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Novel detection strategies for drug discovery

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The Human Genome Project is expected to increase the number of potential drug targets from the current figure of 500 to ~3000–4000. This increased number of targets, and increasing knowledge of signaling-pathway networks and their complexities, sets new demands for efficiency on HTS assay technologies. Assessment of the total efficacy of a given drug candidate requires not only the classical assays, but also a wide variety of assays related to signaling cascades and cellular functions. Discrete functional assays traditionally involved Ca^{2+} flux, kinases and cAMP, but today extend to the whole signaling network, from ligand binding to expression. This review discusses emerging novel non-radioisotopic assays, such as ligand-stimulated GTP-binding, the inositol triphosphate assay, cellular receptor trafficking, and protein–protein interactions.

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▼ The complex cascade of reactions, from the point of ligand binding to eventual phenotypic change, involves secondary messengers, enzymatic reactions, receptor trafficking, protein–protein interactions, compartmentalized scaffolding reactions [1], and transcription control (Fig. 1). This complexity means that the overall estimation of potential drug efficacy is a staggering task [2]. In addition, there is constant debate as to the benefits of discrete, isolated target screening over cell-based screening [3]. In primary screening, a well-characterized, functional, non-radioisotopic, cell-free assay that can differentiate agonist from antagonist, and give a well-defined response, is the preferred approach [3].

Receptor–ligand interaction

Receptor targets represent ~60% of the total number of drug targets. Competitive ligand-binding assays are a direct way of screening for potential agonists or antagonists that bind to the receptor. Receptor-ligand-binding assays use a variety of labels and technologies ranging from radioisotopic to non-isotopic, and from separation-based heterogeneous to mix-and-measure homogeneous assays. Radioactive tracers and scintillating surfaces in the form of plates (e.g. FlashPlates™ from PerkinElmer; http://www.perkinelmer.com) or beads [e.g. the Scintillation Proximity Assay (SPA) from Amersham BioSciences; http://www.amershambiosciences.com), are still frequently used in HTS, although they are being increasingly replaced by technologies based on fluorescence or luminescence [4].

Fluorometric HTS technologies include fluorescence polarization (FP) assays (available from PerkinElmer; PanVera, http://www.panvera.com; and Molecular Devices, http://www.moleculardevices.com), various microvolume-based assays (e.g. FMAT® from Applied Biosystems; http://www.appliedbiosystems.com), and fluorescence correlation and fluorescence intensity distribution analysis (FIDA®; Evotec OAI, http://www.evotecoai.com). This is in addition to various lifetime-based heterogeneous and homogeneous methods such as DELFIA® and LANCE® (PerkinElmer), HTRF® (CisBio; http://www.cisbiointernational.com), and fluorescence-lifetime imaging (FLIM) technologies used in cellular assays [5].

ELISA-type heterogeneous assays are still applied in HTS because of their robustness. Time-resolved fluorometric assays, such as DELFIA®, have widespread applications in HTS not only because of their sensitivity but also because they can use small hydrophilic labels and have a multi-label option [5–7]. The multi-label option enables the simultaneous assay of more than one target, or the addition of an internal control to the assay. The major drawback of this and other heterogeneous assays is the requirement for extensive washings and prolonged incubations, making their automation more demanding.

The FMAT® technology measures cells or beads on the bottom of microtitre-plate wells using a

