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Review

Receptor–ligand binding assays: Technologies and Applications

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Abstract

Receptor–ligand interactions play a crucial role in biological systems and their measurement forms an important part of modern pharmaceutical development. Numerous assay formats are available that can be used to screen and quantify receptor ligands. In this review, we give an overview over both radioactive and non-radioactive assay technologies with emphasis on the latter. While radioreceptor assays are fast, easy to use and reproducible, their major disadvantage is that they are hazardous to human health, produce radioactive waste, require special laboratory conditions and are thus rather expensive on a large scale. This has led to the development of non-radioactive assays based on optical methods like fluorescence polarization, fluorescence resonance energy transfer or surface plasmon resonance. In light of their application in high-throughput screening environments, there has been an emphasis on so called "mix-and-measure" assays that do not require separation of bound from free ligand. The advent of recombinant production of receptors has contributed to the increased availability of specific assays and some aspects of the expression of recombinant receptors will be reviewed. Applications of receptor–ligand binding assays described in this review will relate to screening and the quantification of pharmaceuticals in biological matrices.

Keywords: Receptor–ligand binding; Assay technologies; High-throughput screening; Quantitative receptor assay; Recombinant receptor expression

Contents

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1. Introduction

Many biochemical processes, essential for the functioning and survival of cells (and the organism), are regulated by hormones, neurotransmitters, cytokines and other "messenger" molecules. This regulation proceeds by interaction of these naturally occurring molecules with receptors that are either embedded in the cell membrane (membrane-bound) or present in the cytoplasm (soluble receptor) or the nucleus of the cell. The membrane-bound receptors can be subdivided into G-protein coupled receptors (GPCRs), ion channels and receptors with a single transmembrane segment. GPCRs interact with GTP-binding proteins and consist of seven-transmembrane helices. Ion channels are homo- or hetero-oligomeric receptors that are composed of several subunits arranged in a ring that forms the ion channel containing the ligand-binding sites. Nuclear or soluble receptors are represented by the group of steroid receptors (e.g. the estrogen receptor) and the non-steroidal receptors (e.g. Vitamin D receptor) that regulate biological functions by controlling gene expression. This class of receptors consists of a DNA-binding and a ligand-binding domain.

Changes in receptor density and a disturbed balance in the (in)activation of these receptors give rise to the development of disease. For example, Parkinson's disease is related to a change in dopamine D2 receptor density [1]. Because of the involvement of these receptors in disease development, they are important targets in drug discovery. It is thus not surprising that, for example, drugs that interact with G-protein coupled receptors make up 50% of all available therapeutics [2,3] and are therefore of major interest.

Receptor screening methodologies can be based on either the determination of a functional response (e.g. cell proliferation), the production of second messengers (e.g. Ca^{2+}) or the interaction of a ligand with its receptor [4]. With respect to functional assays, it is possible to simultaneously monitor several signaling events such as enzyme activation (e.g. adenylate cyclase) and the mobilization of Ca^{2+}, and to differentiate between agonistic and antagonistic properties. There is a trend towards the development of cell-based assays (e.g. to replace animal studies), which has been facilitated by recombinant DNA technology using reporter gene systems. Nevertheless, it is sometimes still costly and difficult to obtain stable eukaryotic cell lines [5]. Binding of a ligand (agonist or antagonist) to its cognate receptor is the initial and indispensable step in the cascade of reactions that finally cause a pharmacological effect [4] and many successful and widely used techniques are thus based on measuring ligand binding. Various assay formats to measure the interaction of a ligand with its receptor will be covered in this review, but many of the detection technologies discussed here are also applicable to cell-based assays [5]. Description of the different technologies will be accompanied by an overview of labels that can be utilized in receptor binding assays. Next to this, the review will shortly address the use of recombinant expression of receptors in host organisms and describe the criteria to develop quantitative receptor assays in biological matrices.

2. Theoretical aspects of receptor–ligand interactions

Receptor binding assays have their origin in the competition between an analyte [A] and a labeled ligand [L^*] for binding to a certain receptor [R]. The relationship between the labeled ligand, the receptor and its complex is given by Eq. (1) and follows the law of mass action assuming reversible binding.

$$[R] + [L^*] \underset{k_{-1}}{\overset{k_{+1}}{\rightleftarrows}} [RL^*] \tag{1}$$

The ratio k_{-1}/k_{+1} refers to the dissociation constant K_d, which is inversely proportional to the ligand affinity towards the receptor. At equilibrium, the K_d can be determined as shown in Eq. (2), and represents the amount of ligand that saturates 50% of the binding sites ($[L^*] = K_d$).

$$K_d = \frac{k_{-1}}{k_{+1}} = \frac{[L^*] \times [R]}{[RL^*]} \tag{2}$$

Saturation of the receptor binding sites at a high concentration of labeled ligand (>10 × K_d) defines the total number of specific binding sites B_{max}, which can be derived from Eq. (3). B_{max} is usually expressed in picomoles per milligram protein or as a true concentration in the assay medium.

$$[RL^*] = \frac{[L^*] \times B_{max}}{[L^*] + K_d} \tag{3}$$

Introducing a competing analyte leads to the formation of two receptor complexes (see Eq. (4)). The analyte will displace a certain amount of labeled ligand, which depends on both the concentration and the affinity of the analyte.

$$[R] + [L^*] + [A] \rightleftarrows [RL^*] + [RA] \tag{4}$$

If the analyte concentration is varied and both the receptor concentration and labeled ligand concentration are kept constant, inhibition curves can be constructed. From these curves, the IC_{50}-value, which represents the analyte concentration that displaces 50% of the bound labeled ligand, can be determined. This IC_{50}-value is related to the affinity constant K_i of the analyte

as described by the Cheng–Prusoff equation [6] (Eq. (5)).

$$IC_{50} = K_i \times \left(1 + \frac{[L^*]}{K_d}\right) \quad (5)$$

The validity of the Cheng–Prusoff equation is based on a.o. the assumption that the labeled ligand binds with only a small fraction to the receptor. This means that the free and total labeled ligand concentration is approximately equal. The equation, nevertheless, loses, its validity if ligand depletion occurs as a consequence of a high receptor concentration or a high affinity of the labeled ligand for the receptor [7].

Receptor–ligand binding assays can be used in multiple ways. First, they can be applied as a tool for basic research concerning the receptor itself by determining receptor distribution and identifying receptor subtypes. Second, screening of new chemical entities and the discovery of endogenous ligands is facilitated by the utilization of receptor–ligand binding assays, despite the fact that receptor–ligand binding assays do not predict the intrinsic activity (agonistic or antagonistic) of these compounds [8]. Finally, these types of assays can be used in a quantitative way to determine an unknown amount of analyte that is present in a biological matrix with high sensitivity and reproducibility by comparing the displaced amount of labeled ligand with standard curves that contain known concentrations of the analyte [9].

3. Overview of labels available to measure receptor–ligand interactions

Most of the assay technologies described here, require labeling of either the ligand or the receptor. Radio-isotopic labels such as ^{3}H, ^{125}I and ^{32}P can be used to label the ligand without having an effect on the affinity of the ligand towards the receptor. Because of the disadvantages of disposal of radioactive waste, relatively long read times (10,000 counts: 25 min for radioactivity versus <50 ms for fluorescence [10]), costs, health hazards, the requirement for special licences, etc., efforts have increased to develop new technologies based on either colorimetric, fluorescence or (chemo-/bio-) luminescence detection systems. An overview of non-radioactive labels available to measure receptor–ligand binding interactions is given in Table 1.

Table 1
Spectral characteristics of some non-radioactive labels commonly used to measure receptor–ligand interactions

Detection	Label/substrate	Abs (nm)	Em (nm)	QY (%)	ε ($cm^{-1}\,M^{-1}$)	Mw (Da)	Solvent	Notes
Color	TMB	450	–	–	59000	240	–	Chromogen for HRP
Fluorescence	Alexa Fluor dyes	350–750		>80	15000–250000	410–1400	pH 7/MeOH	Bright, pH insensitive, photostable
	Bodipy FL-propionic acid	505	511	High	91000	293	MeOH	Small Stokes shift, narrow emission bandwidth
	Methoxycoumarin-COOH	336	402	Moderate	20000	220	pH 9	pH insensitive
	CyDyes	548–774		>4	>120000	658–1130	–	Bright, pH insensitive, photostable
	Dansyl-SE	335	518	Moderate	4200	462	MeOH	Weak fluorescence in aqueous solutions
	Fluorescein	490	514	19–74	88000	332	pH 9	Sensitive to pH, photobleaching & quenching, broad emission bandwidth
	NBD-SE	466	535	30–75	22000	392	MeOH	Low fluorescence in water, QY↑ & E_m↓ in aprotic solvents
	Rhodamine Green-SE	504	532	High	78000	508	MeOH	Photostable, pH insensitive
	Texas Red-SE	583	603	High	112000	817	MeOH	Emission at longer wavelenght
	Europium-cloride·$6H_2O$	337	613	<70	17000	366	H_2O	Long fluorescence lifetime, large Stokes shift, narrow emission bandwidth, absorbance and luminescence weak unless chelated
Chemoluminescence	Luminol	355	411	Low	7500	177	MeOH	Enzymatic, signal time min-range, substrate HRP
	Lucigenin	368/455	505	60	36000/7400	511	H_2O	Acridium ester, non-enzymatic, signal time sec-range
Bioluminescence	Luciferin	328	560	High	18000	280	pH 7	Substrate luciferase

It should be noted that the fluorophores presented here are only a small selection of fluorophores available (most of the spectral data were obtained from the Handbook of Fluorescent Probes and Research Products, nineth ed., R.P. Haugland, Molecular Probes, 2002). Abs = absorbance/excitation wavelength; Em = emission wavelength; QY = quantum yield; ε = molar extinction coefficient; Mw = molecular weight; TMB = tetramethylbenzidine; HRP = horse-radish peroxidase; MeOH = methanol; COOH = carboxylic acid; Bodipy = borondipyrromethine; SE = succinimidyl ester; NBD = 7-nitrobenz-2-oxa-1,3-diazole.

Receptor assays based on color development make use of enzymes such as alkaline phosphatase (AP) and horseradish peroxidase (HRP). In the case of HRP, a colored product is formed upon incubation of the enzyme with a suitable chromogenic substrate in the presence of hydrogen peroxide. Assay types based on color development are not very sensitive and fast in comparison to assays that make use of fluorometric or chemiluminescent detection. Moreover, some enzymes show a lack in stability and the final assay setup is rather complex.

Fluorescence is becoming increasingly popular as a detection principle due to dyes with enhanced brightness (fluorescence intensity = molar extinction coefficient × quantum yield), greater photostability and improved physical properties (e.g. pH stability, water solubility) of the fluorophore [11]. The choice of the fluorophore to label ligands is critical in assay development. According to Baindur and Triggle [12], a suitable fluorophore should possess the following characteristics: a high quantum yield (QY > 0.3), a high extinction coefficient (ε) to enable sensitive detection in aqueous media, photostability and a high excitation wavelength to reduce autofluorescence. Moreover, the molecular size, the use of a spacer and the position of the fluorophore on the ligand are of major importance. Via structure–activity relationships (SARs), it is possible to determine the key positions for labeling a ligand by avoiding regions in the molecule that are involved in binding. In most cases, a spacer between fluorophore and ligand is necessary to reduce steric hindrance caused by the attachment of a bulky fluorophore. The introduction of a spacer or the length of the spacer can, however, also bring about a negative effect on the affinity, so that a range of labeled ligands need to be tested to find the optimal one. Sometimes, it is possible that the parent compound does not show any affinity towards the receptor, but that labeling creates a fluorescent ligand with high affinity as shown for the benzodiazepine ligand, desethylflumazenil [13], but this is exceptional.

Disadvantages such as, e.g. photoinstability (fluorescein) and small Stokes shift (Bodipy) can be overcome by using more stable dyes with larger Stokes shifts, like Alexa Fluor® labels. The family of Alexa Fluor® dyes covers a broad range of excitation and emission wavelengths that can be adapted to most of the available detection techniques. Moreover, these dyes are intensely fluorescent, photostable, insensitive to pH changes and soluble in water. Detection methods based on fluorescence still have numerous disadvantages. First, the fluorescent signals can be quenched by other compounds in the assay mixture, plastic materials or the biological matrix. Second, fluorescence emission can be scattered by particles and finally autofluorescence from proteins or other compounds in the matrix can give rise to high background signals. The long lifetime lanthanides (e.g. europium; decay time > 0.5 ms) present a group of fluorescent dyes which can overcome the problems associated with short lifetime fluorophores (decay time < 50 ns). These lanthanides are used in time-resolved fluorescence, where after excitation a delay time is introduced prior to measuring their emission to eliminate interferences due to short-lived fluorescence background signals and scattering. This allows measurements with higher sensitivity and precision. The slow fluorescent decay times of lanthanide chelates makes them useful in a range of other assay formats as described in Sections 4.2.1 and 4.2.2.

