REVIEW

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High-throughput screening assays for the identification of chemical probes

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High-throughput screening (HTS) assays enable the testing of large numbers of chemical substances for activity in diverse areas of biology. The biological responses measured in HTS assays span isolated biochemical systems containing purified receptors or enzymes to signal transduction pathways and complex networks functioning in cellular environments. This Review addresses factors that need to be considered when implementing assays for HTS and is aimed particularly at investigators new to this field. We discuss assay design strategies, the major detection technologies and examples of HTS assays for common target classes, cellular pathways and simple cellular phenotypes. We conclude with special considerations for configuring sensitive, robust, informative and economically feasible HTS assays.

A variety of strategies (**Fig. 1**) have been used for HTS assays, including the measurement of catalytic activity from a purified enzyme[1], a reconstituted complex[2,3], a cellular extract[4,5] or a phenotype[6–9] in intact cells. Configuring assays to function within the constraints imposed by HTS differentiates an HTS assay from traditional laboratory assays, as outlined in **Table 1**. The 96-well microtiter plate, originally designed to facilitate serological studies in virology[10], was later recognized as a suitable replacement for cuvette, tube and dish-based assays. Combined with spectrophotometric plate readers, the microtiter plate quickly became the standard for performing parallel assays for rapid preliminary evaluation of compound bioactivity. During the 1990s, the tremendous increase in chemical libraries and assays with sensitivities amenable to miniaturization further drove the parallel processing concept that today is practiced in the 96-, 384- or 1,536-microwell plate formats, among others. 'High throughput' is a relative term, but it is generally defined as the testing of 10,000 to 100,000 compounds per day, accomplished with mechanization that ranges from manually operated workstations to fully automated robotic systems[11,12]. Assays designed for the purpose of HTS attempt to integrate biological fidelity with enabling assay and screening technologies. Subsequently, understanding the pharmacological impact of the assay design is critical to the identification of biologically relevant compounds from HTS.

Assay design for HTS

An effective HTS strategy considers both the primary and subsequent secondary assay designs carefully. First, is the nature of the response to be measured clearly defined: does the signal increase, decrease, change in nature or location or belong to part of a more complex response? Can the response be measured via a single parameter, or is a multiparametric output possible? For example parameters that provide counter-screen information can guide active compound selection by differentiating selectivity among related targets[13], or by distinguishing the inhibition of a cellular pathway from a cytotoxic response in a cell-based assay[14–16]. Second, is the stimulus dependent only on the compound being tested, or is the response to the compound conditioned by another stimulus, such as a fully efficacious concentration of agonist (when screening for an antagonist), a subefficacious concentration of agonist (when screening for a potentiator) or a permissive temperature? In conditional assays, compound activities independent of the conditional stimulus can be false positives or can act outside the pathway of interest (**Fig. 1d**). Third, does the duration of the response occur within seconds upon application of the stimulus, thereby requiring a rapid read (for example, a calcium transient), or within minutes or hours of the stimulus, thereby permitting endpoint or multiple time point measurements? These parameters will define the basic format of the primary HTS.

Follow-up assays, also referred to as 'secondary screens' or 'counter screens', should be planned before the HTS. It is essential to view the primary HTS as the initial step of an integrated process. Follow-up tests validate compounds targeting the intended biological interaction and assist in the elimination of compounds that generate a positive signal via other mechanisms. False positives can be anticipated based on the assay design (for example, fluorescent compounds in an assay with a fluorescent readout) or eliminated by testing in an independent or 'orthogonal' assay that uses a different methodology or biological readout of target activity. As an example, cyclic AMP (cAMP), an important cellular second messenger, can be measured with an output response signal either directly or indirectly proportional to cellular [cAMP] (**Fig. 2**). cAMP assays showing inversely proportional responses to changes in [cAMP] in principle can function in an HTS-confirmatory assay sequence in which selected candidates identified in the HTS are then tested for initial validation of activity. Such 'preliminary confirmation' strategies using HTS assays in sequence (or parallel) can greatly lower the retesting of false positives in substantially lower throughput secondary assays that are generally more complex and designed to be a more physiological measure of the biological system under study[17].

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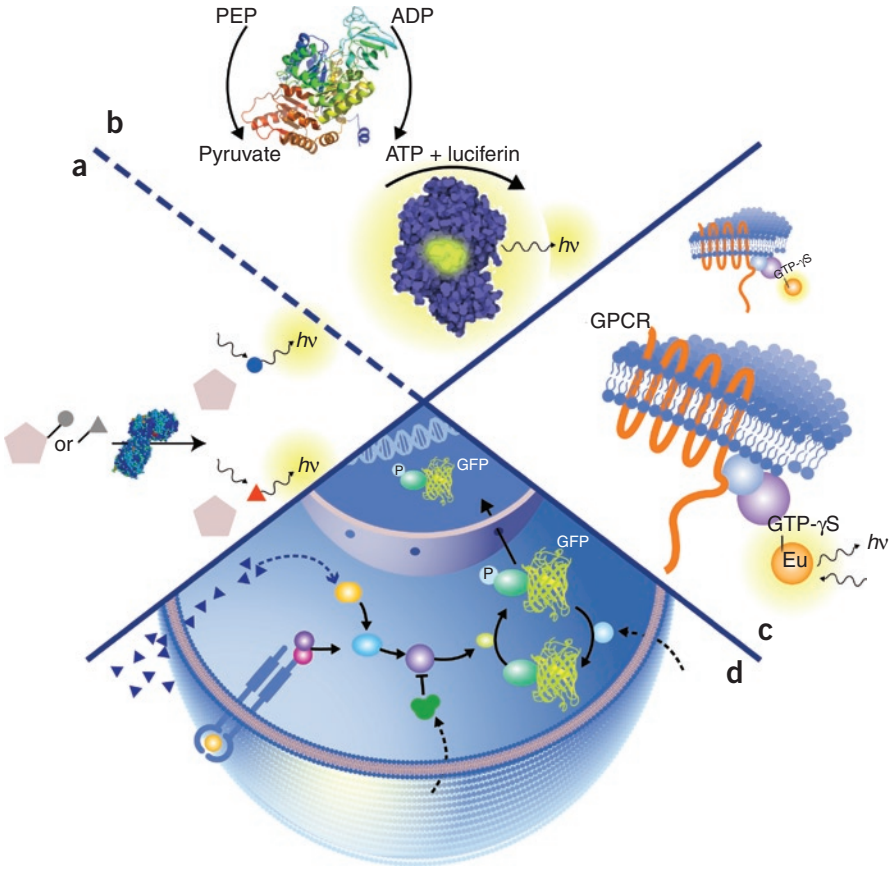

Figure 1 Assay categories and methodologies. (**a**) An isolated enzyme catalyzes turnover of a profluorescent blue- or red-emitting substrate. (**b**) A coupled-enzyme assay in which the ATP product, generated by the pyruvate kinase–catalyzed reaction (ribbon structure), is detected by a "reporter" enzyme (firefly luciferase) to create a luminescent output (*hν*). (**c**) A membrane preparation is used to measure the activity of a GPCR through the ligand-dependent activation of associated G proteins as measured by the binding of a fluorescent europium (Eu^{3+})-labeled GTP-γS analog. (**d**) A phenotypic assay designed to probe a signaling pathway dependent on coactivation of an extracellular receptor (blue) and an intracellular protein (yellow). The intracellular protein is activated by a pharmacological agent (▲, blue dashed arrow) as a prerequisite to revealing potentiators (compounds that are active only in the presence of the costimulus) of the pathway, as measured by the nuclear translocation of a GFP-labeled protein. Such potentiators could act by inhibiting proteins that repress the pathway (black dashed arrows).

Regardless of the approach taken, the HTS assay protocol must be simplified from its original form (**Table 1**), and the following parameters must be optimized: (i) sufficient sensitivity to enable the identification of compounds with low potency or efficacy. (ii) Reproducibility and stability of the biological response between wells and plates. This parameter is dependent on both assay reagents and hardware (such as dispensers, incubators and detectors). (iii) Accuracy of the positive and negative controls as compared with the known pharmacology of the target. (iv) Economic feasibility (frequently measured as cost per well).

