, acetylation, glycosylation, ubiquitination, nitrosylation, methylation and lipidation [153]. Cellular signaling is frequently initiated by phosphorylation at amino acids serine, threonine, and tyrosine by protein kinases [154]. Further, these modifications are recognized by specialized protein modules such as 14-3-3, forkhead associated (FHA) and BRCA1 C-terminal (BRCT), effectively rewiring the downstream cellular responses [155]. Phosphorylation-dependent PPIs are vital events, and any defect in these interactions leads to abnormal phenotypes [156]. For example, nuclear protein BACH1 in its phosphorylated form directly interacts with the highly conserved C-terminal BRCT repeats of tumor suppressor protein BRCA1, and any mutation within the BRCT repeats disrupts the interaction, leading to defects in DNA repair, and is reported in breast and ovarian cancer [157]. Our group and others have a long-standing interest in targeting these phosphoprotein-binding modules for therapeutic intervention [158,159]. Protein kinases phosphorylate specific Ser/Thr/Tyr residues located in the conserved region commonly observed in the unstructured region of proteins [159]. The phosphorylated Ser/Thr/Tyr and the residues that span the site of phosphorylation, called "hot spot", confer the most binding energy and specificity for interaction with specialized protein modules such as 14-3-3, BRCT and FH [160–162]. Thus, these phosphorylation-mediated interactions in hot spots are targets for small-molecule and peptidomimetics modulators. A suitable synthetic fluorescent-labeled phosphopeptide (tracer) derived from substrate protein is crucial in successfully developing a high-throughput FP assay. A typical phosphopeptide is designed with the region spanning approximately −5 to +5 residues from the site of phosphorylation (pSer, pThr or pTyr) that acts as a hotspot region and binds with phosphopeptide-binding protein modules. The size and molecular weight of phosphopeptides change upon binding to protein modules and, thereby, are a factor in determining the dynamic range of FP assays [23]. Also, the choice of fluorophores can influence the dynamic range and quality of the assay (Table 1) [163]. Usually, dyes with red-shift spectra might help to avoid blue/green fluorescence interference from compound libraries. Based on understanding of the peptide interaction, fluorophore labeling of phosphopeptides can be done either at the N- or C- terminal ends.