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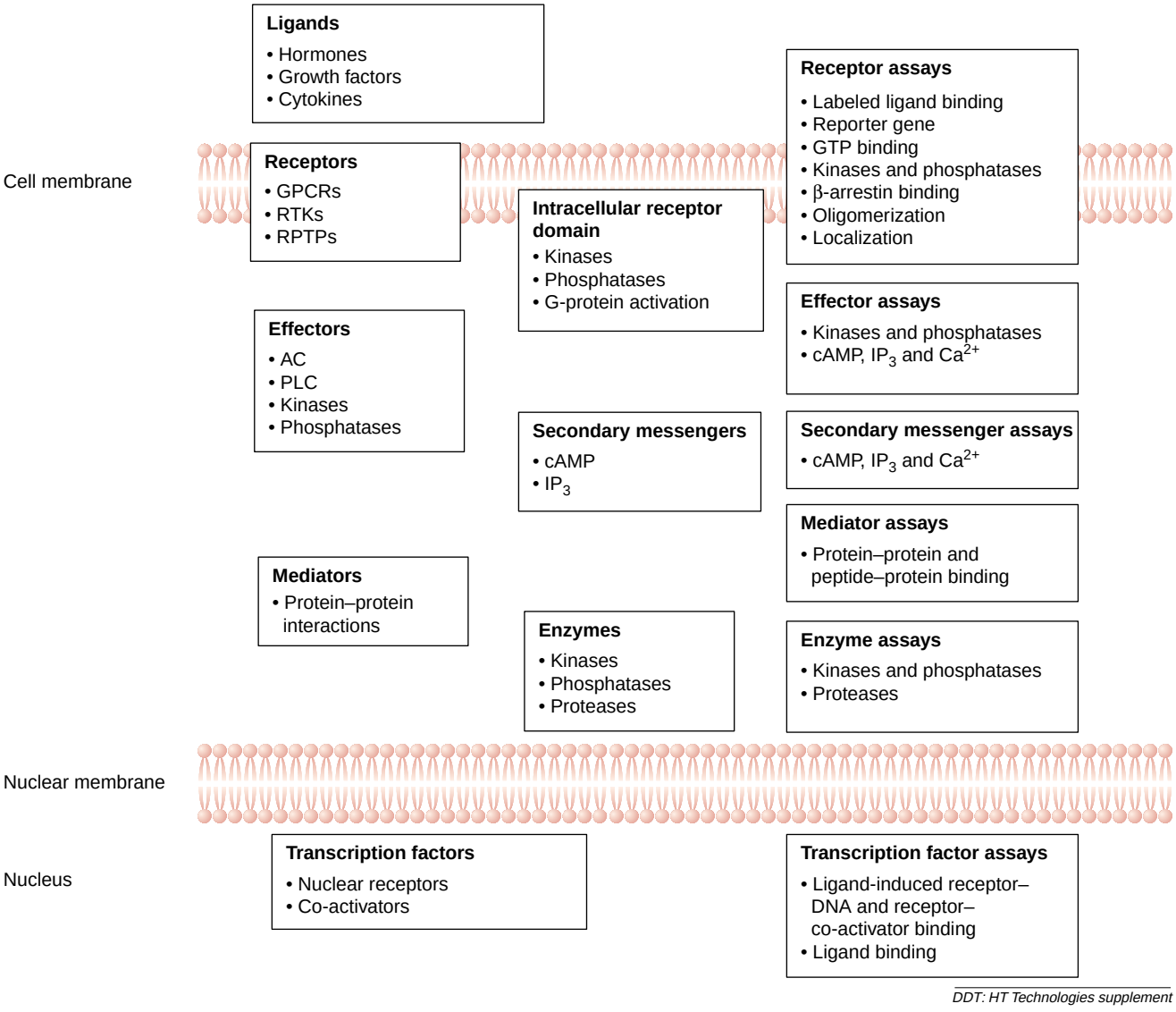

Figure 1. Schematic representation of signal transduction cascades and the type of assays used to study them. Abbreviations: AC, adenylate cyclase; GPCRs, G-protein-coupled receptors; IP3, inositol-1,4,5-trisphosphate; PLC, phospholipase C; RTKs, receptor tyrosine kinases; RPTPs, receptor protein-tyrosine phosphatases.

semiconfocal optical system, thereby avoiding background fluorescence of unbound tracers, and making physical separation unnecessary. An application of this technology has been described for receptor bindings with fluorescent peptide ligands [8]. Although FMAT® assays are easy to set up, they have slow reading times, high assay variation and expensive instrumentation.

In a similar way to FMAT®, and in contrast to fluoresence resonance energy transfer (FRET), polarization technologies have the advantage that they require only a single fluorescent label [9]. Given that FP is a homogeneous technology, there are certain demands on binding efficiency and receptor density. (A reasonable density would be >1 pmol mg^{-1}, and high affinity is required to ascertain a sufficiently high percentage of binding to enable discrimination of polarized signal from the signal of the free-tracer and background.) Currently used fluorochromes also set limitations on ligand size (<5000 Da) and the concentration of compounds in the library [10].

Fluorescence intensity distribution analysis generally uses labeled peptide ligands [10]. A disadvantage of this technique is that small-molecule ligands labeled with conventional fluorophores, such as BODIPY®-TMR (Molecular Probes, http://www.probes.com), tend to stick nonspecifically to receptor preparations, thereby deteriorating assay performance. Although FIDA®