An alternative detection technique for non-radioactive receptor binding assays is based on the generation of chemoluminescence or bioluminescence upon oxidation of luminol catalyzed by peroxidase or luciferin catalyzed by luciferase, respectively. Next to the enzymatically derived signals, there are also non-enzymatic systems that make use of, e.g. acridinium esters like lucigenin. The acridinium esters are characterized by a fast signal development and a high light output upon addition of hydrogen peroxide in an alkaline environment [14,15]. The luminescence signal can be detected with outstanding sensitivity through the use of a photomultiplier tube or a charge-coupled device (CCD) down to 10^{-18} to 10^{-21} mol [16]. There is little or no background from the matrix or scattering, which is an enormous advantage over the fluorescence systems. The use of luminescent compounds with a different kinetic profile or different emission wavelength makes multiplexing with this type of labels possible [15,16]. Nevertheless, as in the case for fluorescence, a disadvantage of luminescence is the possibility of inhibition or enhancement of the signal by matrix components [14]. Moreover, in the case of bioluminescent assays, it is a prerequisite to make use of highly purified reagents.

4. Receptor–ligand binding assay technologies

Receptor–ligand binding assays may be classified according to the need for separation of bound from free ligand or the detection technique. Indexed by the first criterion, the assay types are heterogeneous, homogeneous and non-separating homogeneous. Heterogeneous assays require separation of the free from the bound fraction of the ligand by either filtration, centrifugation or dialysis before measurement. A homogeneous assay requires no separation or washing steps before measurement, resulting in the development of the so called mix-and-measure or mix-and-read assays, which is often an advantage when it comes to assay automation and miniaturization. In the non-separating homogeneous assay, the signal is centered on or around a solid phase which contains the immobilized receptor or ligand. In this assay format, there is also no need to physically separate the free from the bound fraction [17]. The ideal assay should be specific, sensitive, easy to perform, reliable and reproducible, cheap, rapid and suitable for automation. Furthermore, there is a preference for non-radioactive assay formats to reduce health risks and environmental pollution as well as costs. Next to this, the possibility to quantify multiple analytes in a single assay becomes more and more important, so the system should preferably be capable of multiplexing [18].

Next to the formats mentioned above, it is possible to determine the ligand binding properties via affinity chromatography using immobilized receptors and interfacial optical assays, e.g. total internal reflection fluorescence (TIRF) and surface plasmon resonance (SPR). The latter has the advantage that no label is required. An overview of the different receptor-binding assay technologies is given in Tables 2 and 3.

Table 2
Advantages and disadvantages of receptor–ligand binding technologies I

Assay format	Principle	Receptor(s)[a]	Advantages	Disadvantages	Ref.
Radioactive					
Filtration assay	Radioactive	Various	Labeling step does not change affinity, robust	Requires separation, radioactivity, medium throughput	[27,179]
SPA/flash plate	Energy transfer	α-AR, IL-5, GRF	Mix-and-read, labeling step does not change affinity, high-throughput	Radioactivity, receptor immobilization, lower sensitivity	[29]
Non-radioactive					
Filtration assay	Fluorescence	BDZ-R, ER	Fluorescent	Requires separation, labeling can affect affinity, medium throughput, autofluorescence interference	[41]
FRET	Energy transfer	M_1-R, $GABA_A$, insulin	Mix-and-read, fluorescent	Dual labeling, distance constraints, correct dipole orientation, E_m (donor) = E_x (acceptor), background	[50,57]
FP	Light polarization	ER, $5HT_3$, δ-OR	Mix-and-read, one label, ratiometric measurement, ease of automation, facile to miniaturize	Labeling can change affinity, suitable ligands Mw < 5 kDa	[10,50]
FMAT	Single cell measurement	NK-1, IL-1, IL-5	Mix-and-read, multiplexing, mimimal background, miniaturization	Requires receptor immobilization, high assay variation, expensive instrumentation	[66]
AlphaScreen™	Energy transfer	ER_α	Mix-and-read, time-resolved	Donor and acceptor beads, receptor immobilization	[73]
Flow cytometry	Single cell measurement	Cytokines, β_2-AR	Mix-and-read, multiplexing; sensitive (10–100 pM), minimal background, high throughput (1000 particles/s)	Immobilization of ligand or receptor requires fluorescent labeling of receptor or ligand, limitations in sample handling	[18,75]

Ref. = reference; α-AR = alpha-adrenergic receptor; IL = interleukin; GRF = growth hormone releasing factor; BDZ-R = benzodiazepine receptor; ER = estrogen receptor; M1-R = muscarinic receptor; GABA = gamma-aminobutyric acid; 5-HT = 5-hydroxytryptamine; δ-OR = delta-opioid receptor; NK = neurokinin; β_2-AR = beta2-adrenergic receptor.
[a] These are examples of receptors for which this type of receptor–ligand binding technology has been described in literature.

Table 3
Advantages and disadvantages of receptor–ligand binding technologies II

Assay format	Principle	Receptor(s)[a]	Advantages	Disadvantages	References
FCS	Diffusion mediated intensity fluctuations	$GABA_A$, $5HT_{3A}$, nACh-R, EGF	Molecular level (on living cells), homogeneous, minimial background due to confocal optics, real-time kinetics, time-resolved, miniaturization (μHTS), assay time <10 s	Limited accessibility and complexity of instrumentation, stringent optical requirements	[50,85,89]
SPR	Refractive index (mass-dependent)	β_2-AR, rhodopsin, IL-2, nACh-R	No labeling, sensitive, real-time kinetics; no interference light absorption/scattering; automation	Receptor or ligand immobilization, sensitivity dependent on molecular weight of analyte, costs, high protein density, correct orientation	[108]
TIRF	Refractive index (mass-independent)	$5HT_3$	Real-time kinetics, high sensitivity, combined with microfluidics, surface specificity	Receptor immobilization, labeling of ligand	[91,116]
Microarray	Optical intensity changes	β_2AR, neurotensin	Multiplexing, real-time kinetics, number of detection principles	Labeling ligand or receptor, receptor immobilization	[101]
QAC	Retention volume	Pgp, OR, nACh-R	Hyphenation with different detection principles, re-use of column, ligand identification (QAC-MS), multiplexing	Receptor immobilization, receptor denaturation/release, non-specific binding to the support, throughput limited by serial nature chromatography	[135]

nACh-R = nicotinic acetylcholine receptor; EGF = epidermal growth factor; Pgp = P-glycoprotein; OR = opioid receptor.
[a] These are examples of receptors for which this type of receptor–ligand binding technology has been described in literature.

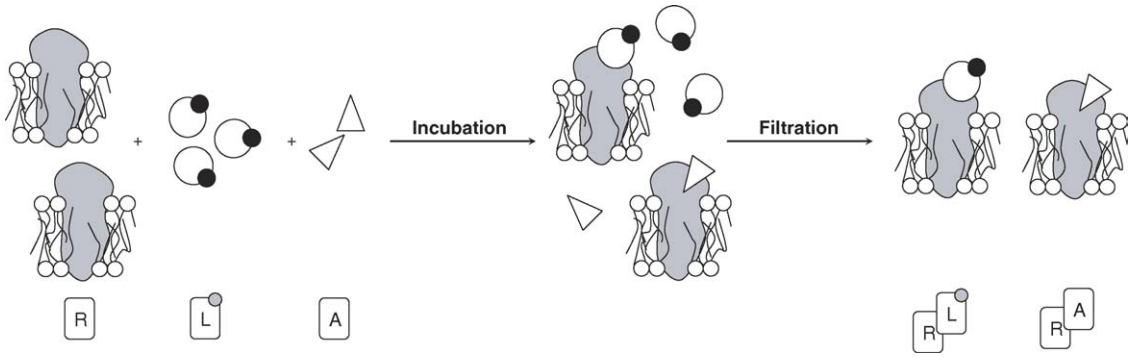

Fig. 1. Principle of a heterogeneous receptor–ligand binding assay. After incubation of the labeled ligand (L•) and the analyte (A) with the receptor (R) of interest, it is necessary to separate the free fraction from the bound fraction. This can be done by centrifugation, dialysis or filtration. The ligand can be labeled with radio-isotopes (e.g. ^{3}H), fluorophores or chemoluminescent probes.

4.1. Radioactive receptor–ligand binding technologies

4.1.1. Radioreceptor assay (RRA)

Most conventional receptor–ligand binding assays are heterogeneous and have been developed using radioactively labeled ligands for binding to a membrane-bound receptor. The first quantitative radioreceptor assay was developed by Lefkowitz et al. [19] based on the same principle as described originally for radioimmunoassays [20]. The principle is based on the competitive interaction between a labeled ligand and an analyte for the same receptor binding site. The principle of these displacement assays is depicted in Fig. 1. Selected examples of radioreceptor assays are the measurement of benzodiazepines [21,22], neuroleptics [23,24] and opioids [25,26].

In choosing the appropriate radioligand for a radioreceptor assay, several criteria have to be met. First, the radioligand should be selective and possess a high affinity for the respective receptor. Second, the radioligand should have a high specific activity and moreover it should be radiochemically pure. Furthermore, the radioligand should be chemically stable and resistant to enzymatic degradation. Finally, the most potent enantiomer of the radioligand is preferred, to avoid interference and complicate analysis as a consequence of the presence of the less active enantiomer [27].

A major advantage of radioligand binding assays is sensitivity, specificity and ease of use. The assay requires only one labeling step, which often does not reduce the affinity towards the receptor. Many high affinity receptor ligands are commercially available allowing to set-up an assay rather quickly. The major drawback of these assays is, however, the use of radioactivity and the need to separate free from bound ligand, which make these assays labour-intensive and relatively slow. Moreover, these assays require that the dissociation of the ligand proceeds much slower than the time to perform the separation (e.g. filtration) step.

To overcome the need to separate the free from the bound fraction radioactive homogeneous assays have been developed, based on scintillation proximity.

4.1.2. Scintillation proximity assay (SPA)

SPAs [28] are radioactive assays, where the receptor is immobilized on a solid surface (bead) and the ligand is labeled with a radioactive isotope. The bead contains a scintillant, which emits light as a result of energy transfer. The bead is a 5 μm I.D. polyvinyltoluene microsphere with a polyhydroxy surface coating, which can be easily dispersed in aqueous solution and is stable in numerous organic solvents (e.g. DMSO, MeOH up to 20% (v/v)) [29]. Immobilization of the receptor is based on either the interaction of glycoproteins and glycolipids with wheat germ agglutinin (WGA) [30–33], by capturing anti-receptor protein antibodies on an anti-IgG coated surface [34], via the streptavidin–biotin interaction [34] or via antibodies directed against the receptor [34,35].

The β-particle and Auger electrons, emitted from the radioisotopes ^{3}H and ^{125}I, respectively, travel over a limited distance in an aqueous environment (<8 μm [^{3}H], <12 μm [^{125}I] [36]), resulting in detection of β-particles and Auger electrons only if the radioactive ligand is in close proximity to the receptor, which is immobilized on the scintillant beads [4,29] (see Fig. 2). The isotopes of interest in the SPA technique are [^{3}H] and [^{125}I] [29], with a preference for ^{125}I because of its higher specific activity [36]. The ^{125}I gamma emission path length is more than 15 cm, resulting in almost no energy absorption by the scintillant beads or assay buffer and thus no interference [37].

The Flashplates or Scintiplates are based on the same principle, but the scintillant is now coated on the inner surface or placed on the entire plastic surface of the wells of a microtiter plate to which the receptor has been immobilized. The mix-and-read-format makes this scintillation proximity assay easy to automate, which enhances assay reliability [4]. Nevertheless, use of radioactivity remains a disadvantage making this technique also very expensive. Another potential difficulty is the need to immobilize the receptor on a solid surface, where it should remain stable and maintain its affinity.