Validation of an assay protocol determines its suitability for HTS and involves two parts: performance and sensitivity[18] (**Fig. 3**). Several statistical parameters are used to evaluate assay performance. Signal window and *Z* factor (**Table 2**) are calculations that gauge the fold response between maximum and minimum output signals and the precision of this response within a plate and across plates. The signal window measures the statistically significant difference between the maximum and minimum signal, but it is not as reliable as *Z* factor for predicting assay performance[18]. The *Z* factor's unitless scale (ranging from 0 to 1) allows comparison of different assays and screens using the control wells (*Z*′) and sample wells (*Z*) of the plate. Comparison of *Z*′ and *Z* in assay validation has been used to determine compound screening concentration, as assays in which $Z' > Z$ predict percent of actives greater than the <1% target value for single concentration screening paradigms. If compounds are absent from the sample field (for example, DMSO only), a low *Z* score may be diagnostic of a faulty reagent dispense or contamination problem in the sample wells, for example.

An equally significant component of assay validation is assay sensitivity—the accuracy and reproducibility of potency measurements of control compounds or conditions. The minimum significance ratio (MSR, **Table 2**) is used to measure the reproducibility of potency values and defines the statistically significant potency range that can be measured in an assay[19,20]. During HTS, the MSR can be calculated from titrations of control compounds on some or all assay plates in the screen to track assay sensitivity variation, which is often an indicator of reagent stability.

Detection of biological responses in HTS

The photon, which is created by either a fluorescent or luminescent mechanism, is the principal output in HTS assays. As the most widely used HTS detection modality (**Table 3**; see **Supplementary Table 1** online for additional detail), fluorescent energy is highly tunable, which allows the development of a variety of fluorescent sensors[21]. Fluorescence can be bright and occur on a timescale ranging from 10^{-9} s (for typical organic fluorophores) to 10^{-4} s (for lanthanide cryptates), thereby allowing for light's many optical properties to be exploited by a number of detection methods[22]. For example, the application of plane-polarized light in fluorescence polarization (FP) can be used to measure a probe's rotational perturbations, thereby enabling simple assay designs dependent on a single labeled ligand (ref. 23, and ref. 24 and other articles in the same issue). Alternatively, time-resolvable fluorescent trivalent lanthanides such as Eu^{3+} and Tb^{3+} can be used to increase assay sensitivity by suppressing background fluorescence from assay components and library compounds[25,26]. Further, fluorescent probes can transfer energy to other chromophores or fluorophores in assays that measure distance-dependent fluorescence or fluorescence quenching via resonance energy transfer (FRET or QFRET) mechanisms[25–27].

For cellular assays in particular, chemical[28,29] and genetically encoded[27] fluorescent probes allow customized, multiplexed or direct measurement of events from biological components present at low concentrations[30]. Coupled with scanning microplate cytometers[31] or microscope-based imaging systems, these probes are moving HTS from population-averaged outputs toward the enumeration of individual cellular events[8,9], thereby enhancing the information content obtainable from each well.

Table 1 Differences in allowed parameters between laboratory "bench top" and HTS assays

Parameter	Bench top	HTS
Protocol	May be complex with numerous steps, aspirations, washes	Few (5–10) steps, simple operations, addition only preferred
Assay volume	0.1 ml to 1 ml	<1 μl[a] to 100 μl
Reagents	Quantity often limited, batch variation acceptable, may be unstable	Sufficient quantity, single batch, must be stable over prolonged period
Reagent handling	Manual	Robotic
Variables	Many—for example, time, substrate/ligand concentration, compound, cell type	Compound[b], compound concentration
Assay container	Varied—tube, slide, microtiter plate, Petri dish, cuvette, animal	Microtiter plate
Time of measurement	Milliseconds to months	Minutes to hours
	Measurements as endpoint, multiple time points, or continuous	Measurements typically endpoint, but also pre-read and kinetic
Output formats	Plate reader, radioactivity, size separation, object enumeration, images interpreted by human visual inspection	Plate reader—mostly fluorescence, luminescence and absorbance
Reporting format	"Representative" data; statistical analysis of manually curated dataset	Automated analysis of all data using statistical criteria

[a]Special reagent dispensers required. [b]Ideally available in milligram quantity with analytical verification of structure and purity.

The absence of excitation light energy in the generation of a luminescence signal greatly reduces compound interference in HTS (**Fig. 4**). Although the signal strength can be significantly lower than fluorescence, the background is negligible, which allows luminescence-based assays to have an enormous dynamic range, on the order of 1,000 times more sensitive than equivalent fluorescence-based assays[32]. Both chemiluminescence and bioluminescence use a chemical reaction to create light. In the case of bioluminescence, an enzyme (typically from the firefly *Photinus pyrali*) generates light through its action on a luciferin substrate, analogs of which have been used to develop assays for cytochrome P450s, proteases and monoamine oxidases[32]. Further, the active site of the *P. pyrali* luciferase has been mutated to spectrally vary the luminescence[33], thereby allowing the use of multiple luminescent sensors in an assay[15].

Luciferase reporters are prevalent in HTS assays because of convenient detection, ample sensitivity and their ubiquitous occurrence in academic research. Dual luciferase reporter assays with distinct substrate specificities, kinetics or emission maxima have been useful for identifying activities specific to the signaling pathway of interest. Frequently, the second reporter is used to normalize gene expression or discriminate cytotoxic compounds[34,35]. Luciferase reporters can be combined with other detection formats as well, for instance in tandem with a green fluorescent protein (GFP) reporter[14], β-galactosidase or Alamar blue[16] to assess cytotoxicity.

The β-lactamase reporter offers particular advantages for HTS. For example, the β-lactam–coupled coumarin-fluorescein substrate, CCF4/AM, allows a ratiometric read that helps minimize variation in cell number or substrate concentration because the emission maxima of the cleaved and intact β-lactamase substrates (460 and 530 nm, respectively) are distinct (reviewed in ref. 36). In addition, the uncleaved substrate can be used to assess cytotoxicity, as fluorescence is detected only if the substrate is taken up by living cells and de-esterified[37,38].

A hybrid system involving both bioluminescence and fluorescence is represented by BRET-based assays[39] (**Table 3** and **Supplementary Table 1**). Here luciferase luminescence stimulates a proximal fluorescent protein emission, thereby bypassing the need for exogenous excitation light. However, this technique may be more challenging to optimize for HTS assays because of lower S:B ratios and higher background from weakly associated target proteins[40].

In addition, radioactive decay in conjunction with the scintillation proximity assay (SPA) format is applicable to a host of targets[41], and low-cost absorbance assays have proven reliable in measuring analytes (for example, H^+ or P_i), cellular staining or cell growth. However, in microtiter plates, absorbance measurements can have low S:B ratios owing to the short path length that results from low volumes, though several assays have been implemented in 1,536-well plates[42,43].

Variations on the use of these technologies in the design of assays are impressive, and a discussion of each is beyond the scope of this paper. Assays suitable for HTS comprise a subset of these and must meet the requirements outlined in **Table 1**. The next section highlights the well-exploited assay formats.

Assays for specific targets

Historically, screening assays have been performed on a limited set of targets, many involving purified proteins. Approximately two thirds of therapeutic targets are comprised of enzymes and receptors[44]. More recently, HTS labs are increasing attention on cell-based high-content screens (HCS) that analyze biological events at subcellular resolution[8,9]; a 2006 survey indicated that roughly half of HTS assays are cell-based and that the use of HCS is rapidly growing, particularly in secondary screening[45]. The types of proteins for which drugs have been successfully developed are relatively restricted. About half of experimental and marketed drugs target five main protein families: G protein–coupled receptors (GPCRs), kinases, proteases, nuclear receptors (NRs) and ion channels[46]. Because of the great interest in these protein classes for drug development, many approaches and technologies (**Table 3** and **Supplementary Table 1**) have been developed to assay these targets, and in some cases they are broadly applicable to other target classes as well.

Enzymes. Enzymes are comprised of six categories: oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases. They catalyze a large number of chemical transformations. In cases in which the enzyme, cofactor and substrate can be obtained in an isolated, active form, the development of an HTS assay generally hinges on the availability of reagents and technologies to enable substrate or product detection. Here, protein kinases and proteases will serve to illustrate various approaches to HTS assay design for enzymes.

Protein kinases. Protein kinases mediate the most prevalent post-translational modification in cells, phosphorylate an array of targets in signaling cascades and cycles, and are important for therapeutic intervention, particularly oncology[47]. In this section we discuss biochemical approaches to protein kinase assays, as cell-based assays are covered in the later sections on cellular signaling. The biochemical approaches can be divided into two categories: generic assays independent of subfamily (for example, tyrosine versus serine/threonine) and antibody-based formats that detect an epitope within the phosphorylated product. The latter are generally used for a particular subfamily or specific protein kinase.