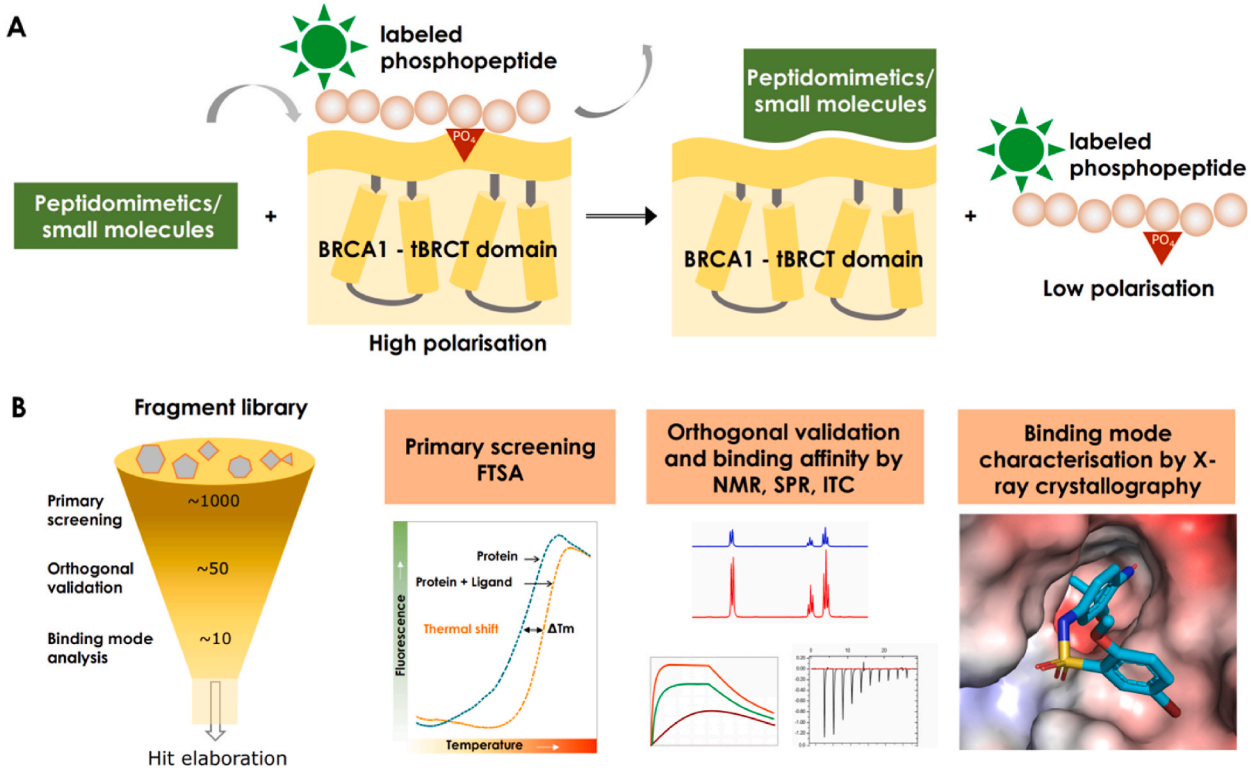

Fig. 5. A. An illustration of the FP-based HTS screening of peptidomimetics/small molecules towards the BRCA1 – tBRCT domain. B. A fragment screening pipeline with FTSA as a primary screening tool. 1D NMR, SPR and ITC are employed for orthogonal validation and the availability of co-crystal crystal reveals the binding mode fragments for Hit elaboration.

As a case example, we have identified bioactive inhibitors for BRCA1-tBRCT domain interaction with its cognate BACH1 phosphopeptide using an N-terminal TAMRA-labeled serine phosphorylated BACH1 peptide (12-mer spaning −6 to +5 pSer site; GGSRSTpSPTFNK) [158]. The non-labeled BACH1 phosphopeptide was used as a positive control that efficiently displaced the labeled phosphopeptide leading to a loss in FP values (Fig. 5A) [158]. A similar approach has been made in finding inhibitors targeting the 14-3-3 phosphobinding protein module [164,165]. In general, FP assays are a popular choice in HTS for targeting PPIs due to their ease of design [23,166–169].

3.3.2. Acetylation-dependent interaction

One of the predominant types of histone modifications is acetylation, which is catalysed by 'writers' histone acetyltransferases (HATs), cleared by 'erasers' histone deacetylases (HDACs) and recognized by 'readers' bromodomains [170]. Acetylation can locally disrupt the condensed state of the chromatin fiber, increasing the accessibility of DNA for protein factors [171]. These epigenetic regulators are involved in critical cellular processes such as nucleosome assembly, DNA damage repair and transcriptional regulation [172]. The aberrant levels and activities of histone acetyl modification proteins are correlated with several human diseases and therefore make them an important class of drug targets [170–173]. HATs transfer a negatively charged acetyl group from acetyl-CoA to the lysine residues at the ε-amino group on histone proteins generating free coenzyme A (CoA-SH). The familiar fluorometric-based assay measures the free CoA produced by the HAT on reacting with a sulfhydryl-scavenging probe, such as MMBC (LGC Biosearch Tech.) and CPM (Abcam, Thermo Fisher Scientific), to form a highly fluorescent CoA-MMBC/CPM adduct quantified by fluorescence intensity [174–176]. Both these probes are non-fluorescent until they react with thiols, giving a clear activity profile of target proteins. Similarly, there are simple two-step fluorometric assays available such as FLUOR DE LYS®HDAC (Enzo Life Sciences), Amplite® Fluorimetric HDAC activity assay kit (AAT Bioquest) and Calbiochem® HDAC assay kit (MilliporeSigma) that measure the removal of an acetyl group from lysines by HDACs. In addition, the TR-FRET assay developed by PerkinElmer LANCE Ultra epigenetic kit has been used in HTS discovery against HATs, HDACs and bromodomains [174,177,178]. Cayman Chemical and BPS Biosciences offer TR-FRET HTS bromodomain kits [179,180].

3.3.3. Fragment screening using FTSA to target acetylation reader protein

In the FBLD approach, fewer low-molecular-weight ligands (~150 Da) are screened against the target of interest. Although FBLD hits from initial screening have low binding potency, ranging from micromolar to millimolar concentrations, this approach offers a more effective and fruitful optimization campaign [181]. In general, FTSA has been employed alone or in combination with biophysical techniques such as ligand-based nuclear magnetic resonance (STD and WaterLOGSY NMR), surface plasmon resonance (SPR) and MST for fragment screening and hit identification (Fig. 5B) [182]. Protein-based NMR technique HSQC and isothermal titration calorimetry (ITC) are used for orthogonal validation of hits. Finally, the binding mode of fragments is resolved by a high-resolution crystallographic structure providing a fruitful way forward for fragments to be developed into a potent chemical molecule (Fig. 5B) [183].