Scintillation proximity assays have been developed for a range of receptors including the α_1- and α_2-adrenergic receptors (α-AR) [33,34], the human interleukin-5 (hIL-5) receptor [32], the growth hormone releasing factor (GRF) [31] and the epidermal growth factor (EGF) receptor [35]. Gobel et al. [33] compared the SPA with a conventional filtration binding assay for the α_1-AR and showed that SPA exhibited a reduced number of counts than the filtration assay, which is due to the central localization of the energy transfer with only 50% of β-particles travelling into the SPA bead. Moreover, the assay sensitivity

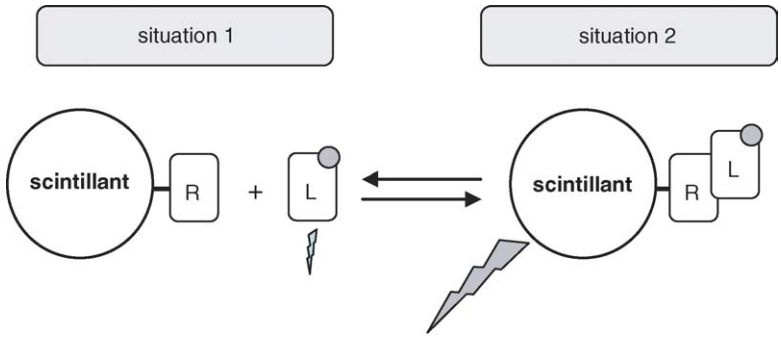

Fig. 2. The scintillation proximity assay is based on the emission of light as a result of energy transfer from the radioactive decay of the ligand to the bead that contains a scintillant. The emission of light only proceeds if the labeled ligand (L•) and receptor (R) are in close proximity (approximately 10 μm, situation 2). Otherwise, the energy of the radioactive ligand is absorbed by the buffer (situation 1).

is also limited by the receptor binding capacity on SPA beads. This relatively low signal to background ratio requires thus the use of membrane or soluble preparations with a high receptor density. Moreover, SPA is rather time-consuming (18 h) in comparison to a filtration assay (90 min) because of the time necessary to reach equilibrium of the receptor-bead interaction and to allow the beads to settle down in the microtiter plate. The latter is necessary to avoid signals from ligand that is not bound to the beads and can be accelerated by centrifugation. The relative long incubation time may also be a problem for rather instable receptors.

A Scintiplate assay was developed for the recombinant α_{2B}-AR [34] with the advantage over SPA that no bead separation step was required. The assay sensitivity in the Scintiplate is, due to the limited receptor binding capacity, lower as compared to the SPA beads [30], but can be improved by washing and drying the plate before measurement. These washing steps eliminate variations in counting efficiency as well as quenching, and moreover reduce non-specific binding [34].

The filtration assay being more labor intensive, costly and with less output of plates per day has made SPA the preferred format for high-throughput screening, despite its lower sensitivity. Nevertheless, radioactivity remains to be the major disadvantage in the use of these assays. This resulted in the still continuing development of receptor–ligand binding assays based on fluorescence or chemo-/bioluminescence.

4.2. Non-radioactive receptor–ligand binding technologies

Many different strategies have been chosen towards the development of non-radioactive assays ranging from the development of heterogeneous filtration assays to homogeneous mix-and-measure assays, such as those based on fluorescence resonance energy transfer (FRET), fluorescence polarization (FP) and flow cytometry. Most of these assays require some type of labeling to measure the ligand–receptor binding. It is thus pivotal to find labeling conditions that do not interfere with the molecular interaction. In the case of a non-radioactively labeled ligand, the ligand should demonstrate similar binding characteristics (e.g. receptor specificity, affinity) and similar or improved sensitivity as its radioactive analog [38].

4.2.1. Heterogeneous non-radioactive receptor assay

One of the first receptor assays that made use of fluorescence was described by McCabe et al. [39] for the benzodiazepine receptor using a fluorescein-labeled ligand. The assay required the use of high amounts of labeled ligand and high amounts of receptor and had considerable background fluorescence. Takeuchi et al. [40] and Janssen et al. [41] tried to overcome the use of radioactivity and the presence of background fluorescence by developing a heterogeneous receptor assay combined with reversed-phase high-performance liquid chromatography (RP-HPLC) and a fluorescence detector. The method by Takeuchi et al. [40] measured the free fraction with RP-HPLC directly after centrifugation. This necessitated a large amount of receptor material in order to reach a level of specific binding that allowed to measure a significant decrease in the fluorescence signal. The procedure from Janssen et al. [41] required the use of a dissociation step of the bound fraction after filtration to recover the bound fluorescent label from the receptor before measurement with RP-HPLC. Measurement of the bound fraction instead of the free fraction is favorable with respect to the precision of the assay. This assay demonstrated to be as sensitive and specific as its radioactive counterpart, and did not require large amounts of label or receptor.

Another way of reducing the significant background signal is presented by Takeuchi et al. [38], who made use of time-resolved fluorescence (TRF), by labeling the benzodiazepine ligand with a europium chelate. After centrifugation, supernatants were transferred to a microtiter plate and the fluorescence was enhanced and stabilized, before measurement, by addition of a fluorescence enhancing ligand.

Examples of other heterogeneous non-radioactive receptor assays are based on the use of enzyme-labels with the critical note that these assays are tedious and time-consuming. The assays make use of either a ligand coupled to an enzyme [42] or measure receptor–ligand binding based on enzyme activity via an indirect route [43–47]. An example of the latter is described by Mahoney [44] for the platelet-derived growth factor receptor (PDGF-R). In this heterogeneous assay, the amount of biotinylated ligand bound to the receptor, which is immobilized on a microtiter plate, was determined through addition of neutravidin-HRP. The excess of neutravidin-HRP was removed

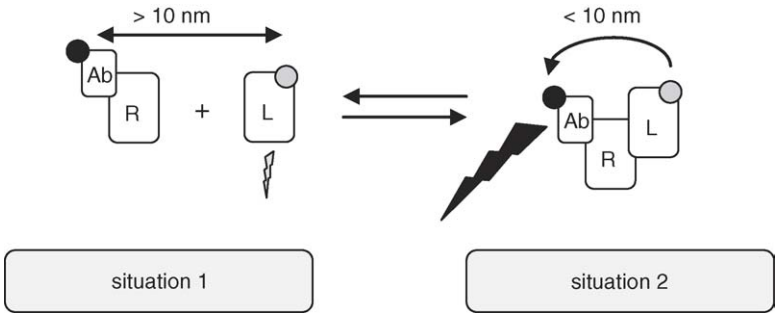

Fig. 3. Fluorescence resonance energy transfer (FRET) is based on the transfer of excitation from a donor to an acceptor molecule without emission of a photon when both come into close proximity (approximately 10 nm), as shown in situation 2. The efficiency of FRET depends on the inverse sixth power of the distance between donor and acceptor. In this case, the receptor (R) is labeled via a fluorescent antibody (Ab•, acceptor) and the ligand is labeled with a fluorophore (L•, donor). If long lifetime lanthanide chelates are used as donors, interfering background fluorescence can be largely reduced by performing a time-resolved measurement (TR-FRET).

by washing and absorbance was measured after addition of substrate.

4.2.2. Fluorescence resonance energy transfer (FRET)

Most of the mix-and-measure assays make use of the principle of fluorescence resonance energy transfer (FRET, Förster [48]), which is based on energy transfer between donor and acceptor molecules that need to be in close proximity (see Fig. 3). As an example, a Cy-labeled antibody directed against the receptor may serve as the acceptor molecule, while the donor is a europium labeled ligand. Upon exitation of the donor, energy is transferred via dipole–dipole interaction to the acceptor molecule, without the emission of a photon. The acceptor emits light provided that the ligand is bound to the receptor–antibody complex [49]. For this assay format to work, it is critical that the antibody does not block the ligand-binding site.

A distance smaller than 10 nm and an overlapping absorption spectrum of the acceptor with the emission spectrum of the donor are essential for efficient energy transfer. Next to strict distance constraints [10,50], the utility of this technique to measure receptor–ligand interactions in a homogeneous assay format is limited by the requirement of labeling both the donor and acceptor molecule.

An example of the use of fluorescence resonance energy transfer is the determination of the ligand–receptor binding properties between the EGFP (enhanced green fluorescent protein)-labeled human M1 muscarinic receptor (hM_1-R) and the Bodipy-labeled antagonist pirenzepine [51]. This homogenous assay was used to identify new muscarinic ligands, but additionally proved to be well suited to examine the binding pocket of the receptor. In the latter case, EGFP was fused to the N-terminus of the human M1-muscarinic receptor via a linker of variable length that did not alter its binding properties. The study showed that the shorter the distance between the EGFP domain and the hM1 receptor, the higher the efficiency of energy transfer between the Bodipy-labeled antagonist pirenzepine and EGFP. This means that the binding pocket is deeply buried within the transmembrane core of the protein. In another example, the binding and structural properties of segments of the ligand-gated ion channel $GABA_A$ receptor α_1-subunit were determined via resonance energy transfer between a Bodipy-labeled benzodiazepine ligand (Bodipy-FL Ro-1986 (didesethylflurazepam)) and intrinsically fluorescent tryptophan residues of the receptor protein [52].

The problem with interferences from background fluorescence can be solved by using donor molecules with a long excited state lifetime like lanthanide chelates, as already mentioned before. Time-resolved fluorescence resonance energy transfer (TR-FRET) has been described by Stenroos et al. [49] for the determination of the binding properties of the human cytokine interleukin-2 towards the recombinant human interleukin-2 receptor α-subunit. The interleukin-2 receptor is involved in growth and differentiation of B- and T-cells, and is a single pass transmembrane protein. The receptor was labeled via a Cy5-labeled specific monoclonal antibody and the ligand with a europium chelate. TR-FRET assays are sensitive, can be miniaturized and display reduced autofluorescence, but there is a limited choice of donor/acceptor pairs. Moreover, due to steric hindrance, it is more complex to label donor and/or acceptor without interfering with binding itself.

The luminescent variant of FRET where energy transfer occurs between a luminescent donor and a fluorescent acceptor is called bioluminescence resonance energy transfer (BRET). The enzymatic oxidation of a substrate results in the emission of energy from the donor, which means that no excitation light is needed in contrast to FRET. Besides, the enzyme reaction does not produce a background signal and the assay is therefore more sensitive than FRET [53].

BRET has been mainly used in protein–protein interaction research for example in studying the β_2-adrenergic/β-arrestin interaction [54] and the determination of insulin receptor activity [55,56], where the latter is governed by a conformational change in the β-subunits of the receptor, bringing them into close proximity. Because of the fact that there is no requirement of a light source, the instrumentation for BRET assays is simpler and cheaper [57], which makes these assays very valuable in high-throughput screening. Nevertheless, because of the large fluorescent and bioluminescent probes, it is necessary to consider which label to choose, how to label the ligand and/or receptor (with or without spacer), and to assess the effect of labeling on the binding properties.

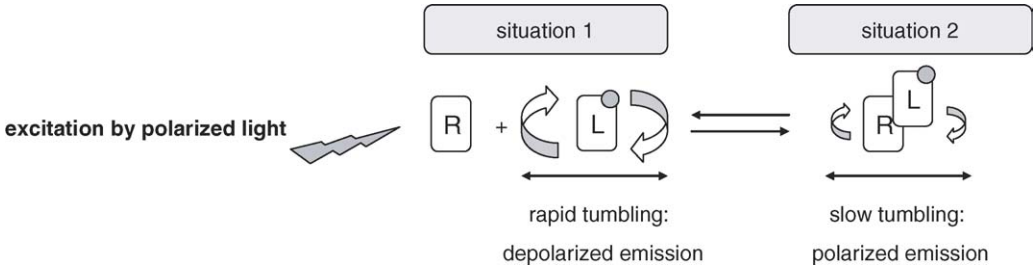

Fig. 4. Fluorescence polarization measures the change in polarization of light emitted from a fluorescent labeled ligand ($L^{\bullet}$) as a consequence of a change in mobility of the labeled ligand. Excitation of a fluorescent low molecular weight ligand by polarized light results in depolarized emitted light due to rapid rotation of the ligand (situation 1). Upon binding of the fluorescent ligand to a high-molecular weight receptor (R), the rotational speed decreases and the emitted light stays partially polarized (situation 2).

4.2.3. Fluorescence polarization (FP)

Another fluorescence based platform, which can be easily adapted to high-throughput screening, is based on fluorescence polarization or fluorescence anisotropy. Both terms are used synonymously to describe molecular interactions in solution [58,59]. This technique measures the change in rotational speed of a ligand during its excited lifetime upon binding to its receptor. The fluorescent labeled ligand is excited by polarized light and polarization of the emitted light is determined. The emitted light is largely depolarized in the presence of a small and therefore rapidly rotating ligand, while rotational speed and thus depolarization are significantly reduced when the ligand is bound to a protein, for example, a receptor. Thus, polarization of the excitation light, the lifetime of the excited state and the mobility of the fluorophore all determine the degree of polarization of the emitted fluorescence. The principle of fluorescence polarization is schematically demonstrated in Fig. 4.

The advantage over the above mentioned resonance energy transfer techniques is the requirement of just one labeling step. Moreover, FP is a very simple technology, which requires only a filter fluorometer with two polarizing filters. Two intensity measurements are performed with the filters parallel (I_p) and perpendicular (I_s) to each other. The difference between the two values divided by their sum represents the degree of polarization. Due to this ratio, FP can correct for fluctuations in lamp intensity or quenching of fluorescence [10].