Protein kinases catalyze phosphoryl group transfers from ATP to hydroxyl groups of amino acid side chains of serine, threonine or tyrosine. An easily implemented assay format that has been used successfully in HTS detects ATP depletion via luciferase, an ATP-dependent photoenzyme[48,49]. A disadvantage of this approach is that 50% conversion of the ATP is required to achieve a two-fold S:B. This may require increased enzyme levels for sufficient signal, thus decreasing sensitivity and increasing reagent costs. Alternatively, kinase activity can be measured through ADP production. One assay format uses an ADP-specific antibody and labeled ADP to generate an FP competition binding assay to monitor ADP product formation[50]. A coupled enzyme strategy for ADP detection that yields a fluorescent readout has also been described[51].

A number of methods measure kinase activity by the detection of phosphorylated peptide. Several formats use micron-sized beads or particles to partition the product phosphopeptide from ATP substrate or peptide. Radiometric filter binding remains the standard for kinase selectivity studies. For HTS, biotinylated ^{33}P-phosphopeptide is typically captured by streptavidin-coated SPA beads[41] or scintillant-treated microtiter plates (for example, FlashPlates). In immobilized metal-ion affinity-based fluorescence polarization (IMAP), transition metal-chelated particles bind fluorescently labeled phosphopeptides with high affinity, thereby enabling an antibody-independent FP format for the detection of phosphopeptide products[52]. Recently IMAP has been adapted to FRET-based systems in which size limitations on the polypeptide substrate (typically <10,000 MW) are relaxed[53]. A similar system has been developed based on fluorescence quenching through an iron-phosphate interaction[54]. However, for HTS the ratiometric nature of IMAP is an advantage of this system over fluorescence quenching.

Antibody-based formats that detect a specific phosphorylated peptide or sequence capitalize on the many phosphospecific antibodies available and on nonseparation technologies, including TR-FRET, ALPHA and FP. Such assays are advantageous in targeting kinase activity dependent on, for example, hierarchical phosphorylation or kinase cascades[55]. Also, multiplexed readouts of kinase activity and cellular phosphoproteins are possible with these formats[56].

A unique system that assays the kinase inactive state relies on ATP binding and uses enzyme fragment complementation (EFC) for detection[57]. Here the general kinase inhibitor, staurosporine, is linked to a enzyme donor (ED) fragment of β-galactosidase that when displaced from the kinase ATP pocket restores β-galactosidase activity through α complementation with the acceptor enzyme (EA) component[58].

A final general point, illustrated with kinases, relates to assay designs that recapitulate as closely as possible the native target to uncover new mechanisms of compound action. As most biochemical kinase assays have used only the catalytic domain for HTS, ATP site–directed inhibitors are the primary class of agents identified. However, with a TR-FRET–based HTS of Akt-family kinases, Barnett *et al.*[59] discovered a pharmacologically unique class of compounds using the full-length kinases. These compounds stabilized the inactive conformation of Akt1 and Akt2 via a site formed only in the presence of the pleckstrin homology (PH) domain of the kinase[59], thereby allowing a unique mode of selectivity between Akt1 and Akt2 versus Akt3, which is not inhibited by the chemical class owing to its lack of a PH domain.

Proteases. As well-established drug targets[60], proteases have received considerable coverage in terms of assay formats and reagent kits. Profluorescent or FRET-based substrates are commonly used to assay proteases as purified proteins or in cell lysates. Typically, probes are coupled to a two-to-five-amino-acid peptide comprising a protease cleavage site and become fluorescent after liberation by protease cleavage[13,61]. Though ideally suited to HTS, a shortcoming of this approach is that the fluorogenic peptide is often insufficient in length to cover the entire binding pocket.

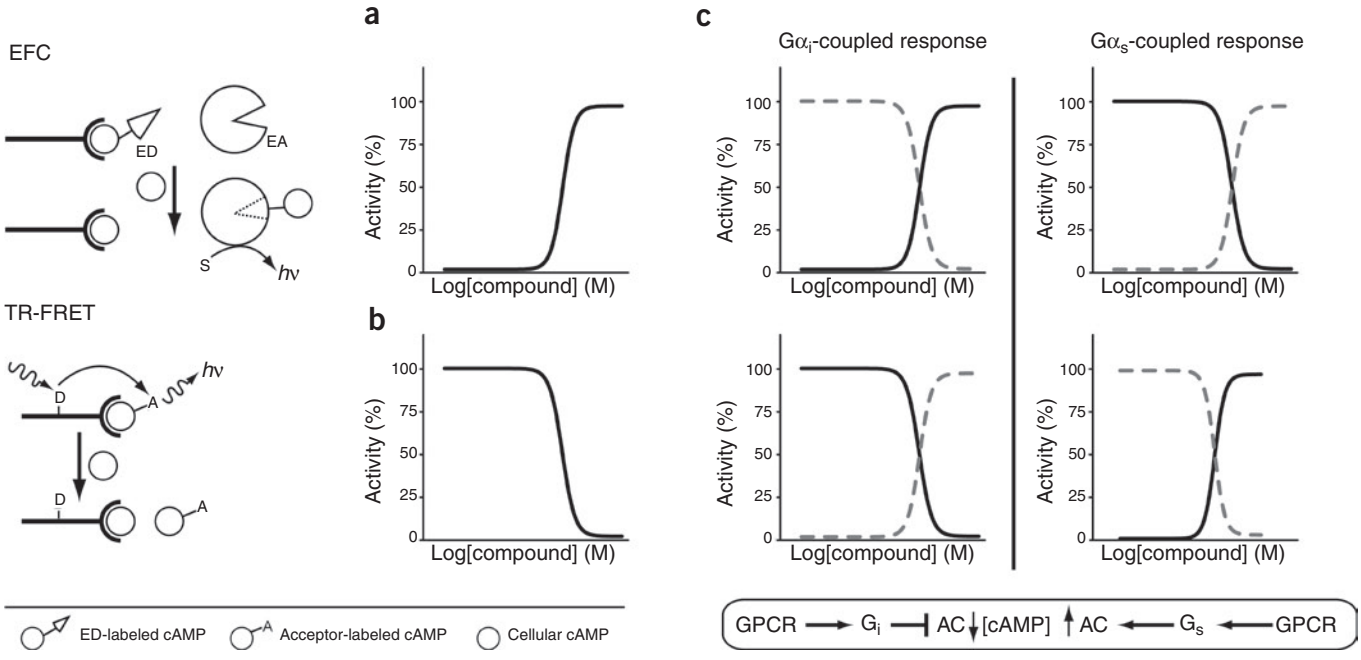

Figure 2 Influence of assay design on the measured biological response. (**a**) An EFC-based competitive immunoassay illustrates an output signal that is directly proportional to intracellular [cAMP]. (**b**) A TR-FRET output signal is indirectly proportional to intracellular [cAMP]. (**c**) The resulting responses for hypothetical assay of G_i-coupled (left) and G_s-coupled (right) GPCRs are shown for the EFC (top) and TR-FRET assays (bottom) for compounds acting as either agonists (dotted lines) or antagonists (solid lines). Assays having opposing signal outputs in response to the same analyte; here [cAMP] is an example of an assay pair in which one could function in HTS and the other in follow-up confirmation and false positive detection.

Table 2 Equations for determining assay performance or sensitivity

Parameter	Equation	Comment
Coefficient of variation	$\%CV = \frac{\sigma}{\mu} \times 100$	A measure of the precision relative to the mean value, calculated for the maximum and minimum signals. An acceptable limit is <15%.
Signal to noise	$S{:}N = \frac{\mu_{max} - \mu_{min}}{\sigma_{min}}$	A measure of the signal strength. This equation is sometimes given as[a]: $\frac{\mu_{max} - \mu_{min}}{\sqrt{\sigma_{max}^2 + \sigma_{min}^2}}$
Signal to background	$S{:}B = \frac{\mu_{max}}{\mu_{min}}$	Usually calculated using control compounds, acceptable value >2-fold.
Signal window	$SW = \frac{\mu_{max} - \mu_{min} - 3(\sigma_{max} + \sigma_{min})}{\sigma_{max}}$	The significant signal between max and min controls, acceptable value >2-fold[b].
Z' factor	$Z' = 1 - \frac{(3\sigma_{max} + 3\sigma_{min})}{\lvert\mu_{max} - \mu_{min}\rvert}$	Representation of the SW using a score where a value >0.5 represents an acceptable assay. Z' is measured in the absence of library compounds; Z is in the presence.
Minimum significance ratio	$MSR = 10^{2\sigma_d}$	Smallest potency ratio between two measurements that is statistically significant (with 95% confidence).
Cheng-Prusoff	$K_i = \frac{IC_{50}}{(1 + [S]/K_m)}$	Used to derive the K_i from the IC_{50} under conditions of competitive inhibition.
Cheng-Prusoff (ligand-binding)	$K_i = \frac{IC_{50}}{(1 + [L]/K_d)}$	Used to derive the K_i from the IC_{50} under conditions of competitive binding.