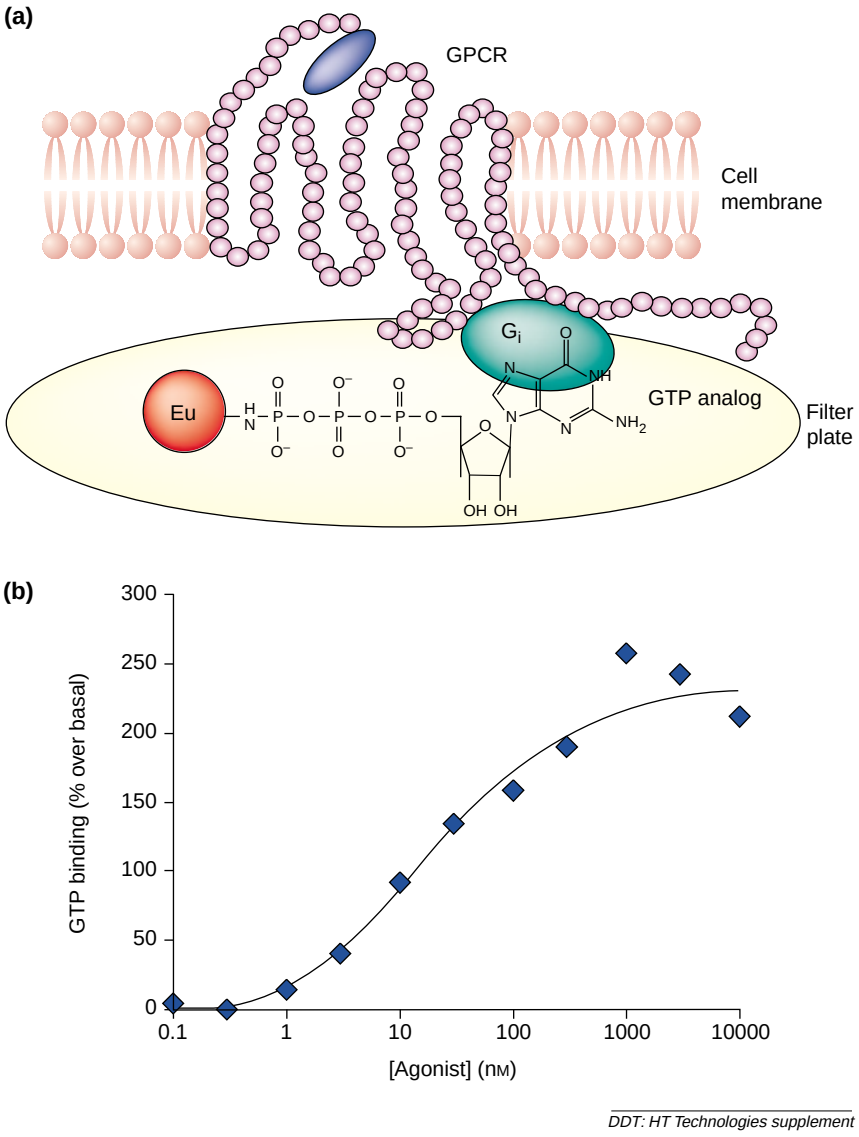

Figure 2. (a) The principle of the Eu–GTP binding assay. Membrane preparations are first incubated with agonist in Acrowell plates for 10 mins before adding Eu–GTP. After 30 mins incubation, the filter wells are washed three times. Fluorescence is measured with a time-resolved fluorometer (Victor^2; PerkinElmer, http://www.perkinelmer.com). (b) The dose-response curve, obtained using α_{2A}-adrenergic receptor membrane preparation, different concentrations of adrenaline as an agonist, and Eu-GTP as the label. Stimulation of G protein is expressed as Eu–GTP binding exceeding basal binding activity. Abbreviation: GPCR, G-protein-coupled receptor.

enables miniaturization down to 1 µl, it requires a reasonably long reading time (2–10 s) and expensive instrumentation [10].

Functional assays

G-protein activation

Traditionally, agonist-stimulated G-protein activation is measured using a non-hydrolysable GTP analog [^{35}S]GTPγS. However, because of high base-line binding and the specific requirement for labeling with GTP analog, the replacement of [^{35}S]GTPγS with a non-radioactive alternative has remained a challenge. Recently, a time-resolved fluorometric GTP-binding assay has been described [11,12]. The assay is based on stimulated binding of a GTP analog (labeled with an Eu-chelate) to membrane receptors, which are subsequently separated by filtration (Fig. 2). The assay has been demonstrated with α_2-adrenergic receptor, which couples through a G-protein-inhibiting adenylate cyclase (G_i).