The FP technique displays a number of disadvantages, like the lack of precision at low nanomolar concentrations. As for all other technologies, the sensitivity of FP is dependent on the affinity of the ligand, the quantity of the receptor and the intensity of the fluorophore. To achieve the required precision for a successful FP assay, the receptor concentration should be at least 1 pmol/mg protein and the ligand affinity must be below 5 nM, as stated by Gagne et al. [60]. Besides, FP is limited to low molecular weight ligands (<5 kDa), if fluorophores are used with short excited lifetimes. This limit can be extended by using fluorophores with longer lifetimes [10,17,60–62].

The efficiency of FP has been considerably increased (HEFP), due to technological advances such as confocal optical design and the use of high-intensity continuous Xe arc lamps, with possibilities to perform assays in as little as 5 μl volumes, instead of the conventional 40–100 μl. Despite these small volumes, it is not necessary to increase the concentrations of assay components to maintain a specific signal window because of the ratiometric measurement of the polarization signal [63]. Moreover, evaporation had little effect on the signal or stability of the signal over time, as demonstrated by Kowski et al. [63]. This means that fluorescence polarization is better suited to assay miniaturization, and therefore a reduction in reagent costs, than fluorescence and scintillation proximity assays.

The FP technology has been applied to, e.g. the soluble estrogen receptor [63], the G-protein coupled delta-opioid receptor [64] and the ligand-gated ion channel serotonin $5HT_3$ receptor [64,65].

4.2.4. Fluorometric microvolume assay technology (FMAT)

Fluorometric microvolume assay technology or microvolume fluorometry (MVF) makes use of a scanner that measures multiwell plates. In this mix-and-measure assay, the peptide or small molecule ligand is labeled with a fluorophore and the receptor should be either expressed on cells or immobilized on beads. The FMAT scans a 1 mm^2 area at the bottom of the multiwell plate (either 96-, 384- or 864-well with a clear bottom and black sidewalls), where the generated images indicate the size and amount of bound fluorescence. The capillary based scanner uses as an excitation source a Helium–Neon (He–Ne) red laser (E_x = 633 nm) and makes simultaneous detection of two independent red dye emissions, e.g. Cy5 and Cy5.5, possible via two photomultiplier tubes with bandpass filters for the respective labels (multiplexing). Multiplexing minimizes reagent consumption and increases the throughput [66].

Mellentin-Michelotti et al. [66] demonstrated the use of FMAT in the determination of neuro-peptide (e.g. substance P) binding to the G-protein coupled NK1 receptor on live cells. The mix-and-measure format is achieved by discriminating between cell-associated and free fluorescence during data processing, where cell-associated fluorescence is detected as localized areas of concentrated fluorescence at the bottom of the well. To avoid interference from autofluorescence of the cells, long wavelength emitting red dyes are used, which results in a very sensitive assay.

Martens et al. [67] described a receptor–ligand assay for the IL-1 and IL-5 receptor, where the receptor is immobilized on a bead or expressed on the surface of a cell, and the localized fluorescence intensity is measured after incubation with the fluorescent ligand. Another possibility is to perform the assay in solution with addition of antibody-beads to capture

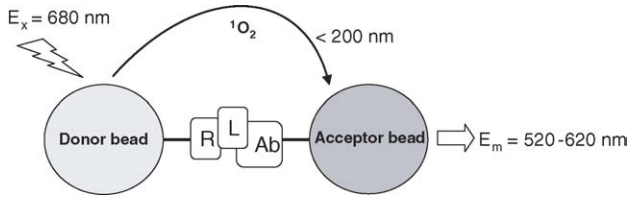

Fig. 5. Schematic representation of the AlphaScreen™ measuring principle. Upon excitation at 680 nm, ambient oxygen is converted to singlet oxygen ($^1O_2$) by a photosensitizer present in the donor beads (phthalocyanine). If the acceptor bead is in close proximity (<200 nm), the singlet oxygen transfers its energy to thioxene derivatives present in the acceptor bead leading to emission of light at 520–620 nm. Proximity is in this case established through the interaction of a receptor (R) immobilized on a donor bead with its antibody-captured ligand (Ab-L) present on the acceptor bead.

the receptor following incubation with the fluorescent ligand. Multiplexing can be achieved by immobilization of the receptor on beads of different sizes, since the analytical software is capable of discriminating fluorescence coming from beads of different diameters. A 96-well plate is scanned within 4 min and a 864-well plate within 30 min. Laser-scanning imaging is an analogous technique to FMAT, which can also be used for multiplexing to measure ligand–receptor interactions [68].

4.2.5. AlphaScreen™

A different format of a homogeneous bead-based assay, called AlphaScreen™ (Amplified Luminescence Proximity Homogeneous Assay [69]) makes use of singlet oxygen ($^1O_2$, half-life 4 μs) production on donor beads, and a chemiluminescent reaction on the acceptor beads as depicted in Fig. 5. This assay allows to probe interactions over longer distances than FRET and BRET, up to 200 nm, due to the fact that singlet oxygen travels farther in solution before it falls back to the ground state. Both donor and acceptor beads are coated with a layer of hydrogel, which can subsequently be derivatized with a variety of capturing molecules such as streptavidin for biotinylated receptors. Next to the possibility to provide a functionalized surface, the hydrogel reduces non-specific binding and self-aggregation of the beads. Upon excitation of the donor beads at 680 nm, the photosensitizer phthalocyanine, present within the donor beads, converts ambient oxygen to singlet oxygen molecules, which results in a very high signal amplification. These singlet oxygen molecules go undetected, if there are no acceptor beads present within a range of 200 nm.

However, if the donor beads, containing the receptor, come into close proximity with a high affinity ligand immobilized on acceptor beads, energy transfers from the singlet oxygen molecules to a thioxene derivative thereby generating chemiluminescence within the acceptor beads. A fluorophore present in the same acceptor bead is excited by the energy emitted by the chemiluminescent molecule and the emitted light is measured in a time-resolved manner as a consequence of long lifetime fluorescence. The beads used in the AlphaScreen™ are ∼250 nm in diameter, which is much smaller than the beads used in SPA and FMAT, which are 2–10 μm and 6–20 μm, respectively. This is advantageous to avoid clogging in liquid handling devices [70–73] (“A Practical Guide to Working with AlphaScreen™, PerkinElmer Inc., 2003). An example of a receptor–ligand binding assay, which made use of the AlphaScreen™ methodology, was described for the ER_α-receptor by Rouleau et al. [73].

4.2.6. Flow cytometry

Flow cytometry [74] sequentially sorts and counts single microscopic particles, either cells or beads suspended in a stream of fluid, based on optical signals, such as fluorescence. Bead-based flow cytometric assays make use of cell-sized polystyrene/latex or dextran microspheres with diameters in the μm-range. The beads can be either filled with two fluorophores of varying concentrations and wavelengths (Luminex® approach, [18]) or can be different in size [75], which allows for multiplexing. In the Luminex® approach, two lasers are used to identify the bead and quantify the fluorescence associated with the immobilized interacting partner. Each bead set is therefore unique to the analyte being measured [18]. Flow cytometry requires that one of the interacting partners is immobilized on the bead and the other is provided with a fluorescent tag, in order to measure and quantify the receptor–ligand binding. The volume that is illuminated by the laser is in the picoliter (pL)-range, which reduces the background noise, resulting from light scatter, fluorescence impurities or free probe. The ratio of specific signal to background together with the number of binding sites per particle and the affinity of the fluorescent probe for these sites determine the sensitivity of flow cytometry [76,77].

An interesting example of this technology was shown by Simons et al. [78], who solubilized the β_2-adrenergic receptor and demonstrated an agonist-induced conformational change, which allowed the receptor to bind to its cognate G-protein. Flow cytometry was used to discriminate between agonist and antagonist binding using the solubilized β_2-adrenergic receptor fused to green fluorescent protein (β_2AR-GFP). The antagonist dihydroalprenolol (DHA) was immobilized on activated sulfhydryl dextran beads while the hexahistidine-tagged G_s-protein ($G\alpha_s\beta_1\gamma_2$) was adsorbed on dextran beads bearing chelating Ni^{2+} ions. As soon as the DHA-bead interacts with β_2AR-GFP in the sample, the bead became fluorescent. Addition of agonist or antagonist decreased the fluorescent signal due to competition for binding. Addition of β_2AR-GFP to the G_s-protein-beads together with an agonist resulted in the fluorescence of the G_s-protein-beads, whereas no fluorescence was observed in the case of addition of β_2AR-GFP together with an antagonist (Fig. 6).

Multiplexing of this procedure is possible, as described both by Simons et al. [78] and by Waller et al. [75]. In the latter case, the assay makes use of large DHA-beads (34 μm) and small G_s-protein-beads (13 μm), which are discriminated based on the difference in light scattering (see Fig. 6). If an agonist is present, this will result in decreased fluorescence at the DHA-beads, but increased fluorescence at the G_s-protein-beads. If an antagonist is present, this will result in decreased fluorescence at the DHA-beads, but also no fluorescence at the G_s-protein-beads. Thus, decreased fluorescence at the DHA-beads is shown if either an agonist or antagonist is present as a result of competition with DHA for the receptor protein. Increased fluorescence at the G_s-protein-beads is only demonstrated if full or partial agonists

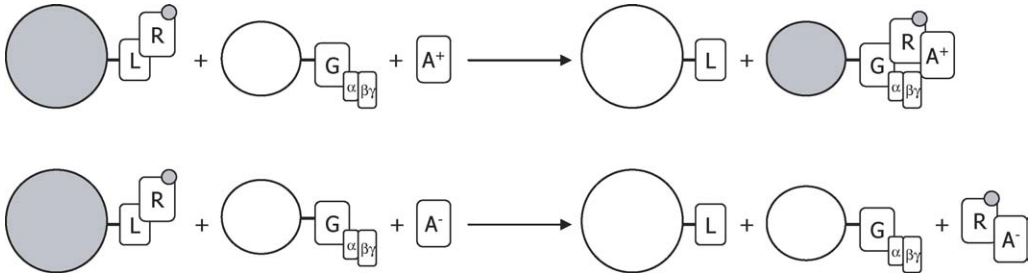

Fig. 6. Multiplexed flow cytometric set-up to determine the affinity and intrinsic activity of analytes. Ligand- and G-protein-beads (L and $G_{\alpha\beta\gamma}$) of different sizes are discriminated based on different light scattering. If an agonist is present (A^+, situation 1), this will result in a decreased fluorescence at the ligand-bead and an increased fluorescence at the G-protein-bead. If an antagonist (A^-) is present (situation 2), this will result in a decreased fluorescence at the ligand-bead, and no fluorescence at the G-protein-bead. The receptor ($R^\bullet$) is fused to, e.g. enhanced green fluorescent protein (see text).

are presented. The receptor is namely not able to bind to the G_s-protein-beads spontaneously or in the presence of an antagonist [75]. Simons et al. [78] demonstrated the same results, but made use of Texas-Red™ colored G-protein beads and blank DHA-beads. Despite these promising results in studying ternary complex formation by flow cytometric analysis, it remains difficult to set-up such assays for screening purposes, especially due to the necessity of the immobilized G-protein to be oriented correctly for recognition.

Summarizing, flow cytometry is an interesting alternative to the above mentioned assay formats, since it can measure and quantify molecular interactions in a sensitive and specific manner, combined with high throughput of samples, multiplexing and the possibility to perform kinetic analyses, in a non-separating homogeneous assay format [76]. Moreover, it has been shown that flow cytometric analysis can be integrated in a miniaturized assay format (microfluidics) [79]. While established assays are as such cost effective, initial set-up of an assay is time-consuming and thus rather expensive [18], due to the requirement of immobilization and fluorescent labeling of the interacting partners. An overview of bead-based assays, like the non-radioactive AlphaScreen™, flow cytometry and FMAT is given by Meza [70].