σ, s.d. of the assay signal; μ, mean of the assay signal; σ_d, s.d. of the difference in log potency; max, maximum signal; min, minimum signal. [a]This is a redundant description of the signal window equation. [b]Some assays, such as those with ratiometric data, may perform adequately with as little as a 20% change in signal (for example, see ref. 111) or with $0 < Z' < 0.5$.

Although there are numerous ways to measure endoprotease activity, assays for exoproteases that recognize C- or N-terminal residues are far less available[62]. The hydrolytic activity of the protease has been used to process simple chromogenic esters and amides, but assays based on these substrates generally lack sensitivity for miniaturized HTS assays. One approach is to use protease-mediated "epitope unmasking," in which cleavage by the exoprotease reveals a binding site for a detection reagent. Here, sensitive time-resolved FRET (TR-FRET)-based formats, for example, can be applied[63].

Cellular FRET-based methods have been developed using fluorescent proteins or dyes linked by a protease consensus sequence. When expressed or taken up by cells, cleavage of the probe can be measured by a change in fluorescence upon activation of proteases such as caspase-3 (ref. 64) and hepatitis virus (HCV) NS2/3 (ref. 65). Similarly, cells expressing a fusion of GFP and alkaline phosphatase coupled through an NS3/4A cleavage site were used to monitor the activity of this HCV protease by the detection of secreted alkaline phosphatase[66]. In an approach based on cell viability, procaspase-3 was modified to contain a β-secretase cleavage motif that triggered apoptosis in 293T cells upon expression of β-secretase. β-secretase inhibitors were shown in this assay to promote cell survival[67].

Nuclear receptors. NRs are transcription factors that are characterized by common modular features including a DNA-binding domain (DBD) and a ligand-binding domain (LBD). NRs are regulated by endobiotic ligands such as hormones and metabolites, or through xenobiotics[68]. The human genome encodes ~48 NR members of known and unknown function (orphan NRs) that are actively being studied in drug discovery and basic research. Consequently, the ligand or DNA-binding properties of isolated domains, and the cellular translocation and gene transactivation properties of NRs, have been featured in assays designed for HTS (ref. 69 and other articles in the same focus issue).

Classical competition binding assays for NRs can be divided into radiometric and fluorometric assays. SPA formats have been developed for NRs such as peroxisome proliferator-activated receptor[70], and FP has been used for well-characterized receptors such as the steroidal NRs, in which a fluorescent conjugate of a known receptor ligand and a purified LBD are prepared[71].

Using GFP fusions of NRs[8], cell imaging assays have been described that measure ligand-dependent, cytoplasm-to-nucleus translocation of the glucocorticoid receptor (GR)[72] or engineered NR chimeras[73]. Translocation assays using GFP fusions of GR have been applied to 1,536-well systems (see ref. 8; PubChem BioAssay ID (AID) 450). Additionally, EFC assay formats have been described to measure NR translocation[72,74] (PubChem AID 451). However, none of the aforementioned assays can distinguish the functional effect of active compounds on NR transactivation.

To distinguish between agonist and antagonistic activity of compounds, the targeting of NRs has been addressed with TR-FRET ligand-dependent coregulator recruitment assays[75], constructed with either coactivators or corepressors to identify agonists and antagonists. Additionally, cell-based reporter gene assays provide among the most sensitive means to screen for functional activity. These assays fuse NR response elements to reporters such as luciferase[76], β-lactamase, and secreted alkaline phosphatase (ref. 69 and other articles in the same focus issue).

G protein–coupled receptors. GPCRs are targets of intensive drug development by the pharmaceutical industry, and numerous, well-established HTS assay methods exist[77,78] and continue to be developed[79]. Technologies to measure ligand-receptor binding[41,80] and the functional consequence of receptor activation[77] have enabled screens that target the rich pharmacology of this receptor superfamily, including assays to differentiate full, partial and inverse agonists, antagonists and allosteric modulators[81], and to identify ligands for orphan GPCRs[8,82].

GPCRs broadly orchestrate a wide range of cellular events and processes for which HTS assays have been configured, including guanine nucleotide exchange[5,83], modulation of second messenger concentrations[84,85], ion channel activity[86], protein phosphorylation[56], protein trafficking[8,87,88], pigment dispersion[89], gene transcription[6] and cell proliferation[90], among others. The assays have different advantages and limitations, but collectively they illustrate the sophistication possible in HTS assay design given the current state of the art. For example, GPCRs that activate intracellular Ca^{2+} stores can be assayed using calcium-sensitive dyes (for example, Fluo-3 and Fura-4) and rapid-inject

imaging platforms[84]. However, GPCRs not naturally coupled to [Ca^{2+}] modulation could not be assayed by this means until the development of so-called universal adaptors, molecular engineered G proteins[91] or promiscuous G proteins[92], to reconfigure such GPCRs to stimulate Ca^{2+} signaling. Today GPCR second messengers can be assayed by the direct measurement of cAMP[85] (**Fig. 2**) or inositol phosphate species[57,93].

Assays based on GPCR internalization are independent of G protein subtype, unlike second messenger formation, and have been applied successfully to analyze a number of GPCRs using either standard microtiter plate readers[94] or fluorescence microscopy[8,9]. The use of the adaptor protein β-arrestin 2, fused with GFP, permits an assay design that generally does not require modifications to the target receptor's N or C terminus. Such phenotypic assays enabled with automated high-content image analysis are bridging cell biology with HTS in a way that will have a major impact on chemical biology, as discussed below.

Ion channels and transporters. Ion channels are membrane-spanning proteins that regulate electrochemical gradient-driven movement of inorganic ions such as Na^+, K^+, Ca^{2+} and Cl^- into or out of cells. HTS technologies aimed at this target class are under rapid development[95]. The existence of multiple responsive states, including closed, open and inactive, to which drugs respond differently, add to the pharmacological diversity and assay development challenges[96].

Ligand binding, ion flux, and fluorescent probe-based assays are the established methods for ion channel HTS (**Table 3** and **Supplementary Table 1**). Competition binding measures the affinity of the ligand-channel protein interaction but is dependent on the availability of radiolabeled ligands. As with GPCRs, binding assays do not detect the functional consequence of compound interactions with ion channels. For these reasons, cell-based functional assays are more relevant to ion channel screening at both the primary and secondary levels.

The calcium-sensing fluorophores Fluo-3, Fluo-4 and Fura-2 have been used extensively in HTS for voltage- and ligand-gated channels and for GPCR-induced intracellular calcium release[95]. The fluorescence intensity of these probes inside cells increases proportionally with the elevation of intracellular free calcium concentration[97]. The addition of opaque dyes to the assay buffer eliminates the need for cell washing steps, as only intracellular fluorescence of adherent cells is detected in bottom-read measurements, thereby improving compatibility with HTS protocols[95]. Voltage-sensing probes track changes in membrane potential and have been used in several assay formats, such as FRET-based and positional voltage sensors[98].

Ion flux measurements using radioactive tracers such as $^{86}Rb^+$, $^{45}Ca^{2+}$, $^{22}Na^+$ and $^{36}Cl^-$ are established assays of ion channel activity. However, the medium removal steps and considerable radioactivity limit this approach for HTS. Atomic absorbance spectrometry using nonradioactive tracer ions has recently permitted significant improvements to the ion flux assay for HTS[99].

Patch-clamp electrophysiology remains the standard for measuring ion channel activity, as the potency of certain compounds, especially those with state- or voltage-dependent mechanisms, may appear significantly less potent when using other assay methods (for example, fluorometric or rubidium ion flux). Automated patch-clamp instruments represent a remarkable development in technology, increasing throughput from approximately ten compounds per week to hundreds[95,100]. However, further improvements are needed in seal quality, throughput, and cost before this technology can replace other formats such as fluorescence-based assays for HTS.

The broad examination of drug candidates for ion channel activity has become critical in the assessment of a candidate's safety profile. In addition to the functional inhibition of ion channels, the folding and trafficking of ion channels represents another avenue for the development of HTS assays analogous to those developed for GPCRs. For example, an ELISA-based assay has been reported that measures the amount of cell surface hERG channel to determine drug effects on hERG channel trafficking[101].