Secondary messengers

G-protein activation is mediated by effectors such as adenylate cyclase and phospholipase C (PLC). Inhibition or activation of adenylate cyclase results in a decrease or increase in the intracellular concentration of cAMP. Recently, several homogeneous nonisotopic cAMP assays have been introduced (including FP kits from Molecular Devices and PerkinElmer). The oxygen-channeling assay – a proximity assay based on oxygen-radical production on donor beads, and chemiluminescence reaction on acceptor beads – has been commercialized for HTS as AlphaScreen™ (PerkinElmer). As with HTRF® [14], the AlphaScreen™ assay [13] enables highly sensitivity cAMP assays (25–50 fmol per well).

Phospholipase C activation results in an increase of intracellular IP_3 concentration. The development of a nonisotopic assay for IP_3 has long been a challenge, partly because of the high sensitivity required (down to the pmol concentration), and partly because of the cumbersome sample treatment. However, an AlphaScreen™ assay has been recently introduced for IP_3 [15]. This competitive assay is based on the use of an IP_3 binding protein and a labeled IP_3 analog. Only 1000–2000 cells are required in the assay, compared with ~50,000 cells per well in Ca^{2+} flux assays.

Protein phosphorylation and dephosphorylation

Tyrosine and serine and/or threonine kinases are important mediators in signal transduction and constitute a large target-group

for drug development. Time-resolved fluorometric technologies use Eu-labeled anti-phospho-amino-acid antibodies for detection of phosphorylated peptides, heteropolymeric peptides or protein substrates. DELFIA® kinase assays [16,17] enable the use of minimal concentrations of enzyme but, being a heterogeneous technology, require longer incubations, washings and enhancement. The respective FRET technologies – LANCE® and HTRF® – have been widely applied in kinase screenings [18]. AlphaScreen™ kinase assays can also be performed with various kinds of substrates. It uses unlabelled anti-phospho-amino-acid antibodies together with generic anti-IgG beads [19]. A competitive kinase assay format has been applied in FP in which a pre-formed complex of a phospho-specific antibody and a fluorescent phospho-peptide is added to the kinase reaction (PanVera) [20]. Although this FP assay enables both a kinetc and an endpoint assay, and uses unlabeled phospho-specific antibody, it requires careful optimization of the fluorescently labeled phosphopeptide tracer. Recently, a generic, non-antibody FP kinase assay platform has been introduced that uses a peptide substrate labeled with a fluorochrome, and beads containing chelated metal ions to capture phosphorylated molecules (IMAP™; Molecular Devices) [21]. Compared with the other non-isotopic kinase assays, which use physiological concentrations of ATP, this assay format uses sub-physiological ATP concentrations (1–30 μM) to avoid bead saturation.

Detection of specific kinase activities in living cells has recently been studied based on genetically encoded fluorescent reporters [22,23]. These reporters consist of fusions of cyan fluorescent protein (CFP), a phosphotyrosine binding domain [e.g. Src homology domain 2, (SH2)], a substrate for the relevant kinase, and yellow fluorescent protein (YFP). Phosphorylation of the reporter results in a conformational change and a subsequent change in FRET. This change can be detected by measuring both yellow and cyan emissions.

Protein phosphatase assays, such as FP phosphatase [20], mostly rely on kinase assay reagents and produce decreasing signal upon increasing enzymatic activity. They are therefore not optimized for monitoring of a minor hydrolysis in phosphorylated substrates. An energy transfer (TR-FRET) assay, based on the use of a masked biotinylated-PDZ-domain peptide ligand and Eu-labeled PDZ domain, has been used for the detection of a phosphatase activity [24]. The substrate, a binding-incompetent phosphorylated peptide, is cleaved by a phosphatase to create a peptide product, which is recognized by PDZ and produces an increasing energy transfer signal. A time-resolved fluorescence-quenching assay (TR-FQA) has been designed that gives an increasing signal upon dephosphorylation. This assay is based on a phospho-specific antibody labeled with a quencher and an Eu-labeled phosphorylated peptide (J. Karvinen, personal communication).

Receptor trafficking

In addition to G-protein coupling, the efficacy of a potential drug can be measured using alternative approaches [25]. Correlating receptor–ligand interaction with the efficacy of an agonist (or antagonist, partial agonist or allosteric agonist) can be a much more complicated process and factors such as receptor trafficking, internalization, desensitization, di- or oligomerization, docking and other interactions with modulating proteins have to be taken into consideration [25].