4.2.7. *Fluorescence correlation spectroscopy (FCS)*

Fluorescence correlation spectroscopy is a technology that determines receptor–ligand binding in a homogeneous fashion by measuring intensity fluctuations as a result of differences in diffusion rate of individual dye-labeled ligands free in solution or bound to a high-molecular weight receptor. Since fluctuations of the fluorescent signal are governed by the number and quantum yield of the fluorescent molecules, it is necessary to reduce the irradiated volume such that individual molecules can be measured. This is achieved by using diffraction-limited laser beams and confocal detection optics, in combination with pinholes in the image plane, generating observation volumes in the order of femtoliters (μm^3) [80,81]. Laser systems in FCS are based on Helium–Neon (He–Ne)-, Argon (Ar)- and Argon–Krypton (Ar–Kr)-lasers with excitation wavelengths λ_{ex} (He–Ne) of 543 nm and 633 nm, λ_{ex} (Ar) of 488 and 514 nm and λ_{ex} (Ar–Kr) of 568 and 647 nm. The tiny volumes, in which the few diffusing fluorophores with concentrations in the nanomolar range are excited allow to follow the motion of single molecules measured with single-photon detectors. Via autocorrelation analysis of the fluctuations in the obtained signal, it is possible to determine the number and brightness of molecules and their characteristic diffusion time, which is related to the size and shape of the molecules [82]. The experimental set-up for FCS and its fundamental principles have been described and the reader is referred to these publications for details [80,81,83–85]. By measuring the average correlation time of the fluorescent labeled ligand in solution, it is possible to discriminate between free and bound labeled ligand. Binding to the receptor results in a change in mass and thus in a reduced rate of diffusion, which in turn leads to an increased average time spent in the irradiated volume, resulting in multiple photons being emitted from the labeled ligand/complex within a given time. As these photons are emitted from the same labeled ligand/complex moving by Brownian motion in the volume, they are correlated in time. The average correlation time is thus a measure of the fraction of ligand that is bound to the receptor and will be at its maximum if the fluorescent labeled ligand saturates the receptor (see Fig. 7).

Receptor–ligand interactions have been monitored by FCS for the $GABA_A$ receptor [86], the nicotinic acetylcholine receptor (nACh-R; [87]), the 5-HT_{3A} receptor [88] and the single transmembrane EGF receptor [82]. An example of the application of FCS is the study of biomolecular interactions of the $GABA_A$ receptor with the ligand muscimol labeled with Alexa 532 (Mu-Alexa) [86]. To this end, neuronal cells containing the $GABA_A$ receptor from rat hippocamus were cultured on coverslips. The affinity of muscimol for the receptor was hardly influenced by labeling with Alexa 532 as determined by a radioreceptor assay. The FCS detection element was calibrated on the basis of a defined concentration of Rhodamine (Rho) 6G corresponding to the spectral line (514 nm) of the argon ion laser. From the diffusion coefficient of Rhodamine 6G, it was possible to determine all diffusion coefficients via Eq. (6) [88].

$$D_x = \frac{\tau_{Rho}}{\tau_x} \times D_{Rho} \quad (6)$$

where D is the diffusion coefficient, τ is the correlation time, x is the ligand to be measured.

In order to determine the binding parameters K_d and B_{max}, the cells were incubated with 300 μl droplets of labeled muscimol in increasing concentrations. Background fluorescence of endogenous cell components was taken into account as artefact

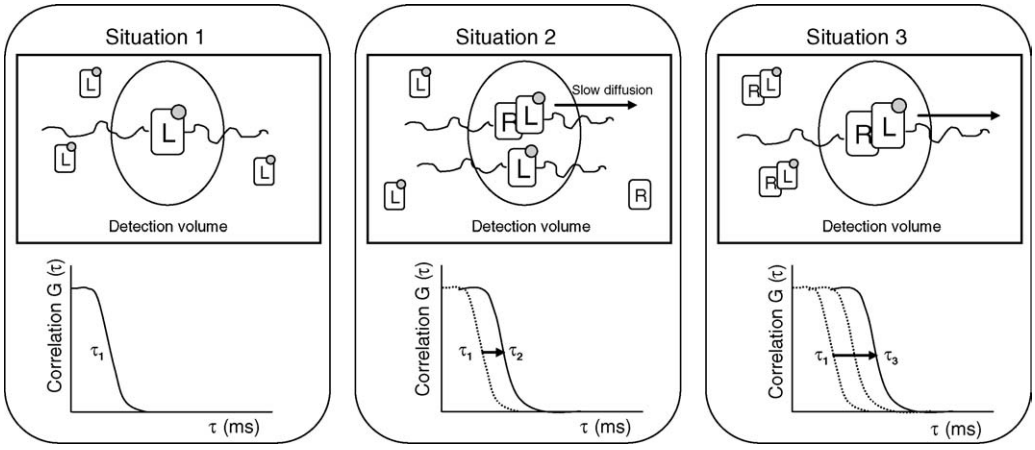

Fig. 7. Principle of fluorescence correlation spectroscopy: in situation 1, only free labeled ligand (L•) is present, which results in a low average correlation time (τ_1) as a consequence of rapidly diffusing molecules. At a 1:1 free ligand to ligand–receptor complex (RL•, situation 2), there is an increase in mass resulting in a decreased diffusion rate and thereby an increased averaged correlation time (τ_2), reaching a maximum (τ_3) as the labeled ligand saturates the receptor (situation 3).

in the determination of the ligand–receptor binding constants. The correlation time for freely diffusing Mu-Alexa in solution was determined in independent experiments and kept constant during all fitting procedures. Upon addition of increasing concentrations Mu-Alexa to the $GABA_A$ receptor on the slide, the diffusion time constant (τ) increased eventually reaching a maximum as a consequence of saturation of Mu-Alexa binding to the $GABA_A$ receptor on the neuronal cells. The specificity of binding was demonstrated by competitive displacement with a 1000-fold excess of muscimol hydrobromide [86]. It should be noted that it is crucial to keep the neuronal cells healthy during FCS-analysis. Non-living cells lack the fluidity of the lipid bilayer resulting in immobility of the ligand–receptor complexes. Buffer composition and pH are therefore important parameters during the binding experiments. Moreover, it is necessary to properly position the focus of the laser beam on the membrane surface and not to let the cells move on the coverslide during FCS-analysis [89]. The binding constants obtained using FCS were comparable to those found in the classical radioreceptor assay. As this assay was performed on living cells, it was not only possible to study receptor–ligand binding properties, but also the mobility of the receptor–ligand complexes in the cell membrane resulting, for example, from interaction with the cytoskeleton. For more details, the reader is referred to the article by Meissner and Häberlein [86].

The possibility to study receptor–ligand interactions at the molecular level on a living cell is one advantage of FCS if compared to traditional receptor assays, which make use of cell membrane preparations or solubilized and reconstituted receptors. Extracting a receptor from its natural membrane environment can, however, lead to structural alterations resulting in altered ligand affinities. Another advantage of FCS is that there is no need to immobilize a ligand or receptor as in the case of biosensors (see next section) or bead-based assays. However, as for all fluorescence-based techniques, the ligand has to be covalently labeled with a fluorophore and this labeling may affect the ligand's affinity towards the receptor. Moreover, the fluorescent labeled ligand should be water soluble, have a high quantum yield and a good photostability [86,90]. The latter is especially crucial in order to withstand the enormous power of the laser focused on a tiny volume. FCS can also detect the interaction of ligands with rapid binding kinetics. This is an advantage over filtration assays, which are only possible if the dissociation half-time of the receptor–ligand complex is significantly longer than the time required for the separation process [86]. Moreover, FCS is especially suitable for high-throughput screening where it combines assay times on the order of seconds with nanoliter sample volumes.

4.2.8. Receptor-based biosensors (chip-based assays)

All biosensor technologies, such as surface plasmon resonance (SPR) and total internal reflection fluorescence (TIRF) require the immobilization of either the receptor or the ligand on a surface. Several surfaces have been used, such as metaloxide, glass, quartz and gold surfaces. Immobilization of receptor proteins on a solid support has often proven to be difficult due to the loss of functional integrity, especially for membrane proteins that consist of several subunits with transmembrane spanning domains [91]. Receptors can be covalently attached by chemical cross-linking or non-covalently deposited on the surface. Covalent immobilization may lead to irreversible structural alterations and moreover gives rise to random orientation of the proteins on the surface [92]. Non-covalent immobilization of the receptor can be achieved via adsorption, via incorporation in lipid bilayers, via an affinity tag, such as hexahistidine or biotin or via antibody capturing. Crucial parameters to consider are the receptor's structural integrity and the orientation of the receptor protein so that the ligand can be bound without steric restrictions [93–95]. In addition to the requirement of unchanged receptor affinity and specificity, the receptor should not denature or be released from its support during analysis and the support should demonstrate limited non-specific ligand binding. Moreover, immobilization of the receptor protein onto a solid support is a slow, tedious process that needs to be optimized for every receptor [96].

For transmembrane receptor proteins to be successfully immobilized onto a solid support, the presence of their natural environment, the lipid bilayer, is often required. Direct immobi-

lization of the lipid bilayer onto the surface and the subsequent reconstitution of the receptor protein in the immobilized membrane often results in low receptor densities and thus a poor signal, as a result of denaturation of the receptor on the surface. Different approaches towards integrating a correctly oriented and structurally integer transmembrane receptor into a lipid bilayer have been described. The addition of thiolipids (e.g. PE-SH) that consist of a hydrophilic polyethylelene glycol spacer, gives rise to a hydrophilic layer between membrane and the chip surface, which was shown to favor maintenance of receptor's functional integrity [93,97]. Another approach to spatially separate the lipid membrane from the glass (quartz) surface of the chip makes use of a hydrophilic polymeric cushion (hydrogel), such as polyethylene imine (PEI) or dextran. The hydrogel creates additional space for the protruding loop domains of the transmembrane protein, thereby maintaining receptor functionality [98]. However, the incorporation of receptor protein into a lipid bilayer results in random orientation of the receptor. Moreover, the use of lipid bilayers limits the access to one surface of the receptor. Capturing methods with immobilized affinity tags or antibodies give rise to more evenly oriented receptor surfaces also facilitating research towards agonist-induced interactions between immobilized receptor and G-proteins. Capturing a receptor on a surface via affinity tags normally requires that the receptor is genetically engineered and produced in recombinant form (see Section 5). In addition, the receptor should be purified and remain stable in a functional form upon capturing via an affinity tag or antibody [99]. Some of the immobilization technologies are presented in Fig. 8.

An important chip-based approach to be mentioned here are the microarrays, which consist of grids that contain small amounts of receptor proteins in high density [100]. Microarrays form a suitable platform for the simultaneous determination of ligand binding towards multiple receptors [101]. A range of detection principles can be adapted to microarrays but fluorescence is most widely used. Performing many assays in parallel is relevant for high-throughput screening during drug discovery and can result in significant savings in reagents as compared to microtiter plate assays. Immobilization of integral membrane proteins onto a support is, however, a difficult task as outlined above and constitutes the major obstacle of applying microarrays to receptor binding assays. Accurate application of many different proteins onto a solid surface, using, e.g. jet or contact printing in a high-throughput fashion, remains difficult as protein activity and stability depends on the used surfaces [102,103]. Moreover, as miniaturization results in problems of evaporation and thereby denaturation and inactivation of proteins, it is important to add humidifier during preparation of the arrays, their storage and shipment and during analysis. The problem of evaporation can be reduced by using matrix slides (prepared by photolithography) or nanowells instead of glass slides. These chip formats are, however, more expensive than glass slides [103].

A microarray format for a GPCR, the human neurotensin receptor (subtype 1) was described by Fang et al. [101,104]. The membrane-associated neurotensin receptor was spotted onto γ-aminopropylsilane-coated slides and each array was incubated with the fluorescent labeled ligand with or without a competitive ligand. After careful removal of the solutions with a pipette tip attached to a vacuum pump, the slides were washed and after drying imaged in a fluorescence scanner. In an analogous manner, the β_1-, β_2-, and α_{2A}-adrenergic receptor subtypes were spotted on a microscope slide and incubated with a fluorescent β-selective antagonist. By addition of competitive ligands with known selectivities towards the β_1- or β_2-adrenergic receptor, fluorescence intensities were differentially decreased, demonstrating the possibility of multiplexing using this GPCR microarray [101].

4.2.8.1. Surface plasmon resonance (SPR). Surface plasmon resonance [105,106] is a method that allows to follow molecular interactions without the need for labeling. SPR measures changes in refractive index and thus in resonance angle at which polarized light is reflected from a surface, which is in turn related to a change in mass or layer thickness [107,108]. Thus, if polarized light strikes a gold layer at the interface between media of different refractive indices (e.g. glass and buffer) at a fixed wavelength and above a critical angle (total internal reflection), the photons are absorbed into surface plasmons (electron den-

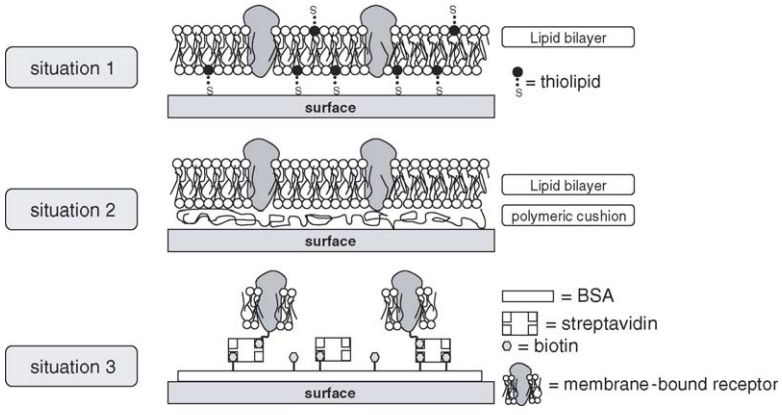

Fig. 8. Immobilization strategies for the analysis of biomolecular interactions of membrane receptors with their ligands via receptor-based biosensors. Situation 1: the lipid membranes are tethered to the surface via thiolipids; situation 2: a lipid bilayer is adsorbed on a spongy matrix, formed by a hydrogel polymer; situation 3: BSA-biotin is deposited on a gold or glass surface to immobilize a biotinylated receptor through a streptavidin interface.