Transporters, another category of transmembrane proteins, have an important role in ion, membrane potential and nutrient homeostasis. Perturbations in transporter function can result in disease and altered drug absorption, distribution, metabolism, excretion and toxicity (ADMET) properties, leading to drug resistance, for example. As a case in point, multidrug-resistance (MDR) transporters (for example, P-glycoprotein) belong to the family of ATP-binding cassette (ABC) proteins that extrude xenobiotic substances from the cell, including many drugs. Assays based on ATPase activity and fluorophore transport have been described[102,103]. Typical assay formats for amino acid and neurotransmitter transporters rely on radiolabeled substrates (for example, ^{3}H-glycine) and involve multiple assay steps including medium aspirations. However, new assay methods are being developed that are suitable for HTS such as yeast heterologous expression systems[104] and anion sensors[105].

Assays for pathways and networks, and cellular phenotypes

The complexity of components and their interactions within cellular networks offers a rich source of new targets to assay using technologies to examine protein interactions, expression, distribution and stability. For instance, signaling mediated by Hedgehog, Wnt and Notch proteins uses unconventional proteins implicated in developmental and disease processes, making these pathways important therapeutic targets[106] that are assayable with HTS automated fluorescence microscopy methods[8]. The following sections describe additional cellular assays but reveal only a glimpse of the breath of cellular processes amenable to testing by HTS assays.

Transcriptional readouts. Reporter gene expression is a mainstay for detection in cellular signaling assays[6] and is useful for broadly identifying compounds that modulate the pathway and isolate new targets within. However, the purposely promiscuous nature of these assays is disadvantageous if the objective is a probe of a particular component of the pathway, as 'target decryption' can be a formidable undertaking. A separate critical consideration resides in identifying a cell type that appropriately reflects the cellular context of the signaling pathway being assayed by the reporter; both the types and amounts of cell surface receptors and intracellular signaling components can vary widely among the commonly used engineered cell lines.

Protein interactions and redistribution. Several innovative enzymatic and image-based methodologies have provided new ways to identify compounds that modulate movement and associations of protein components within living cells. These approaches fall into several general categories: reporter complementation, resonance energy transfer and protein redistribution. Low-affinity associations form the basis of protein complementation assays (PCA), in which two proteins known to associate are separately fused to N- and C-terminal halves of a bifurcated reporter protein. Upon interaction of the two sentinel proteins, the halves of the reporter (now in close proximity) reconstitute a functional enzymatic or fluorescent reporter. Commonly used PCA reporters include β-lactamase, GFP, YFP and luciferase[27,40,107]. This methodology is attractive because it can be adapted to many protein-protein interactions and is amenable to screening and profiling, as demonstrated with YFP-based[108] and β-lactamase[109] reporters. Alternatively, high-affinity β-galactosidase α complementation has been used to monitor protein movement. Here the ω fragment is targeted to a specific subcellular

Table 3 Assay technologies

Technology	Description	Refs.
AAS	Atomic absorption spectroscopy for measurements of ion transport/flux (primarily focused on K^+ channels with Rb^+ as tracer); 96-/384-well microtiter plates enabled with, for example, the ICR 12000.	99
Absorbance	Follows Beer's Law; trans- or epi-absorbance; via fluorescence attenuation.	42,43
ALPHA	Amplified luminescent proximity homogeneous assay; donor-acceptor beads of ~200 nm diameter; λ_{ex} 680 nm (laser); singlet oxygen reacts with acceptor bead within ~200 nm distance; λ_{em} 520–620 nm.	56,149
Bioluminescence	Renilla or firefly luciferases used in reporter gene assays; analogs of D-luciferin used to expand biochemical assays for proteases, P450s, monoamine oxidases.	32
BRET	Bioluminescence resonance energy transfer; useful for protein-protein interactions; Renilla luciferase donor luminescence (480 nm) stimulates fluorescence of GFP/YFP acceptor; follows dipole-dipole interaction $1/r^6$ (~5 nm maximum).	39,150
Bla/CCF4	FRET-based assay using β-lactamase and β-lactam-containing substrate (for example, CCF4); λ_{ex} 395 nm, λ_{em} 460/530 nm; ratiometric reporter gene assays and other formats.	36,38,151
DELFIA	Dissociation-enhanced lanthanide fluorescent immunoassay/time-resolved fluorescence; ELISA-like format using lanthanide chelate-labeled antibody; τ_f ~500 μs; separation-based enhanced fluorescence; variations using labeled ligands; extremely sensitive.	25
ECL	Electrochemiluminescence assay. Uses microcarbon electrode plates allowing electrical oxidation of a $[Ru(phen)_3]^{2+}$ label. Multiplex applications in ELISA format.	17
EFC	Enzyme fragment complementation. Based on β-galactosidase α complementation; high-affinity complementation. Applied to both cell-free and cell-based systems.	57,74
FLT	Fluorescent lifetime; time-domain method (~ns) measures the rate of fluorescent decay following excitation; promises to eliminate fluorescence compound interference; only recently applied to some microplate systems.	146
FP	Fluorescence polarization; polarized light reports change in probe ligand τ_c; fluorophores of τ_f ~ 5 ns needed to measure ligand of MW < 1,500 binding to a receptor of MW ≥ 10,000; widely applied, ratiometric measurement.	23,24
FRET	Fluorescence resonance energy transfer; donor-acceptor pair as in EDANS-DABCYL peptides or fluorescent proteins (for example, CFP/YFP). Signal distance dependence ~5 nm maximum.	22,53
Fluorescence	Measurement of fluorescence intensity including the use of chromophores for quenching as in profluorescent protease substrates (~5 nm maximum distance); widely applied; superquenching varies in that nonequimolar amounts of donor fluorophore and quencher are used. Includes kinetic modes of detection with timescales of approximately minutes to hours.	22,152
Fluorescence (rapid kinetic)	Requires fluorescent imaging plate reader or equivalent (for example, FLIPR, FDS6000, VIPR); timescale ~seconds; GPCR functional assays using Ca^{2+}-sensitive dyes, or steady state ion channel activity using voltage-sensitive dyes.	84,98
GFP and variants	Cell-based imaging microscopy/cytometry assays; translocation, redistribution, protein-protein interactions, reporter gene assays; see below.	27,112
HT electrophysiology	High-throughput electrophysiology; measurement of ion channel currents with microaperture arrays such as the planar patch-clamp electrode (for example, PatchXpress 7000A, IonWorks Quattro).	98,99,153
PCA	Protein complementation assay; recombinant split reporters (for example, GFP, luciferase, β-lactamase) fused to interacting proteins; phenotypic assays; low-affinity complementation.	40,108
SPA, FlashPlate	Scintillation proximity assay; nonseparation-based radiometric detection using solid supports; typical isotopes include ^{35}S, ^{33}P or ^{3}H.	41
TR-FRET	Time-resolved FRET; also known commercially as HTRF/LANCE; nonseparation-based format using a lanthanide cryptate donor fluorophore of long fluorescence lifetime (τ_f ~ 500 μs); detection distance ~9 nm max.	25,26,53

τ_f, fluorescent lifetime; τ_c, rotational correlation time.

region and the α-peptide-tagged translocating protein restores β-galactosidase activity once α and ω combine[74].

In the resonance energy transfer methods FRET and BRET (**Table 3** and **Supplementary Table 1**), donor and acceptor (or sensor) proteins are fused to the target proteins of interest. In BRET, a signal is generated when a bioluminescent donor enzyme is brought into proximity with an acceptor fluorophore, the photon emission of which is measured[39]. In FRET, energy is transferred from a donor to an acceptor fluorophore when the two proteins come into close proximity, which is manifested as a change in wavelength of the emitted fluorescence. FRET-based sensors use spectrally distinct fluorescent proteins such as engineered GFP and YFP (reviewed in ref. 27). Intracellular indicators of specific kinase activity have been described using phosphorylation-activated FRET sensors engineered from fluorescent protein pairs[110], or for insulin signaling via PIP_3 recruitment of labeled Akt2 PH domains[111]. Additionally, a cell-based HTS using BRET to detect CCR5-β arrestin association identified inhibitors of CCR5-mediated signaling[94].

FRET, BRET, PCA and similar methods have been combined with subcellular imaging to produce highly successful and versatile HTS assays for monitoring protein movements within cells[9,110]. The development of technology platforms for automated cellular imaging, together with sophisticated object recognition algorithms, has made the screening of cell-based protein redistribution assays routine[8,9]. For instance, assays monitoring the redistribution of Akt1 (ref. 112), MAPK2 (ref. 113) and ERF1 (ref. 114) have been used for HTS. Indeed, MacDonald *et al.*[108] report the use of 49 PCAs to monitor protein redistribution or interactions for profiling small-molecule activities.