Receptor internalization

GPCRs are internalized upon agonist binding. The process includes phosphorylation of the intracellular loop of the receptor, binding of β-arrestin to the phosphorylated receptor, formation of endosomes, and either degradation or recycling of the receptor to the membrane. Assays for β-arrestin binding and localization use recombinant β-arrestin fused with a fluorescent protein (e.g. GFP). Without agonist binding, β-arrestin–GFP is located in the cytoplasm and shows a diffuse fluorescence. After agonist binding, the fusion protein concentrates near the plasma membrane and the fluorescence-distribution change can be detected using, for example, a confocal microscope [26]. A translocation assay, based on β-arrestin fused to a fluorescent protein, has been commercialized by Norak Biosciences (http://www.norakbiosciences.com), Cellomics (http://www.cellomics.com), and Amersham BioSciences in collaboration with BioImage (http://www.bioimage.dk). The functional cell-based ligand binding assay, bioluminescence energy transfer (BRET™, PerkinElmer), measures energy transfer from luminescent *Renilla*-luciferase–receptor fusion protein to β-arrestin–GFP [27].

The actual formation of endosomes can be followed using either receptor–GFP conjugates or a ligand labeled with a fluorochrome, or by using a labeled antibody against the extracellular part of the receptor (or against an epitope tag in the extracellular part of the receptor). G-protein-coupled receptor–GFP conjugates [28] have been used in the ArrayScan system (Cellomics) [29]. In an approach used by Amersham (CypHer), the anti-receptor or anti-tag antibody is labeled with a pH sensitive CyDye [30]. At pH 7.4, this dye exhibits fluorescence that is <5% of the fluorescence at pH 5.0. At the low pH of endosomes, the CyDye on the extracellular side of the receptor exhibits increased fluorescence, which can be measured with a dedicated instrument (e.g. the LEADseeker Cell Analysis System; Amersham Biosciences).

Receptor dimerization and oligomerization

Receptor hetero- and homo-dimerization, or hetero- and homo-oligomerization, is intensively studied as a part of signaling. Enzyme complementation technologies (e.g. HitHunter™ from DiscoverX, http://www.discoverx.com; and ICAST™ from

Applied Biosystems) have been applied to the detection of ligand-induced epidermal growth factor (EGF) receptor dimerization [31], and a respective HTS format has been developed [32]. Energy transfer using FLIM has also been used to study ligand-enhanced EGF receptor dimerization [33].

Downstream signaling reactions

Protein–protein and peptide–protein binding

In protein–protein and peptide–protein binding assays, the interacting proteins or peptides are either labeled or expressed with either a fusion partner [e.g. glutathione-*S*-transferase, (GST)] or an epitope tag (e.g. c-myc peptide). Fusion proteins and proteins with epitope tags enable the use of generic reagents (e.g. anti-GST and anti-c-myc) for detection of the interaction. Both TR-FRET and AlphaScreen™ technologies are widely used and well-suited for screenings of protein–protein and protein–peptide interactions [34–36]. A dual-label heterogeneous peptide–protein binding assay for the simultaneous detection of inhibitors for two binding domains on one protein has been used with a combination of Eu-labeled antibody and Sm-labeled streptavidin [37].

Proteases

Proteases comprise 15–20% of HTS targets today. Conventional substrates for proteases are peptides labeled with a fluorochrome. Peptide substrates carrying a fluorescent Eu-chelate at one end and a quencher at the other end, have been developed by PerkinElmer for improved sensitivity, and require less enzyme or substrate [38]. In intact caspase-3 substrate, the Eu fluorescence is 99.9% quenched, as compared with an Eu-labeled peptide with no quencher [39]. LANCE® technology uses protease multiplexing to enable up to three simultaneous measurements by applying three substrates labeled with either Eu, Tb or Sm, together with a quencher [40]. An intracellular GFP-FRET protease assay has also been developed by Aurora Biosciences (http://www.aurorabio.com) [41]. In this assay, tandem molecules of GFP mutants stably expressed within cells serve as a sensor for a protease activity. The sensor construct consists of CFP, a linker with a protease cleavage sequence, and YFP. When there is no protease activity capable of cleaving the sensor, fluorescence energy transfer takes place between CFP and YFP. Enzymatic cleavage eliminates the energy transfer and changes the fluorescence ratio between CFP and YFP.

Nuclear receptors

Binding of cofactor-derived peptides to estrogen receptor subtypes in the presence of different ligands has been studied using DELFIA® technology [42]. In addition, TR-FRET, FP and AlphaScreen™ technologies have been used to detect protein–protein interactions between coactivator and peroxisome proliferation-activated receptor (PPAR) [43,44]. Time-resolved fluorescence resonance energy transfer has been used to measure the effect of bile acids on the binding of steroid receptor coactivator-1 (SRC-1) to farnesoid X receptor (FXR), an orphan nuclear receptor [45]. Fluorescein-labeled estradiol has been used in FP assays with estrogen receptors α and β to screen compounds able to displace the labeled estradiol [20]. Ligand-binding assays for nuclear receptors are a well-suited target for FP because a purified receptor protein can be used, which minimizes problems of nonspecific interactions of the tracer, and enables polarization analysis because of a large change in the molecular rotation during receptor binding. Fluorescence polarization assays for ligand-binding to nuclear receptors, including estrogen, progesterone and glucocorticoid receptors, have been commercialized by PanVera.