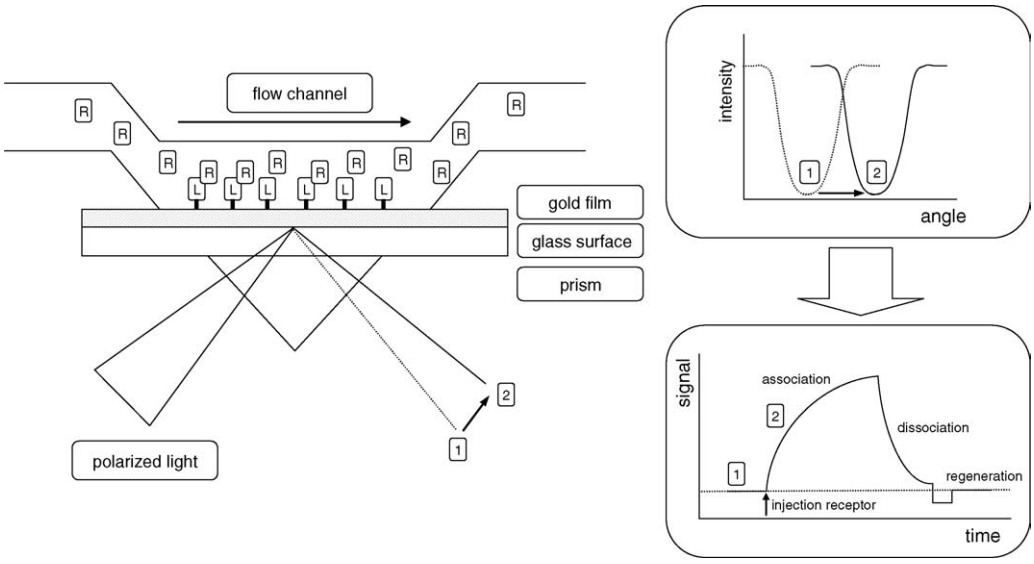

Fig. 9. Schematic representation of an optical device for the analysis of interactions using surface plasmon resonance. One of the interacting partners is immobilized on a modified gold surface and the other flows over the surface of the sensor chip allowing interaction (association) with the immobilized molecules. This gives rise to an increase in mass, and thereby a change in refractive index and the angle of reflected light ($1 \rightarrow 2$). As soon as the injection is stopped and washing continued with buffer alone, the receptor dissociates from the ligand resulting in a decrease of the signal due to a shift in the angle of the reflected light to its original position (position 1). In the described case, the ligand (L) is immobilized and the receptor (R) flows over the sensor surface, but the inverse configuration is feasible.

sity waves) resulting in resonance and no reflection of light. If the refractive index changes at one side of the surface as a consequence of immobilization of the receptor or the ligand, the resonance angle is changed and the intensity of the reflected light is increased.

The surface plasmon resonance device consists of a sensor chip, a flow cell, a light source, a prism and a detector that is positioned at a fixed angle (Fig. 9). The sensor surface consists of a thin gold layer (∼50 nm) fixed onto a glass surface with an interaction layer of, e.g. carboxymethyl-dextran [108,109] or BSA-biotin [110]. The carboxylic acid group on this layer can be activated to immobilize either the receptor or the ligand covalently. Alternatively, affinity capturing methods via biotin, antibodies or fusion tags can be applied, which opens the possibility of regenerating the chip surface, but have the potential disadvantage that the immobilized molecules might be "bleeding" off the surface [94,95]. Other immobilization techniques with respect to membrane receptor proteins have been applied by tethering lipid bilayers via thiolipids or polymeric hydrogel cushions, as discussed above. As surface plasmon resonance is dependent on changes in mass, it is advantageous to attach the molecule with the lowest molecular weight to the surface and measure binding of the higher molecular weight partner. Nevertheless, due to possible difficulties of immobilizing low molecular weight ligands (e.g. loss in binding affinity), it might be necessary to attach the high-molecular weight receptor to the surface and work with a smaller signal [108]. The latter approach has the benefit of requiring less receptor [111], but has an important limitation in that denaturation of the immobilized receptor may occur upon repeated use.

Several receptors have been immobilized onto sensor surfaces, amongst which the β_2-AR [110,112], rhodopsin [97,109], the IL-2-receptor [113] and the nACh-R [93]. For example, in the case of the β_2-AR [110], BSA-biotin was deposited on the gold surface, which absorbed spontaneously and was stable during buffer washes. The available biotin on the surface was subsequently treated with streptavidin or avidin to create a surface that would in turn have a strong affinity for biotinylated ligands or receptors ($K_d = 1$ fM). The β_2-AR containing a FLAG peptide fusion at its N-terminus was immobilized via a biotinylated anti-FLAG M1-antibody in a calcium-dependent manner. By immobilization of the β_2-AR at its N-terminus, the cytosolic C-terminus will be oriented away from the surface, which makes the ligand-binding sites uniformly accessible.

The application of surface plasmon resonance to measure membrane receptor–ligand interactions has been described in detail for the nicotinic acetylcholine receptor. Receptor–ligand binding properties were determined using immobilized nACh-R in tethered membranes. For immobilization, the receptor had to be reconstituted in thiolipid containing liposomes. These thiolipids contain a hydrophilic polyethylene glycol spacer and a terminal thiol moiety in the lipid headgroup, which allows the bilayer to be anchored to the gold surface with a hydrophilic layer between bilayer and surface. Binding affinities of the agonist carbamoylcholine (carbachol) and the antagonist α-bungarotoxin were measured in the presence of an antibody that competes with the ligands for receptor binding. Due to the low molecular mass of the ligands, it was necessary to use such an antibody to measure a significant change in signal intensity. Another possibility to circumvent the small difference in signal was to label α-bungarotoxin with biotin and enhance the signal by addition of streptavidin [93].

The sensitivity of the assay may be increased by immobilizing the ligand on the sensor surface thereby generating a large signal

due to binding of the high-molecular mass receptor as described by Kröger et al. [114] for the nicotinic acetylcholine receptor. In this approach, the biotinylated antagonist α-bungarotoxin was immobilized onto a streptavidin coated gold surface and the receptor was solubilized or reconstituted in liposomes in order to suppress non-specific binding. The liposomes were sterically stabilized by introducing poly(ethyleneglycol)-lipids in order to prevent binding between the liposomes and the surface or proteins. The receptor preparation was preincubated with defined amounts of ligand, e.g. carbachol and subsequently injected onto the biosensor. A high concentration of carbachol added to the receptor suspension gives rise to a small increase in the SPR response because of low receptor binding to the surface and vice versa [114].

The main advantage of SPR is thus the monitoring of molecular interactions in real time without the use of labels. The major limitation is that one of the binding partners needs to be immobilized.

4.2.8.2. Total internal reflection fluorescence (TIRF). This technique is based on the binding of a fluorescent-labeled ligand to an immobilized receptor on a sensor surface. The detection principle corresponds to surface plasmon resonance, but the signal is not dependent on the mass of the ligand, but makes use of the sensitivity of fluorescence. In this case, the fluorescent molecules that are present near the surface (within ∼100 nm) are excited by the electron density waves (plasmons) resulting from polarized light that undergoes total internal reflectance above the critical angle [91,115]. The different attachment procedures and the difficulties related to the immobilization of membrane-bound proteins are similar to those described for SPR.

Schmid et al. [91,116] immobilized the purified histidine-tagged serotonin receptor ($5HT_3$-R) via a nickel(II) nitrilotriacetic acid (Ni-NTA) chelating group on a microscope slide and investigated the binding of the fluorescent ligand (GR119566X-fluorescein) with high sensitivity in a mass-independent manner and in real time with direct discrimination between bound and unbound ligands. Since only those fluorescent ligands that are near the surface and thus bound to the receptor are excited by the electron density waves, the signal is not affected by fluorescent ligands in the bulk buffer solution. The amount of receptor recovered from the surface by specific elution with imidazole was quantified using a radioreceptor assay indicating no loss in affinity. The pharmacological properties of agonists and antagonists towards the receptor were studied by mixing the competing ligands at various concentrations with the fluorescent tracer ligand before measuring binding to the immobilized receptor with TIRF. The obtained results correlated with the affinities determined for the native membrane-bound receptor by radioreceptor assay. The amount of receptor immobilized on the surface (∼454 molecules/μm^2) was large enough to measure receptor–ligand interactions. The surface should not be overloaded with receptor in order to avoid steric hindrance and mass-transport limited binding. TIRF can be combined with microfluidics resulting in a sensitive high-throughput assay for the measurement of binding constants, with minimal receptor and reagent consumption.

4.2.8.3. Chip-based mass spectrometry. Chip-based mass spectrometry combines the use of adsorptive surfaces (e.g. ion exchange, reversed-phase, receptor, antibody) to purify and enrich analytes of interest from biological materials with mass-spectrometric analysis [117–119]. If the sample is applied on a chip with immobilized receptors, ligands with affinity for the receptor are retained and thereby concentrated in a small volume after a washing procedure with appropriate buffers. In the case of matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS), a matrix solution is subsequently added to permit ionization. In most cases, a pulsed UV-laser (N_2; λ = 337 nm) irradiates the sample resulting in desorption/ionization to form gas-phase ions. Ions are discriminated based on their mass-to-charge (m/z) ratio measured in a time-of-flight (TOF) mass analyzer after acceleration in a defined electric field [118]. The advantage of this technique lies in the ability to measure multiple ligands in one sample (multiplexing) as long as they differ sufficiently in m/z and to obtain spectra from numerous samples within a short time requiring little sample preparation and no chromatographic separation. Application of the generally acidic matrix and the use of the UV-laser will, however, likely irreversibly denature the immobilized receptors on the surface, which limits the re-use of the chip.

A quantitative immunoassay based on chip-MS has been shown by Nelson et al. [120] for myotoxin *a* and Mojave toxin from the venom of rattlesnake. To serve as internal standard for quantification, myotoxin *a* was chemically modified to H-myotoxin *a* by converting lysine to homoarginine for discrimination by the mass spectrometer. The antibodies against both toxins were immobilized on protein-A beads. The toxins were extracted using this affinity resin and subsequently eluted on a mass spectrometer target plate.

To determine interaction in terms of binding constants combined with compound identification requires, however, the need to couple the mass spectrometer to, for example, surface plasmon resonance [121–123] or frontal affinity chromatography (see Section 4.2.9.1). In SPR-MS, SPR was used to monitor the biomolecular interaction and the species retained on the chip were subsequently analyzed by MALDI-TOF-MS.

4.2.9. Other techniques

4.2.9.1. Quantitative affinity chromatography (QAC). Quantitative affinity chromatography can be used as a tool for the on-line determination of affinities using receptors immobilized on chromatographic matrices. Receptors, either in membranes, solubilized in mixed micelles or reconstituted in liposomes, have been immobilized on several solid-phase matrices, for example on immobilized artificial membrane (IAM) beads or by steric entrapment in the pore structures of gels (e.g. Superdex® beads). Other possibilities to immobilize receptors are based on the use of chromatographic supports that are covered with capturing molecules such as affinity tags and antibodies.