Protein stability. The addition of degradation and destabilization domains to reporter proteins has been useful for assaying a number of cell processes (such as ubiquitin-mediated degradation and the cell cycle) and enabling screening of unstable proteins such as the *cis*-acting viral NS2/3 protease[65]. For pathways in which a particular protein is controlled through degradation, destabilization domain GFP sensors allow a measure of pathway activity. For instance, fusing the degradation and localization domains of cyclin B1 onto GFP conferred onto the sensor the equivalent turnover and localization as endogenous cyclin B1, thereby allowing high-content screening for cell cycle modulators[8]. Similarly, fusion of the IκBα destabilization domain to luciferase was used to identify modulators of NFκB signaling[15]. Also, coupling of

the degradation domain of ornithine decarboxylase to the fluorescent protein ZsGreen was used to discover new proteosome inhibitors[115]. Fusing only the degradation domain of a particular protein, as opposed to the entire protein, to a sensor is advantageous in that it minimizes the pathway interference that can be caused by overexpression of the full-length protein[8].

Assays for rare disease targets. With over 1,800 genetic associations with human disease now known[116], the number of human diseases for which molecularly based assays could be developed is large. Occasionally a rare disease is caused by mutation of an immediately druggable target, and even more rarely one that may be ameliorated by a clinically approved drug[117]. More commonly however, the genes responsible for rare diseases have obscure or entirely unknown functions. Some common causes of human genetic diseases are listed below and illustrate the challenge of assay development for HTS in this arena:

(i) Nonsense mutations resulting in premature termination and absence of protein expression (for example, phenylketonuria, spinal muscular atrophy (SMA) and cystic fibrosis): assays to identify compounds that compensate for the defect, for example by upregulating an alternative pathway or pseudogene as in SMA[118], or promoting readthrough of premature stop codons[119].

(ii) Missense mutations resulting in a potentially functional but misfolded or mistrafficked protein (for example, cystic fibrosis): assay needs to discover compounds that act as molecular chaperones to facilitate correct folding and trafficking of the protein to its proper location[120].

(iii) Synonymous mutation that introduces a new splice donor or acceptor site, thereby altering splicing and producing an abnormal protein (for example, Hutchinson-Gilford Progeria Syndrome and β-thalassemia): assays to identify compounds that alter splicing[121].

(iv) Motif expansion or duplication mutations that result in expression of a protein with toxic or dominant negative function (for example, Huntington's disease): assay needs to identify molecules that downregulate the expression of the toxic protein or interfere with its function[122].

These urgent and challenging biological problems will be an important frontier for HTS assay development in the next decade.

Assay optimization for HTS

A balance between statistical performance and assay sensitivity facilitates the identification of the broadest range of compound potencies and efficacies from HTS. Assays are generally configured to measure one or more of the following changes: (i) concentration of a substance (for example, enzyme substrate or product, second messenger or reporter gene product); (ii) concentration of a receptor-ligand complex or (iii) distribution of cellular markers. In addition, different methodologies measuring the same biological process can have discordant determinations of apparent compound potency and efficacy[37,123–125], which emphasizes the need for selecting the proper assay format.

In general, four types of assays can be defined based on the acceptable limits of Z' factor and assay sensitivity (**Fig. 3a**). Conditions leading to satisfactory performance (Z' factor > 0.5) should be identified that minimally impact assay sensitivity of a control ligand or stimulus (**Fig. 3a**, quadrant i). With this in mind, the optimal assay will not always have the highest Z' factor, as some very robust assays (Z' scores > 0.8) can have poor sensitivity (**Fig. 3a**, quadrant iii). Conversely, assays with lower Z' factors may have superior sensitivity (for example, **Fig. 3a**, quadrants i and ii) and may be rescued for HTS using replicate or concentration-dependent formats[126]. Because potent compounds can be identified in poorly designed assays, the irrational exuberance of observing such high-potency or high-efficacy compounds is often displaced by the realization that the activity was artifactual in nature, and a careful analysis of the screening results ensues to identify compounds with reliable biological activity, many times with lower (μM) potency. Thus, the need for assay sensitivity is critically important to identify compounds with half-maximal inhibitory concentrations (IC_{50}s) in the vicinity of the screening concentration, as these are false-negative candidates in assays having poor sensitivity.

Ideally, HTS assays are designed with substrate (S) or ligand (L) concentrations below their K_m or K_d, so that the observed potency (IC_{50}) approximates the intrinsic potency K_i (see equations in **Table 2**; this relationship differs for more complex mechanisms or when determining functional activity of receptors such as K_b values[127–129]). Though increasing either [S] or [L] improves the overall Z' factor of the assay, doing so could potentially increase the IC_{50} values above the threshold used to identify actives in the screen (**Fig. 3b**,**c**), thereby preventing them from being identified as positives. Catalytic systems (for example, enzyme assays) should be designed for the minimum substrate conversion needed to achieve an acceptable Z' factor. Although shifts in IC_{50}s can be less than two-fold for enzyme assays operating at conversions upward of 80%[130], high turnover significantly affects the ability to detect inhibitors with potencies near the screening concentration (**Fig. 3d–f**) and lowers the assay sensitivity for enzyme activators.

Many HTS technologies are inherently unable to detect low-affinity complexes (K_d ~ 1 μM) without resorting to high ligand or target concentration, or engineering in a high-affinity interaction. The various assays developed to screen for inhibitors of the protein-protein interaction between the p53 tumor suppressor protein and its negative regulator (DM2) illustrate these issues (see **Supplementary Table 2** online for assay details). In one assay the measurement of a p53-DM2 interaction with a competition-binding FP assay[131] required 1 μM DM2 to allow sufficient complex formation (~50% bound) for a detectable signal. This configuration limits the observable compound potency range, thereby rendering the assay unreceptive to weak compounds and making it difficult to determine the rank order of potent compounds.

Conversely, high-affinity interactions resulting from enhanced avidity can increase the apparent affinity of the binding partners many fold through multiple receptor-ligand interactions. This "Velcro effect" is evident as a rightward shift in IC_{50} values of competing control ligands. For example, returning to the p53-DM2 interaction, Kane *et al.*[132] developed a TR-FRET–based assay for this protein-protein interaction that required only nanomolar protein concentrations, in contrast to the micromolar level needed in the previous FP format. However, enhanced affinity due to multivalent interactions in the resulting complex (**Supplementary Table 2**) decreased the sensitivity of the assay as judged by the higher-than-expected IC_{50} of unlabeled competitor p53 protein. The potential for such avidity effects through surreptitious polyvalent interactions should always be considered carefully when examining the benefits and limitations of the assay[133]. Further, irrespective of affinity, assay conditions requiring a high fraction of bound ligand (for example, most FP-based assays) cannot rely on the Cheng-Prusoff correction to estimate the K_d or K_i of control or test compounds. Rather, accurate approximations can be made using equations describing the complete equilibria of the competing components[134]. In another biochemical format, the thermal shift assay[135], which measures a ligand's ability to stabilize a protein's thermal denaturation, has been used to investigate compound binding to the p53 binding domain of DM2 directly. Though thermal shift technology at present is relatively low-throughput, requiring comparatively large amounts of target protein, it is one of the few function- or ligand-independent methods to screen for small molecule–protein interactions that may provide a means to identify small molecules with protein complex disruption potential (**Supplementary Tables 1** and **2**).