GFP-tagged glucocorticoid receptor (GFP–GR) was recently used in a receptor-translocation assay [46]. Glucocorticoid receptors exist in cytoplasm in the absence of ligand, but are translocated to the nucleus after ligand binding, and this translocation can be traced by GFP fluorescence. A further elaboration of the translocation assay is to create a GFP-tagged chimeric receptor containing the retinoid acid receptor (RAR) and GR [47]. Retinoid acid receptors are located in the nucleus and their subcellular distribution is unaffected by ligand binding. Mackem *et al.* made a chimeric receptor with ligand-binding properties of RAR and subcellular distribution properties of GR, induced by ligand-binding of RAR. In the absence of RAR ligand, the chimeric receptor was in the cytoplasm; after addition of RAR ligand, the chimeric receptor translocated to nucleus, as shown by the distribution of GFP. Live-cell translocation assay technologies are available from Amersham Biosciences and Cellomics.

Reporter gene assays

Reporter gene assays for receptor binding are based on the use of cell lines that are engineered to contain a reporter gene (e.g. luciferase or galactosidase) under the control of response element [e.g. cAMP response element (CRE)]. Agonist binding induces expression of the reporter protein. Although these assays use unlabelled ligands, they have several disadvantages: incubations must be performed at 37°C, continuous cell-culture has to be performed, procedures tend to be long and dimethyl sulfoxide tolerance is low [48]. Additionally, false-positives are a problem, especially in antagonist screening, and toxic compounds complicate agonist screens. An approach using cells that constitutively produce a protein capable of signaling upon agonist binding has been commercialized (AequoScreen™ system; Euroscreen, http://www.euroscreen.be). In this system [49], cells are transfected with an apoaequorin gene, in addition to a receptor [50]. Before the assay, cells are incubated with

coelenterazine, a prosthetic luminophore, which forms a complex with apoaequorin (aequorin) in the cells. When agonist-binding results in the increase of cytosolic Ca^{2+}, aequorin binds Ca^{2+} and coelenterazine is oxidized, resulting in the emission of light. Advantages of the AequoScreen™ system include its high sensitivity (5000–10,000 cells per well required) and short incubation time (10–30 s), which reduce the number of false-positive hits, mainly caused by the toxicity of compounds or solvents. However, this method involves loading the cells with coelenterazine (a process that takes 3–4 h), which has limited stability (maximum stability 24 h), and measurement of flash luminescence after injection of the cells into wells containing compounds.

Concluding remarks

The increasing number of drug targets, and the complexity of signal transduction and cellular function, means there is increasing pressure on system providers to develop relevant, cost-effective and robust tools for primary and secondary screening programs. Numerous new assay technologies have been developed to address that demand. In particular, emerging discrete assays for secondary messengers are expected to provide novel tools for monitoring downstream signaling reactions. However, a technological challenge still remains, which is to develop not only a simple, universally applicable, non-radioisotopic ligand-binding platform, but also, more importantly, *in vivo* functional cellular assays capable of satisfying the increased demand for cost-effective and intelligent ways of identifying new blockbuster drugs.

References

1 McDonald, P.H. *et al.* (2000) β-Arrestin 2: a receptor-regulated MAPK scaffold for the activation of JNK3. *Science* 290, 1574–1577

2 Downward, J. (2001) The ins and outs of signaling. *Nature* 411, 759–762

3 Moore, K. and Rees, S. (2001) Cell-based versus isolated target screening: how lucky do you feel. *J. Biomol. Screening* 6, 69–74

4 Sittampalam, G.S. *et al.* (1997) High-throughput screening: advantages in assay technologies. *Curr. Opin. Chem. Biol.* 1, 384–391

5 Hemmilä, I.A. and Mukkala, V.M. (2001) Time-resolution in fluorometry techniques, labels, and applications in bioanalytical assays. *CRC Rev. Clin. Lab. Sci.* 18, 441–519

6 Inglese, J. *et al.* (1998) Chemokine receptor-ligand interactions measured using time-resolved fluorescence. *Biochemistry* 37, 2372–2377