The IAM surfaces consist of either phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylglycerol (PG) or phosphatidic acid (PA). Both PC and PE are zwitterionic, whereas PS, PG and PA are negatively charged. Single chain phospholipids (PLs) were immobilized

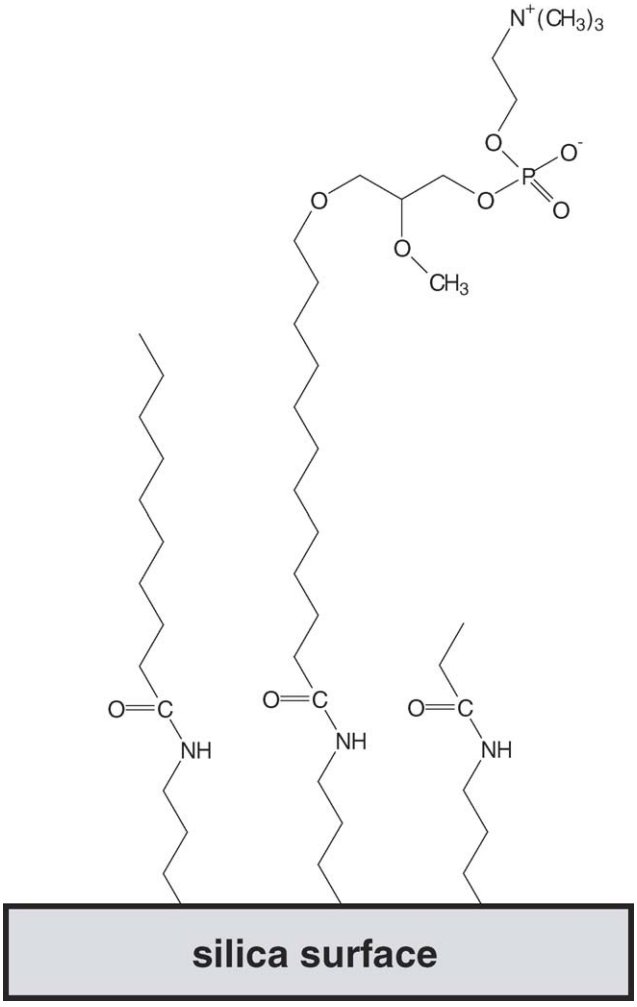

Fig. 10. Immobilized affinity membrane (IAM) chromatographic surface displaying single chain phosphatidylcholine (PC) immobilized on silica propylamine beads. The IAM beads have been "endcapped" with both decanoic (C_{10}) and propionic (C_3) acid anhydrides to block residual surface amines.

on silica propylamine beads with the advantage that binding of neighboring PLs is not sterically hindered by the conformational freedom of the non-bonded acyl chain. This gives rise to an increased density of immobilized PLs on the beads as presented in Fig. 10. PLs containing a polar headgroup (e.g. PG) have been derivatized with protecting groups to eliminate intermolecular bonding and to orient the immobilized phospholipid with its headgroup away from the silica surface. To decrease non-specific binding and to make the surfaces more stable over a broad pH range (pH 2–7.5), the IAM beads have been "endcapped" with both decanoic (C_{10}) and proprionic (C_3) acid symmetric anhydrides to block residual surface amines. Finally, the protecting groups on the polar headgroups of the PLs were removed under acidic conditions to form the IAM surface [124,125].

IAM beads (12 μm particles; 30 nm pore diameter) have been used to determine binding constants towards, e.g. the nAChR [126,127], the μ- and κ-opioid receptor [128], the β-AR [129] and the Pgp transporter [130]. Prior to reconstitution of the receptor on the phospholipid containing beads, the receptor was solubilized using a suitable detergent. This detergent should possess a relatively high critical micelle concentration to make detergent removal via dialysis possible.

Another way of making receptor-based stationary phases is by steric entrapment of receptors, reconstituted in liposomes or present as membrane vesicles, in Superdex® beads (average diameter 34 μm). The matrix of Superdex® consists of a network of agarose and dextran, which makes it more hydrophilic than the IAM beads. The mechanism, as proposed by Lundqvist et al. [131], states that the immobilization in a gel bead occurs by suction upon swelling (SUS) after freeze-thawing (FT). In other words, the proteoliposomes are absorbed into the cavities during rehydration of dry gel beads, where they remain trapped as a consequence of disruption and subsequent fusion to larger proteoliposomes during freeze-thawing. Trapped in the beads, the proteoliposomes are protected from mechanical disruption resulting in a stable chromatographic support. The glucose (GLUT1) [132,133] and nucleoside (NT) [134] transporter from red blood cells have been immobilized in this way. By immobilization in Superdex® beads, it is important to keep in mind, that the pore size of the beads is large enough to let the proteoliposomes or membrane vesicles pass and small enough to keep them trapped in the beads after fusion.

These receptor-based stationary phases are subsequently packed into glass columns and receptor–ligand binding affinities are determined using a radiolabeled ligand with an on-line flow-through scintillation detector. Affinity-based chromatography can also be combined with other detection techniques, like mass spectrometry [135] or fluorescence, where the former has the additional advantage of identifying unknown ligands without the requirement of a label. The limitation of hyphenating affinity chromatography with MS lies in the selection of buffers, which should consist of volatile salts, and should be usually of low ionic strength. Moreover, as ligands are simultaneously detected, it is crucial to avoid ion-suppression, if the ligands co-elute [96]. Overall, the advantages of affinity chromatography lie in multiple ligand screening and the repeated use of the column (dependent on receptor stability and carry-over) as well as in the possibility to enrich ligands. Affinity chromatography requires immobilization of the receptor protein with all its possible difficulties and caveats as already discussed in Section 4.2.8. Moreover, the slow and serial nature of chromatography limits throughput.

QAC can be used in two different modes, the zonal and frontal mode. Overall retention on the column is caused by specific interaction with the immobilized receptor and non-specific interactions with the stationary phase itself, like the lipid bilayer and other membrane proteins [136]. During zonal chromatography, the ligand is applied to a column in a narrow band. As a result, the ligand will be diluted, resulting in the need for sensitive detection methods, especially for high affinity ligands that are strongly retained on the column. The zonal mode can be used for screening of ligands that interact with a receptor, but is not useful for the quantitative determination of binding constants, due to the unknown ligand concentration in the column.

In contrast to the zonal mode, a continuous stream of mobile phase containing a defined concentration of ligand runs through the column during frontal chromatography. Initially all of the

applied ligand will interact with the receptor until all of the receptor binding sites are occupied and breakthrough occurs. A plateau will be reached when a steady state between ligand and receptor has been established in the column [137]. Applying the ligand in the frontal mode provides quantitative information about its affinity to the immobilized receptor and the amount of active binding sites in the column by linear or non-linear regression analysis of the breakthrough curves [136,137]. During frontal analysis, the affinity of the labeled ligand is determined by varying its concentration or that of its competing non-labeled equivalent in the mobile phase. The elution volume (V) reflects the sum of specific (V_{spec}) and non-specific (V_{min}) binding sites of the labeled ligand [137]. To determine the association constants K_{LR}, K_{AR} and the number of binding sites R by frontal chromatographic analysis, Eqs. (7) and (8) can be used [133]:

$$\frac{1}{V - V_{min}} = \frac{1}{V_{min} \times [R] \times K_{LR}} + \frac{1}{V_{min} \times [R]} \times [L] \quad (7)$$

V is the elution volume of [L]; V_{min} is the non-specific elution volume of [L]; [R] is the receptor concentration; K_{LR} is the association constant receptor–ligand; [L] is the ligand concentration.

$$\frac{1}{V_{max} - V_i} = \frac{1 + [L] \times K_{LR}}{V_{min} \times [R] \times K_{LR}} + \frac{(1 + [L] \times K_{LR})^2}{V_{min} \times [R] \times K_{LR} \times K_{AR}} \times \frac{1}{[A]} \quad (8)$$

V_{max} is the total elution volume of [L]; V_i is the elution volume [L] in the presence of [A]; K_{AR} is the association constant analyte-receptor; [A] is the analyte concentration.

When $(V - V_{min})^{-1}$ is plotted against [L] and $(V_{max} - V_i)^{-1}$ is plotted against $[A]^{-1}$, linear plots are obtained. From the slopes and the intercepts binding constants can be calculated. The inverse of the calculated association constants gives the dissociation constants (K_d) for the interaction of ligand and analytes with the receptor in question [133]. A high concentration of active binding sites (R/bed volume) together with a high V_{spec}/V_{min} ratio increases the precision of the determination of these binding constants [137].

4.2.9.2. Affinity selection mass spectrometry. The identification of ligands with affinity for a receptor can be achieved by combining mass spectrometry with SPR or QAC, as already mentioned. These strategies require, however, the immobilization of one of the binding partners. Other affinity selection mass spectrometry-based strategies measure the ligand after size-exclusion chromatography (SEC-MS [138–141]) or pulsed ultrafiltration (PUF-MS [142,143]) or make use of the possibility to directly inject the receptor–ligand complex [144,145].

Direct injection of the receptor–ligand complex on the mass spectrometer has been applied to the cytoplasmic receptor FKBP (FK506 binding protein [144]). The soft electrospray ionization mass spectrometry (ESI-MS) technique was used to form gas-phase ions of the receptor and receptor–ligand complex directly from solution without fragmentation. Addition of a slight excess of FK506 (Tacrolimus) at pH 7.5 to FKBP resulted in an additional signal corresponding to the FK506-FKBP complex. Increased addition of FK506 did not give rise to increased relative intensities, which indicated saturation of the receptor. For this methodology to work and to give quantitative data, it is important that the receptor–ligand complex is not dissociated during the generation of gas-phase ions from solution. This may be achieved by modifying the ion source (higher pressure in the interface) and by choosing buffer conditions that allow both stability of the receptor–ligand complex and successful ionization. These rather restrictive conditions do not allow to measure every receptor–ligand interaction directly by electrospray ionization mass spectrometry. Notably membrane-bound receptors are difficult to maintain in solution without surfactants that interfere with the ionization process. These issues need to be considered in the determination of binding constants.

A more widely applicable strategy to achieve reliable binding data is the separation of the unbound from the bound fraction by size-exclusion chromatography or pulsed ultrafiltration. Annis et al. [138] described a multidimensional chromatography mass spectrometry method for the muscarinic M2 receptor. Atropine was used as the ligand and was incubated to equilibrium with the muscarinic M2 receptor. The sample was subsequently subjected to a rapid (<20 s) SEC stage thereby separating the receptor–ligand complex from the unbound ligand. The receptor–ligand complex was subsequently captured on a reversed-phase chromatography–MS system. The ligand was dissociated from the complex at high temperature (60 °C) and acidic (pH < 2) conditions.

In the case of PUF-MS [142,143], the receptor and receptor–ligand complex were trapped in a chamber fitted with a molecular weight cut-off ultrafiltration membrane. Low molecular weight ligands passed through the membrane and eluted from the chamber. The receptor and the receptor–ligand complex were subsequently captured on a HPLC column and the receptor–ligand complex was destabilized with an organic solvent or due to a pH change. In both SEC-MS as PUF-MS, the eluted desalted ligands were analyzed using mass spectrometry.

4.2.9.3. On-line liquid chromatography with biochemical detection. Another way to hyphenate techniques in order to separate and to determine the biological activity of ligands can be achieved by on-line reversed-phase liquid chromatography (RP-LC) coupled to a biochemical detection system, e.g. receptor affinity detection (RAD), as described by Oosterkamp et al. [146]. The RAD system consists of open-tubular reaction coils, where the fluorescent ligand is mixed with the receptor and a competing analyte. In the heterogeneous set-up of the RAD system (see Fig. 11), it is necessary to separate free from bound ligand, in contrast to the homogeneous set-up, where the change of fluorescence properties (quenching/enhancement) upon binding of the fluorescent ligand to the receptor is measured directly. Despite the fact that the homogeneous set-up is easier, it displays lower sensitivity resulting from background fluorescence of the receptor preparation and is not generally applicable for all fluorescent labels.

Separation in the heterogeneous set-up is done using a short column of restricted-access material (RAM [147]) containing

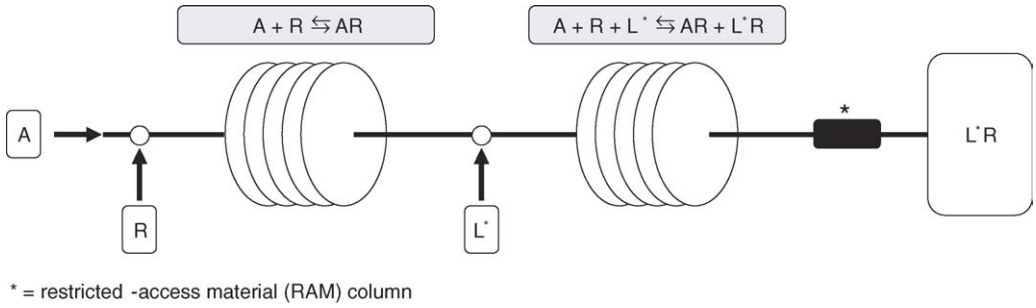

Fig. 11. Heterogenous set-up of an HPLC system coupled to a receptor affinity detector (RAD): in the first reaction coil the analyte (A, eluate from the HPLC) interacts with the receptor (R). The labeled ligand (L^*) subsequently competes with the analyte for the same binding site in the second reaction coil. The bound labeled ligand fraction, separated from free labeled ligand via a restricted-access material (RAM) column, is determined by a fluorescence detector. In the homogenous set-up, the RAM column is omitted.