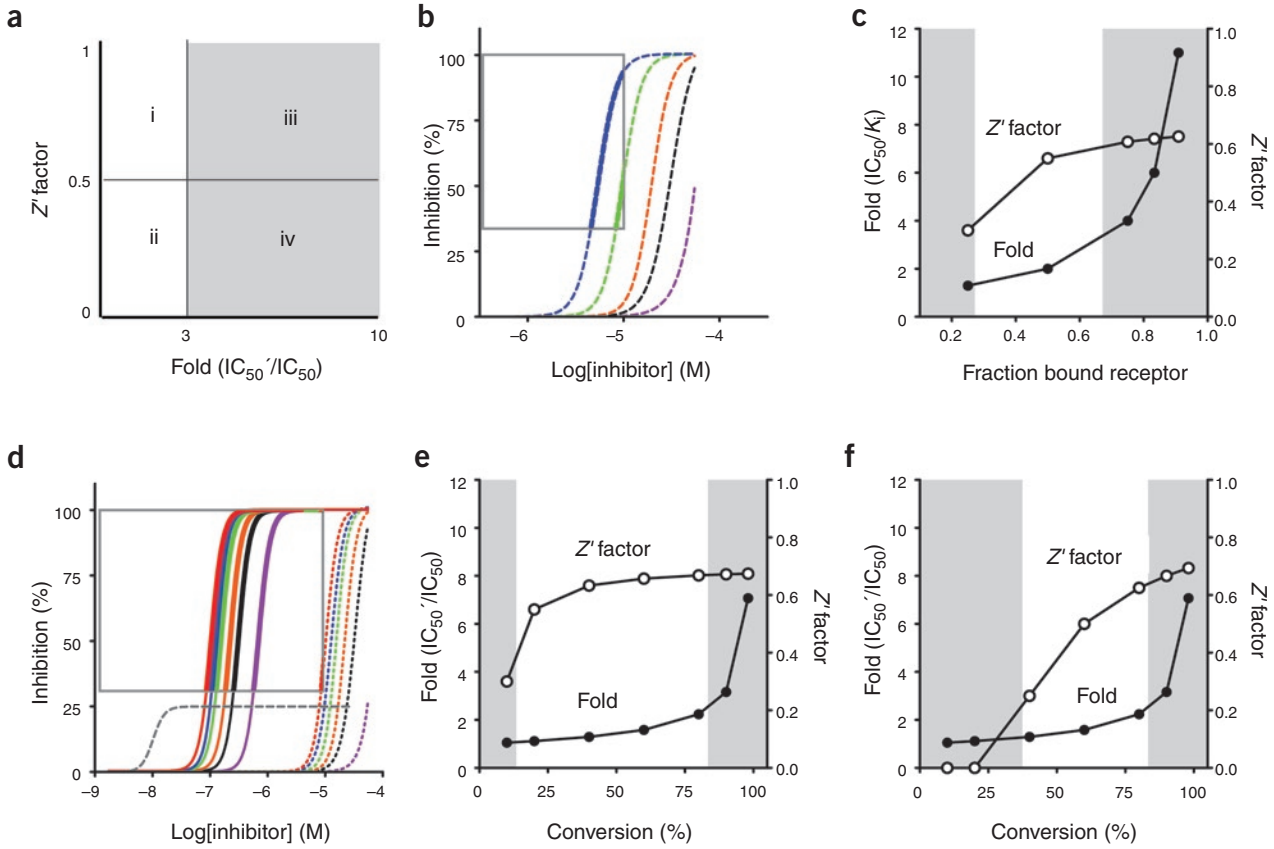

Figure 3 Assay performance and sensitivity. (**a**) Assay performance (Z' factor) is plotted against sensitivity, as defined by the ratio of observed (IC_{50}') and known (IC_{50}) values of a control compound. i, Optimal performance and sensitivity ($Z' > 0.5$, IC_{50}'/IC_{50} < three-fold). ii, Poor performance and good sensitivity ($Z' < 0.5$). iii, Good performance and poor sensitivity ($Z' > 0.5$; IC_{50}'/IC_{50} > three-fold). iv, Poor performance and sensitivity ($Z' < 0.5$; IC_{50}'/IC_{50} > three-fold). (**b**) Effect of varying the [ligand]/K_d ratio for a binding assay. Concentration-response (CR) curves represent [ligand]/K_d ratios as follows: 0.10, blue; 1, green; 3, orange; 5, black; and 11, purple. IC_{50}' was calculated using the Cheng-Prusoff equation (**Table 2**), with a compound K_i = 5 μM and a ligand K_d = 10 μM. The gray box is the "observation window" representing observed activity for a 10 μM screening concentration and a hit cutoff of >30% inhibition. (**c**) A modeled assay in which between ~30% and 70% bound receptor yields optimal performance. (**d**) CR curves for an enzyme assay at different substrate conversions (10%, red; 40%, blue; 60%, green; 80%, orange; 90%, black and 98%, purple) for two reversible inhibitors (solid lines, IC_{50} = 100 nM; dotted lines, IC_{50} = 10 μM). The observation window (gray box) indicates how shifts in IC_{50}' limit the identification of weak inhibitors, including potent, low-efficacy compounds (gray dotted curve). (**e**,**f**) Shifts in IC_{50}' were calculated as described[130]. Two modeled enzyme assays in which either an adequate performance (for example, two-fold S:B and %CV < 5%) is achieved at 10% conversion (**e**) or the equivalent performance is not reached until ~60% substrate conversion (**f**). Grey regions indicate areas of either poor assay performance or poor assay sensitivity (**a**, **c**, **e** and **f**).

Owing to recent advances in high-throughput cellular imaging[9,136], phenotypic assays now represent a viable alternative or complement to biochemical formats for protein-protein interaction HTS assays. Several cell-based assays have been used to investigate the p53-DM2 interaction[108,137], and these may circumvent the affinity and 'enhanced avidity' concerns encountered in systems configured with isolated proteins.

Reagent-coated surfaces provide both opportunities and drawbacks for HTS assays. Formats involving reagent-coated beads or surfaces enable a physical separation of a bound and free ligand, thereby eliminating filtration steps[41,133], or they provide components that comprise the detection system, as in ALPHA[138]. However, two issues should be considered when designing heterogeneous assays using solid supports. One issue pertains to the "avidity" of the interaction mentioned above that can occur in bead-based assays in which two binding partners are coated or tethered to separate beads, which results in multiple binding sites on the solid support (for example, the Velcro effect). The other issue is the capacity of the solid support to 'hold' a reagent or substrate. For example, when presenting an enzyme substrate on a solid support the amount of substrate that can be coated onto a surface can limit the reaction rate, and is often compensated by increasing enzyme concentration to achieve a suitable signal[139]. The net result of increasing target concentration is to increase assay cost and decrease sensitivity[140].

Compound interference within HTS assays

Compound-dependent assay interference is a very common cause of screening artifacts and must be ruled out before any compound activity is attributed to modulating the biological activity of interest. Here the inhibition or activation of an assay signal is not caused by the compound's effect on the target or pathway of interest, but rather it is due to the compound's ability to mimic that effect as a result of its physicochemical properties (for example, light absorbance) or adventitious biological activities (for example, luciferase inhibition)[141,142] (**Fig. 4**). Cheminformatic approaches have been described that attempt to identify or remove from screening collections compounds with such problematic characteristics[143], but currently prediction of such properties is not reliable, and each active compound must be assumed to be an artifact until proven otherwise. This may be particularly true for chemical genomics applications, as the allowed functionalities of a chemical probe are less strict than for a medicinal lead. Thus although the boundaries of a useful chemical series may be broader for chemical

genomics applications than for drug development, the potential for misleading HTS activity is perhaps even greater with 'probe-like' than with 'drug-like' compounds.

Compound fluorescence and absorbance are very common causes of signal amplification or attenuation. Heterocyclic compounds that dominate screening collections most commonly fluoresce in the blue-green range, meaning that common assay sensors, such as GFP and coumarin, as well as the many profluorescent enzyme substrates that excite or emit near the blue end of the spectrum, are susceptible to this type of optical interference. Assay responses can differ in signal strength or brightness and can determine assay sensitivity to certain classes of interferences, such as attenuation of luminescence or scintillation by light-absorbing compounds[142]. Signal brightness becomes particularly important with regard to compound interference when incident light is used to excite a fluorophore. In this case, assays having emitted light signals that overlap with the fluorescence from compounds will show increased false positives (**Fig. 4a**,**b**). The amount of compound fluorescence is dependent on the wavelength of excitation energy used, with blue light being more problematic than red light for typical heterocyclic compound libraries[142,144].