7 Liu, J. *et al.* (1998) A time-resolved fluorometric assay for galanin receptors. *J. Biomol. Screening* 3, 199–206

8 Miraglia, S. *et al.* (1999) Homogeneous cell- and bead-based assays for high throughput screening using fluorometric microvolume assay technology. *J. Biomol. Screening* 4, 193–204

9 Banks, P. *et al.* (2000) Fluorescence polarization assays for high throughput screening of G protein-coupled receptors. *J. Biomol. Screening* 5, 159–167

10 Rüdiger, M. *et al.* (2001) Single-molecule detection technologies in miniaturized high throughput screening: binding assays for G protein-coupled receptors using fluorescence intensity distribution analysis and fluorescence anisotropy. *J. Biomol. Screening* 6, 29–37

11 Ollikka, P. *et al.* (2001) A non-radioactive GTP binding assay based on time-resolved fluorometry. *IBC's Conference on G Protein-Coupled Receptor: Taking Receptors from Orphans to Drugs*, 11–13 June 2001, Scottsdale, AZ, USA

12 Hurskainen, P. *et al.* (2001) Time-resolved fluorometry in bioanalytical assays. *Chem. Today* 5, 22–24

13 Bosse, R. *et al.* (2000) AlphaScreen™: true format flexibility 96→384→1536. cAMP assays. *SBS 6th Annual Conference and Exhibition*, 6–9 September 2000, Vancouver, BC, Canada, Abstract 209

14 Degorce, F. *et al.* (2000) A new HTRF® assay for the direct assessment of cyclic AMP in high throughput screening. *SBS 6th Annual Conference and Exhibition*, 6–9 September 2000, Vancouver, BC, Canada, Abstract 3043

15 Chelsky, D. *et al.* (2001) AlphaScreen HTS assay for IP3. *7th SBS Annual Conference and Exhibition*, 10–13 September 2001, Baltimore, MD, USA, Abstract 10058

16 Gaarde, W.A. *et al.* (1997) Development of nonradioactive, time-resolved assay for the measurement of Jun N-terminal kinase activity. *J. Biomol. Screening* 2, 213–223

17 Mallon, R. *et al.* (2001) An enzyme-linked immunosorbent assay for the Raf/MEK1/MAPK signaling cascade. *Anal. Biochem.* 294, 48–54

18 Biazzo-Ashnault, D.E. *et al.* (2001) Detection of insulin receptor tyrosine kinase activity using time-resolved fluorescence energy transfer technology. *Anal. Biochem.* 291, 155–158

19 Bosse, R. *et al.* (2001) Ultra-sensitive detection of Akt kinase activity using AlphaScreen. *7th SBS Annual Conference and Exhibition*, 10–13 September 2001, Baltimore, MD, USA, Abstract 10057

20 Parker, G.J. *et al.* (2000) Development of high throughput screening assays using fluorescence polarization: nuclear receptor-ligand-binding and kinase/phosphatase assays. *J. Biomol. Screening* 5, 77–88

21 Huang, K-S. *et al.* (2001) Generic kinase assay platform for high throughput screening. *SBS 7th Annual Conference and Exhibition*, 10–13 September 2001, Baltimore, MD, USA, Abstract 10003

22 Ting, A.Y. *et al.* (2001) Genetically encoded fluorescent reporters of protein tyrosine kinase activities in living cells. *Proc. Natl. Acad. Sci. U. S. A.* 98, 15003–15008

23 Sato, M. *et al.* (2002) Fluorescent indicators for imaging protein phosphorylation in single living cells. *Nat. Biotechnol.* 20, 287–294

24 Ferrer, M. *et al.* (2002) A PDZ domain-based detection system for enzymatic assays. *Anal. Biochem.* 301, 207–216

25 Kenakin, T. (2002) Efficacy at G-protein-coupled receptors. *Nat. Rev. Drug Discov.* 1, 103–110

26 Barak, L.S. *et al.* (1997) A β-arrestin/green fluorescent protein biosensor for detecting G protein-coupled receptor activation. *J. Biol. Chem.* 272, 27497–27500

27 Menard, L. *et al.* (2001) Bioluminescence resonance energy transfer (BRET™): a powerful platform to study G protein-coupled receptor (GPCR) activation in intact cells. *7th SBS Annual Conference and Exhibition*, 10–13 September 2001, Baltimore, MD, USA, Abstract 4024

28 Barak, L.S. *et al*. (1997) Internal trafficking and surface mobility of a functionally intact β_2-adrenergic receptor-green fluorescent protein conjugate. *Mol. Pharmacol*. 51, 177–184