In an interesting study, FTSA was used to discover inhibitors for bromodomain-1 of BRD4, in which 3695 fragments were screened using 4-μM BRD4, 6X SYPRO Orange dye and 100 μM of fragments at 1% DMSO. Compounds showing positive (48 fragments) and negative (31 fragments) thermal shifts were considered as initial hits, of which 73 fragments were reconfirmed at higher fragment concentrations. Based on the primary hits, 3200 new compounds were synthesized as "Active" and "Random" sets. Similarly, a "Non-active" set of 3432 compounds was generated from non-hit fragments. Re-screening of the new molecules resulted in 1.59%, 0.81%, and 0.97% hit rates for Active, Random and Non-active sets, respectively. Intriguingly, most of the Active-set hits showed positive thermal shifts. Totally, 108 hits from all sets were re-tested using FTSA for BRD4 protein at 10, 20, and 40 μM concentrations yielding 61 validated hits, which were further tested using TR-FRET assay. Of these, 18 compounds showed at least 50% inhibition at 20 μM and 11 were from the Active set. At least one molecule from each set had IC50 in the low single-digit micromolar range [50]. Another report used FTSA in parallel with SPR and MST assays, and a library of about 1700 compounds was screened against the bromodomain of BRD9. FTSA screening was performed with 10-μM protein, 25x SYPRO Orange, 400 μM fragments with 2% DMSO, and $\Delta Tm \geq 1\ ^{\circ}C$ was considered a hit. MST screening resulted in 124 hits compared to 36 from FTSA and 45 from SPR. About 10% overlap was observed between SPR and FTSA hits; however, the overlap was very low for MST hits with SPR and FTSA hits. The hits identified using FTSA were more consistent in HSQC orthogonal validation. Out of 77 hits reconfirmed by 2D $^{1}H–^{15}N$ HSQC NMR spectra, 34 out of 36 primary hits were from FTSA screen, 38 out of 45 primary hits were from SPR, and 38 out of 124 primary hits were from MST and 7 common hits from all the three methods. Later, X-ray structures were resolved for 55 fragments by crystal soaking, yielding a fruitful fragment optimization strategy [184]. In another successful example, FTSA-based screening was applied to discover CBP/EP300 bromodomain inhibitors from 2000 fragments. The screening was performed with 4 μM of protein, 5X SYPRO Orange dye and 800 μM fragments with 0.8% DMSO, and $\Delta Tm \geq 1\ ^{\circ}C$ was considered for hit selection. The hits re-confirmed using TR-FRET assay were further validated with $^{1}H–^{15}N$ HSQC NMR, ITC and X-ray crystallography [185]. There are other reports as well that successfully employed FTSA-based fragment screening for the identification of bromodomain inhibitors [186–188]. Additionally, FTSA has been utilized to validate the hits identified from computational virtual screening approaches [189,190]. In the study, high throughput docking and MD simulations resulted in 24 virtual hit compounds for bromodomain1 of BRD4. When tested in FTSA with 2 μM of protein, 5X SYPRO Orange dye, 50 μM of compounds with 0.4% DMSO, 4 compounds showed $\Delta Tm \geq 1\ ^{\circ}C$ [191]. Another interesting application of FTSA is in the evaluation of the selectivity of the ligand towards a panel of bromodomain-containing proteins referred to as BROMOscan, wherein a specific ligand is tested using FTSA for binding to multiple bromodomain proteins and the selectivity profiling is determined from Tm shifts [192,193]. FTSA has been actively employed in FBLD for other protein targets such as GTPases, GPCRs and PPIs as well [147,194–197].

4. Challenges and future perspectives

Fluorescence-based techniques such as FI, FP/FA, FRET and TR-FRET are routinely used to measure the binding kinetics of fluorescently labeling ligands and the competitive binding of unlabeled ligands. They have gained usage in the last decade with the advancement in synthetic chemistry of fluorophore dyes, plate readers and ready-to-use assay kits. A search for high throughput screens using fluorescence-based methods on Google Scholar returns over 87,200 hits since 2012. Significantly, FP and TR-FRET are vastly preferred as primary assays to identify hits from large chemical libraries. These also have immense value as an orthogonal assay to validate identified hits, helping to measure binding affinity and inhibition constant to further optimise their potency and selectivity. However, fluorescent compounds and fluorescent impurities in screening libraries can interfere with fluorescence assay readouts [198]. A red-shifted dye labeling fluorescence ligand combined with TR-FRET assay provides the best solution to overcome compound interference arising from auto-fluorescence signal and other short-lived fluorescence sources. Commercially available TR-FRET assays have continued to evolve over the years for important drug target classes. For example, a fluorescently labeled substrate and an antibody selective to the substrate, cryptate-labeled anti-His/anti-GST antibody for capturing His-tag/GST-tag of molecular target binding to fluorescently labeled substrate/ligand, and direct covalent fluorescent labeling of target proteins offer robust development of TR-FRET assays for a vast range of molecular targets.