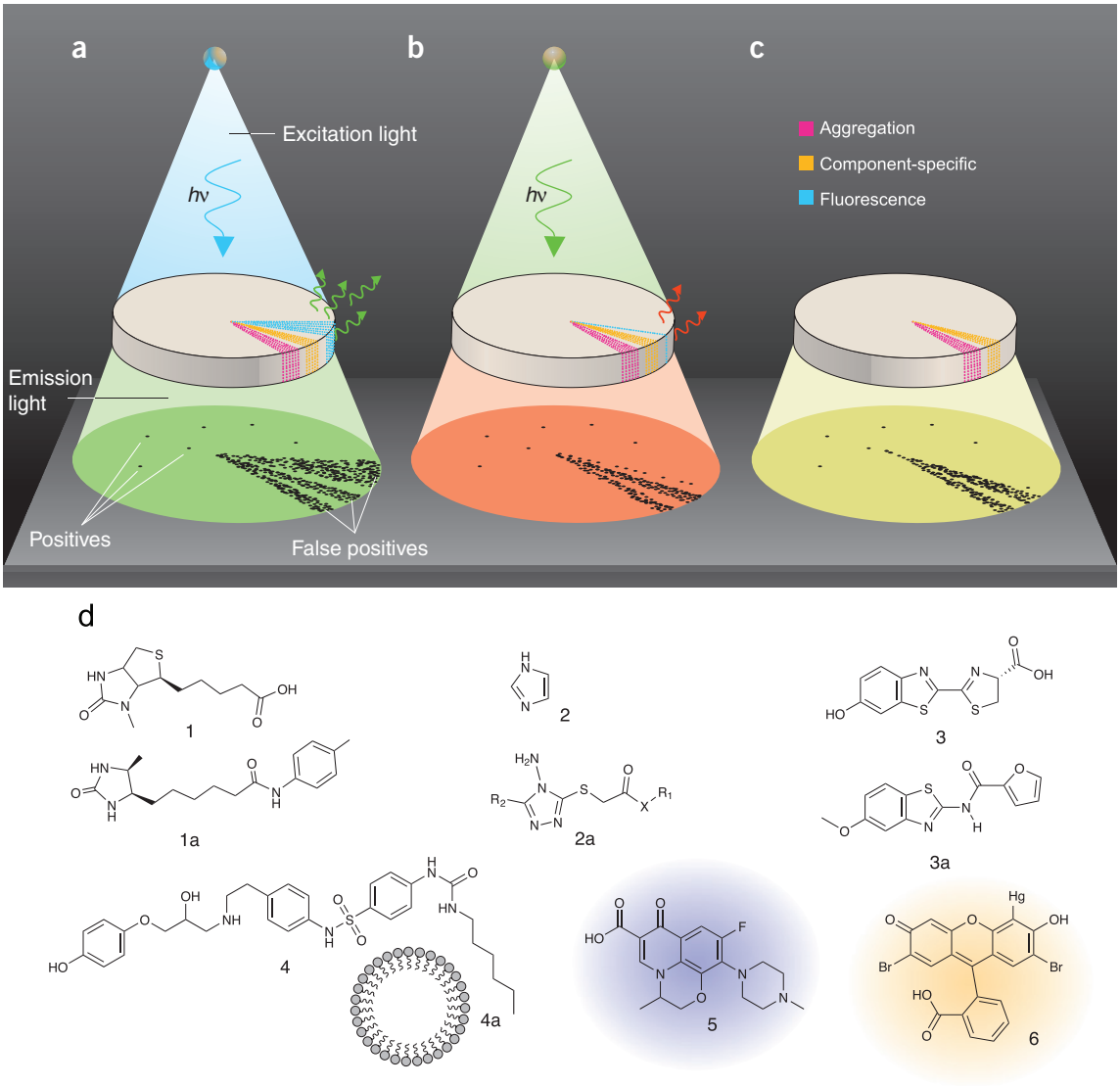

Figure 4 Compound effects on assay outputs. (**a**–**c**) The effect of different types of compound interference within a library (colored slices of the pie charts) on the scoring of actives. Interfering compounds are represented as dark spots arranged as a shadow cast by assay output signal, and individual spots represent genuine actives in assays using different modes of detection (blue or red fluorescence, or luminescence). In assays in which blue spectrum excitation is used (**a**), significant interference from compound emission is possible, whereas for red-shifted excitation light (**b**), interference is typically reduced. Also shown below are example fluorescent compounds **5** and **6**, excited by blue and red light, respectively. (**c**) Luminescence assays do not use an external excitation source, which results in negligible interference by compound fluorescence unless compounds or sample impurities absorb light (for example, inner filter effect by **6**). (**d**) Example molecules that illustrate different interference mechanisms. Biotin (**1**) analogs such as **1a** disrupt biotin-avidin complexes commonly used in assay designs (for example, PubChem AIDs 595 and 632). The imidazole (**2**) derivative **2a** can coordinate with Ni^{2+} and interfere with polyhistidine-tagged proteins at μM concentrations. Nickel-chelated microspheres are used in some instances to tether polyhistidine-tagged proteins (for example, PubChem AIDs 595 and 632), and can thus be disrupted by compounds similar to **2a** at typical screening concentrations. The luciferin (**3**) analog **3a** is an inhibitor of the reporter luciferase. A major source of interference is compound aggregation (see **4**), in which detergent-sensitive 'promiscuous' inhibitors block assays by forming target-sequestering/denaturing compound aggregates (**4a**)[147].

To provide experimental data on the fluorescent properties of the compounds screened in our center, we recently performed spectroscopic profiling of ~70,000 compounds from the Molecular Libraries Small Molecule Repository for eight common wavelengths (unpublished data; PubChem AIDs 587–592). For example, 4-methylumbelliferone (4-MU; excitation 385 nm, emission 502 nm) is a commonly used label in profluorescent substrates for a wide range of hydrolytic enzymes. We found ~2% of the library fluoresced at a level equivalent to at least 100 nM 4-MU, which indicates that a screen using this excitation wavelength would yield many false positives via this mechanism.

Several approaches can minimize interference from fluorescent compounds, and should be used whenever possible. Separation-based formats such as ELISA offer an advantage over solution-phase assays, as compounds are removed before the detection step. However, the necessary separation steps can make ELISA difficult to miniaturize. For homogeneous assays, compound fluorescence can be reduced by using probes that use red-shifted fluorophores[61], time-resolved lanthanide-based fluorescence[25,26], pre-reads or multiple time points that permit background subtraction or rate determinations[145]. Technologies that in principle are minimally affected by compound fluorescence, such as fluorescence lifetime (FLT) spectroscopy[146], are in development, but for now remain unproven in HTS.

Signal attenuation or activation of assay signal can also be caused by compound aggregation, which can result in target denaturation, ligand sequestration or light scattering[147] (**Fig. 4c,d**). Like the optical properties discussed above, aggregation often occurs within the concentration range of the expected biological response and so may be easily mistaken for true modulation of the target biology. Aggregation effects are most prevalent at concentrations >1 μM, the hallmark of which is the elimination of apparent compound activity by the addition of detergents such as Triton X-100, a disruptor of aggregate micellar structures. Such compounds may show steep Hill slopes, but this alone does not distinguish aggregation from biologically relevant, stoichiometric, or cooperative modulation of enzyme activity. The prevalence of aggregation-dependent enzyme inhibition has been observed to range from 2 to 10%, depending on the concentration of detergent and the chemical library tested[147]; importantly, no clear structure-activity relationship has been evident from these studies, making the establishment of aggregation behavior currently entirely empirical. Results of a large aggregation screen recently performed at our center can be found in PubChem (AIDs 585 and 584).

Biological interferences are the second major source of compound-specific assay artifacts (**Fig. 4a**). These are due to inhibition (or, less frequently, activation) of a detection component of the assay such as inhibitors of enzyme reporters (for example, luciferases or β-lactamases) or intermolecular interactions (for example, nickel chelate–polyhistidine tag, small-molecule antigen-antibody, biotin-streptavidin). As an example, inhibition of luciferase by library compounds is common and has been described for the well-publicized compound resveratrol[148]. A recent screen of *P. pyrali* luciferase showed between 2 and 3% of the library was capable of interfering with luciferase detection (unpublished data; PubChem AID 411).

Summary

Given the right compound library, assay design becomes the determining factor for identifying chemical probes using HTS. Before the selection of an assay format for HTS, thorough consideration of its advantages, limitations, and (when possible) prior application in HTS is recommended. The investigator's familiarity with specific technologies should not form the basis of an assay strategy; rather, an unbiased assessment of key factors discussed here will increase the probability of a successful HTS assay. Assay sensitivity and susceptibility to compound interference must also be studied prospectively when designing assays for HTS and planning follow-up analysis of active samples.

In general, the signal output of an assay should be assessed in conjunction with consequential controls. For example, protein-protein interaction assays using purified proteins may have suitable statistical performance factors but show poor sensitivity, which suggests that cellular assays with phenotypic outputs should be considered as alternatives. When investigating novel target biology, effort should be made to leverage existing well-developed technologies where possible. For instance, methods developed to detect ATP and ADP in protein kinase assays[48,51,53] are also useful for lipid[49] and metabolic kinases[126].

As HTS assay technologies, screening systems, and analytical instrumentation such as patch clamping and mass spectrometry evolve, the interfacing of large compound libraries with sophisticated assay and detection platforms will greatly expand the capability to identify chemical probes for the vast untapped biology encoded by genomes. The ramifications will allow a reinterpretation of the 'druggable genome'.

Note: Supplementary information is available on the Nature Chemical Biology website.

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COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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