29 Conway, B.R. *et al*. (1999) Quantification of G-protein coupled receptor internalization using G-protein coupled receptor-green fluorescent protein conjugates with the ArrayScan™ High-Content Screening system. *J. Biomol. Screening* 4, 75–86

30 Adie, E. (2001) The LEADSeeker™ Cell Analysis System for live cell based screening. *IBC's Conference on G Protein-Coupled Receptor: Taking Receptors from Orphans to Drugs*, 11–13 June 2001, Scottsdale, AZ, USA

31 Blakely, B.T. *et al*. (2000) Epidermal growth factor receptor dimerization monitored in live cells. *Nat. Biotechnol*. 18, 218–222

32 Graham, D.L. *et al*. (2001) Application of β-galactosidase enzyme technology as a high throughput screening format for antagonists of the epidermal growth factor receptor. *J. Biomol. Screening* 6, 401–411

33 Gadella, T.W.J and Jovin, T.M (1995) Oligomerization of epidermal growth factor receptors on A431 cells studied by time-resolved fluorescence imaging microscopy. A stereochemical model for tyrosine kinase receptor activation. *J. Cell Biol*. 129, 1543–1558

34 Moore, K.J. *et al*. (1999) A homogeneous 384-well high throughput screen for novel tumor necrosis factor receptor: ligand interactions using time resolved energy transfer. *J. Biomol. Screening* 4, 205–214

35 Mellor, G.W. *et al*. (1998) Development of a CD28/CD86 (B7-2) binding assay for high throughput screening by homogeneous time-resolved fluorescence. *J. Biomol. Screening* 3, 91–99

36 Hill, S. *et al.* (2001) Experience with AlphaScreen for high throughput screening of low affinity SH2 domain protein-protein interactions. *7th SBS Annual Conference and Exhibition*, 10–13 September 2001, Baltimore, MD, USA, Abstract 9013

37 Beresini, M. *et al.* (2001) A tale of two ligands: development of a dual-label, solid-phase assay for inhibitors of exosite peptide binding to Factor VIIa. *IBC's 5th Annual Conference on Assay Development: Developing Robust Assays for Screening through Advanced Technologies*, 28–30 November 2001, Coronado, CA, USA

38 Chen, K. and Wahl, R. (2000) Evaluation of different FRET pairs as a metalloproteinase substrate. *SBS 6th Conference and Exhibition*, 6–9 September 2000, Vancouver, BC, Canada

39 Karvinen J. *et al.* (1999) Homogeneous time-resolved fluorescence quenching assay (LANCE) for caspase-3. SBS *5th Conference and Exhibition*, 13–16 September 2000, Edinburgh, UK

40 Karvinen, J. *et al*. (2001) Multiparametric homogeneous LANCE assay for simultaneous detection of caspases. *SBS 7th Annual Conference and Exhibition*, 10–13 September 2001, Baltimore, MD, USA, Abstract 10097

41 Jones, J. *et al.* (2000) Development and application of a GFP-FRET intracellular caspase assay for drug screening. *J. Biomol. Screening* 5, 307–317

42 ramlett, K.S. *et al.* (2001) Ligands specify coactivator nuclear receptor (NR)B box affinity for estrogen receptor subtypes. *Mol. Endocrinol*. 15, 909–922

43 Zhou, G. *et al.* (2001) Use of homogeneous time-resolved fluorescence energy transfer in the measurement of nuclear receptor activation. *Methods* 25, 54–61

44 Xu, H.E. *et al*. (2002) Structural basis for antagonist-mediated recruitment of nuclear co-repression by PPARα. *Nature* 415, 813-817

45 Parks, D.J. *et al*. (1999) Bile acids: natural ligands for an orphan nuclear receptor. *Science* 284, 1365–1368

46 McNally, J.G. *et al*. (2000) The glucocorticoid receptor: rapid exchange with regulatory sites in living cells. *Science* 287, 1262–1265

47 Mackem, S. *et al*. (2001) A glucocorticoid/retinoic acid receptor chimera that displays cytoplasmic/nuclear translocation in response to retinoic acid. *J. Biol. Chem*. 276, 45501–45504

48 Rees, S. *et al*. (2001) Development of a homogeneous MAP kinase reporter gene screen for the identification of agonists and antagonists at the CXCR1 chemokine receptor. *J. Biomol. Screening* 6, 19–27

49 Le Poul, E. *et al.* (2002) Adaptation of aequorin functional assay to high throughput screening. *J. Biomol. Screening* 7, 57–65

50 Stables, J. *et al*. (1997) A bioluminescent assay for agonist activity at potentially any G-protein-coupled receptor. *Anal. Biochem*. 252, 115–126