Many therapeutically attractive protein targets have not been pursued or there have been few attempts to discover small molecules against them due to large and featureless interacting surfaces. In recent years, fragment-based screening has proven to be a precious tool in identifying small molecules against “undruggable” targets such as PPIs. FTSA has been widely implicated in fragment-based screening due to the ease of its implementation in a high-throughput format. The fragments produce poorly defined thermal unfolding curves and have fluorescence properties that are not amenable to FTSA. Fragment binding can result in both positive and negative thermal shifts. In general, only the fragments that produce positive thermal shifts are pursued for optimization, while those that result in negative thermal shifts are excluded. However, the negative thermal shift fragments were also demonstrated to have potential values [199,200]. Therefore, evaluating both positive and negative thermal shift hits in orthogonal validation methods like NMR, SPR, and MST is worthwhile. During the fragment optimization campaigns, fluorescence-based assays that measure the inhibitory effect of optimized fragments are preferred.

Only a small fraction of functionally significant protein targets encoded in the human genome is accessible to modulation using small-molecule chemical tools, impeding chemical biology and the development of new therapeutics [201–203]. Multiple assessment aspects such as genetic validation, assayability, druggability and safety issues have to be considered to expand the portfolio of targets amenable to chemical modulation. In such a scenario, having a wide repertoire of biochemical and biophysical HTS assays becomes imperative to assess novel biological targets. To this end, the fluorescence-based assays have huge translational potential both in academia and biopharma industries, exploring fundamental biomolecular functions and developing potent and safe chemical probes against novel targets that are different from traditional drug targets.

5. Conclusion

An overview of principles, advantages, and limitations of various fluorescence-based high-throughput techniques such as FP, FRET, TR-FRET, FLT, PIFE, FTSA and MST have been discussed here. These techniques have gained widespread applications in early drug discovery processes due to their sensitivity, robustness, and availability for high-throughput formats. Recent advances in fluorescence-based assays for important drug target classes such as GPCR and GTPases, as well as phosphorylation- and acetylation-mediated PPIs are reviewed, focusing on the commercially available high-throughput assay kits. The recent development in these techniques has facilitated extending their usage for characterization and screening activities against novel targets for developing therapeutics.

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CRediT authorship contribution statement

Vikrant Kumar: Data curation, Writing – original draft, Writing – review & editing. **Puneeth Kumar Chunchagatta Lakshman:** Data curation, Writing – original draft. **Thazhe Kootteri Prasad:** Data curation, Writing – original draft, Writing – review & editing. **Kavyashree Manjunath:** Data curation. **Sneha Bairy:** Data curation. **Akshaya S. Vasu:** Data curation. **B. Ganavi:** Data curation. **Subbarao Jasti:** Data curation. **Neelagandan Kamariah:** Conceptualization, Data curation, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Abbreviations

ANS	8-anilino-1-naphthalenesulfonic acid
BODIPY	Boron-dipyrromethene
BRCA1	BReast CAncer gene 1
BRCT	BRCA1 C-terminal
cAMP	Cyclic adenosine monophosphate
CETSA	Cellular thermal shift assay
CoA-SH	Free coenzyme A
CPM	N-[4-(7-diethylamino-4-methylcoumarin-3- yl)phenyl]maleimide
Cy3	Cyanine dye 3
Cy5	Cyanine dye 5
DAG	Diacylglycerol
DMSO	Dimethyl sulfoxide
FBLD	Fragment-based ligand discovery
FA	Fluorescence anisotropy
FHA	Forkhead associated domain
FI	Fluorescence intensity
FLT	Fluorescence lifetime
FP	Fluorescence polarization
FRET	Förster resonance energy transfer
FTSA	Fluorescence thermal shift assay
Gαq	Gq alpha subunit
GAP	GTPase activating proteins
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factors
GPCR	G protein-coupled receptors
GTP	Guanosine-5′-triphosphate
HAT	Histone acetyltransferases
HDAC	Histone deacetylases
HSQC	Heteronuclear single quantum coherence spectroscopy
HTS	High-throughput screening
IP1	Inositol monophosphate
IP3	Inositol trisphosphate
ITC	Isothermal titration calorimetry
MANT	n-methyl-3′-o-anthranoyl
MMBC	Methyl 10-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-9-methoxy-3-oxo-3H-benzo[f]chromene-2-carboxylate
mP	Milli polarization
MST	Microscale thermophoresis
ns	Nanosecond
PIFE	Protein-induced fluorescence enhancement
pSer	Phosphoserine
pThr	Phosphothreonine
pTyr	Phosphotyrosine
PPI	Protein-protein Interactions
PTM	Posttranslational modification
RhoA	Ras homolog family member A
ROX	Carboxy-X-rhodamine
TF	Transcription factor
Tm	Melting temperature
TR-FRET	Time-resolved Förster energy transfer
SPR	Surface plasmon resonance

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