C_{18}-bonded silica on the internal pore surface so that separation is based on mass difference between free and bound label and hydrophobicity of the free label. The use of a RAM column is limited to soluble receptors to avoid clogging in the column and to small ligands ($M_w < 10$ kDa) that can enter the pores and are retained due to hydrophobic interactions. Moreover, breakthrough of the fluorescent labeled ligand after a number of analyses complicates detection and requires frequent regeneration of the RAM column, especially if organic modifiers are present during the on-line coupling of reversed-phase LC to the RAD system [146,148]. The use of a hollow fiber consisting of a cut-off membrane allows to continuously separate bound from free ligand based purely on size without the need for frequent regeneration [148].

The assay principle has been demonstrated for the soluble estrogen receptor [146] and the soluble urokinase receptor [149], but not for integral membrane receptors. The technique uses either fluorescent labeled ligands [146,150] or fluorescent labeled proteins [151]. The use of a labeled protein requires the immobilization of a competing ligand on an affinity column of sufficient capacity to trap the excess of labeled protein [151].

The response in a continuous-flow RAD system is influenced by dispersion in the reaction coil(s) (reaction time), the concentration of both the receptor and the labeled ligand, the affinity of both the labeled ligand and the analyte and the stability of the receptor–ligand complex. Increasing the reaction time by a delayed addition of the labeled ligand results in better sensitivities and an increase in the receptor concentration gives rise to linearization of the calibration curves due to the interaction reaching its steady state. Adding a higher concentration of label results in an increase of the absolute signal from the receptor-labeled ligand complex, but also to an increase in background noise, if the homogeneous set-up is used [150]. A major problem in on-line coupling of the LC system with RAD lies in the fact that organic solvents used in the LC runs can denature the receptor at prolonged reaction times and thereby decrease the total number of binding sites. This counteracts the increased formation of analyte-receptor and labeled ligand–receptor complex during longer incubation times, as discussed above and requires careful optimization of the final modifier concentration in the reaction coil [146]. Nevertheless, due to the continuous-flow set-up of this technique, reaction times can be set to 1–2 min, instead of an average of 1–2 h in microtiter plate assays and some receptors have been shown to survive such short incubation times under mildly denaturing conditions [146,151]. Moreover, splitting the flow post-column provides the means for a combined analysis of ligands via RAD (biochemical analysis) and other detection systems, for example mass spectrometry (chemical analysis).

4.2.9.4. Microfluidics (lab-on-a-chip). Miniaturization of assay formats has long been a driving force for new technical developments. Miniaturization reduces reagent consumption and often allows to reduce assay times significantly. However, performing miniaturized assays in microtiter plates increases the demands on liquid handling equipment (dispensing, mixing) and evaporation control significantly often rendering the assays less robust. Furthermore, not all assay formats are amenable to miniaturization, as sensitivity decreases in, for example, the scintillation proximity assay.

Microscale total analysis systems (μTAS) provide a versatile, novel analysis platform that integrates sample handling, mixing, separation and detection in a single microfluidics device, where only submicroliter reagent volumes are required. Moreover, several analyses can be done in parallel reaction channels. The reagents are pumped through capillaries or channels via electroosmosis or via pressure driven flow. Detection is most often done based on fluorescence but coupling to mass spectrometry has also been described [152,153]. Chemiluminescence, on the other hand, is one of the most promising detection technologies for microfluidic systems due to high sensitivities (10^{-18} to 10^{-21} mol) and no requirement of an external light source [154].

Buranda et al. [155] demonstrated biomolecular recognition in microfluidic channels, where fluorescently labeled biomolecules of interest were attached to streptavidin-coated beads. In this case, quenching was directly monitored by pumping fluorescent labeled M1 anti-FLAG monoclonal antibodies through the microcolumn consisting of fluorescent FLAG peptide bearing beads. A microfluidics enzyme immunoassay was described by Yakovleva et al. [154]. The silica surface

of the microchip was modified by attachment of protein A or G via a hydrophilic polymer, such as dextran. The detection was based on chemiluminescence using horseradish peroxidase (HRP) mediated oxidation of luminol. The HRP labeled antigen competed with the analyte for the antibody binding sites off-line with subsequent trapping of the formed immunocomplexes on the protein A (or protein G) chip in the microfluidics device. The amount of bound labeled antigen was determined by injection of luminol/H_2O_2/*p*-iodophenol. The applicability of the immunosensor was demonstrated for the pesticide atrazine. The full potential of microfluidics has yet to be realized, especially in the quantitative analysis of receptor–ligand interactions.

4.2.9.5. Nuclear magnetic resonance spectroscopy (NMR). NMR spectroscopy is widely used in elucidating the structure of chemically synthesized compounds and biomolecules. Next to this main application, there are NMR techniques that allow the study of molecular interactions at the atomic level. Despite the fact that NMR is being more and more used in understanding receptor–ligand interactions including, e.g. competitive binding and allosteric effects that are otherwise difficult to study [156], it is fair to say that this technology has limitations due to its poor sensitivity and low throughput. It is beyond the scope of this review to go into detail concerning the different experimental approaches in NMR spectroscopy and the reader is referred to literature reviews by Lepre et al. [157] and Meyer and Peters [156] for further information.

5. The use of recombinant receptor proteins

Receptor assays rely on the availability of well controlled and reproducible sources of receptor protein. One widely available source is animal tissue. However, the presence of other pharmacologically similar receptors and the fact that non-human receptors may show different binding profiles than their human counterparts are potential drawbacks. It is also exceedingly difficult to purify appreciable quantities of receptors from animal tissue, which makes the development of well-defined assay conditions tedious or even impossible.

The limitations of receptor preparations from natural sources has driven the development of heterologous expression systems for human receptors both soluble and membrane-bound. This has greatly facilitated research on single human receptor subtypes. Despite much progress in the expression and purification of recombinant receptors, there is no universal host that provides high expression levels, homogenous receptors and expression of the receptor in its functional form, which testifies to the individuality of receptors [158].

Expression systems can be roughly divided into prokaryotic and eukaryotic host organisms, including insect and mammalian cells, each of which demonstrate advantages and disadvantages as shown in Table 4 and reviewed by several authors [158–162]. The advantages and disadvantages focus mainly on the expression of membrane-bound receptor proteins, as expression of soluble proteins has generally proven to be easier. This is due to

Table 4
Advantages, disadvantages and expression levels for heterologous expression of human membrane-bound receptors in prokaryotic and eukaryotic host organisms

Expression system	Host organism	Advantages	Disadvantages	Expression levels
Bacteria	*Escherichia coli* *Bacillus subtilis*	Low costs Protein homogeneity Rapid growth (generation time 20 min) Ease of plasmid construction	No post-translation modifications No endogenous G-proteins Incorrect protein folding and membrane insertion Different membrane lipid composition	0.2–16 pmol/mg
Yeast	*Saccharomyces cerevisiae* *Schizosaccharomyces pombe* *Pichia pastoris*	Rapid growth (generation time 2 h) Simple media Scale-up (fermentation) High cell densities Post-translational modification	Cell lysis on large-scale difficult due to strength of cell wall Low cholesterol content Low number of endogenous G-proteins	0.13–115 pmol/mg
Baculovirus/insect cells	*Spodoptera frugiperda* (Sf9) *Trichoplusia ni* (High five)	Post-translational modifications Correct protein folding and membrane protein insertion Endogenous G-proteins Relative ease to obtain large amounts	Slow growth (generation time 24 h) Complex culture media/expensive Protein heterogeneity Low abundance of cholesterol Limited number of endogenous G-proteins	0.15–80 pmol/mg
Mammalian cells	CHO-cells HEK-293-cells COS-7-cells	Perform complex post-translational modifications Endogenous G-proteins Comparable membrane lipid composition Correct protein folding and membrane protein insertion	Difficult to scale-up Complex culture media/expensive Slow growth (generation time >24 h) Protein heterogeneity	0.23–200 pmol/mg

CHO = Chinese hamster ovary; HEK = human embryonic kidney; COS = African monkey cells.
The range of expression levels were obtained from the review by Sarramegna et al. [161] on heterologous expression of GPCRs.

a lack of knowledge concerning membrane protein insertion and folding in comparison to folding of soluble proteins. Moreover, it should be noted that high expression levels are only reported for a few receptors, such as the β-adrenergic receptor in *Saccharomyces cerevisiae* (115 pmol/mg [163]) and in CHO cells (200 pmol/mg [164]). As described by Grisshammer and Tate [159], expression levels are dependent on the number of transmembrane regions and the requirement for post-translational modifications necessary for receptor activity. Moreover, in the case of GPCRs, the presence of high and low affinity binding sites can give rise to altered functional expression levels, due to the fact that antagonists label both affinity sites, where agonists label only the high affinity site.

Expression in a prokaryotic host, such as *Escherichia coli*, allows the production of large amounts of biomass. Nevertheless, the production of functional human receptor is often limited by the bacteria's inability to perform post-translational modifications, like glycosylation, and incorrect protein folding. Especially functional G-protein coupled receptors cannot be expressed in *E. coli*, due to the lack of endogeneous G-proteins, which are required for high affinity agonist binding [165].

The use of yeasts, like *S. cerevisiae* or *Pichia pastoris*, as expression hosts is a good alternative between the expression in bacteria (*E. coli*) and mammalian cells. This lower eukaryote is able to glycosylate proteins, while still growing rapidly to high cell densities on simple media. It has to be noted, however, that glycosylation in yeast is not the same as in higher eukaryotes like mammalian cells in culture, which may affect functionality of the receptor. The use of protease-deficient strains and targeted expression to the plasma membrane (e.g. via the N-terminal yeast α-STE2 gene [163]) overcomes some of the difficulties of expressing GPCRs in yeasts. Still altered binding properties are often observed due to the low cholesterol content in the yeast plasma membrane, the low number of endogenous G-proteins and differences in post-translational modifications [166,167]. Co-expression of G_α-proteins in both *E. coli* [165] and *S. cerevisiae* [163] resulted in high affinity agonist binding and G-protein coupling, respectively, indicating that further engineering of these expression hosts holds promise for the expression of a wider range of membrane receptors.

Baculovirus-infected insect cells (e.g. *Spodoptera frugiperda* (Sf9)) express the majority of membrane-bound receptors in a functional form. However, ligand–receptor binding properties might be altered due to the low cholesterol concentration in the membrane and the limited number of endogenous G-proteins. The latter becomes especially important at high expression levels [168]. The doubling time of insect cells is long (24 h) if compared to bacteria and yeast and expression requires complex media, which makes production rather expensive. Moreover, expression at very high levels gives rise to protein heterogeneity as a consequence of incomplete glycosylation and the presence of inactive protein. The latter can be judged from the discrepancy between the amount of protein (e.g. judged by SDS–PAGE with Coomassie staining) and the amount of ligand binding [158,159].

Heterologous expression of receptors in mammalian cells is the most appropriate approach if functional studies are to be performed, due to the close resemblance with the membrane environment receptors naturally occur in. Mammalian cells can perform complex post-translational modifications, contain numerous endogenous G-proteins and have comparable membrane lipid compositions. However, as in the case of insect cells, growth of mammalian cells is time-consuming, difficult to scale up and requires expensive and complex culture media.

Expression of receptor proteins in heterologous hosts opens the possibility of engineering the receptor protein, for example, by fusing affinity tags to the protein, which can be used for detection, purification and oriented immobilization. Most of the affinity tags are fused to the C-terminus of the receptor protein, but depending on the specific application (detection, immobilization, purification), the affinity tag can also be fused at the N-terminal or at both termini. Affinity tags greatly facilitate purification of proteins and thus functional and structural studies as well as the development of receptor binding assays. The fusion tags comprise polyhistidine-metal ion, antibody–antigen and biotin–streptavidin recognition elements. Especially the biotin–streptavidin interaction provides a good basis for robust and efficient immobilization of proteins on a solid surface as demonstrated for the β_2-adrenergic receptor in SPR applications. An overview of the commonly used affinity fusion systems is provided by Nilson et al. [169].

Enhanced green fluorescent protein (EGFP) can be fused at the N-terminus of the receptor, thereby creating a reporter of receptor expression, as demonstrated by Sarramegna et al. [170] for the human μ-opioid receptor (HuMOR) expressed in *P. pastoris*. EGFP was used to quantify HuMOR expression levels, whereas saturation ligand binding experiments with the antagonist [^{3}H]-diprenorphine quantified the level of functional receptor. While antagonist binding reached up to 1 pmol/mg protein, EGFP fluorescence demonstrated expression levels of 16 pmol/mg total protein. In this elegant manner, it was shown that only about 6% of the expressed receptor was functional.

In choosing the most appropriate host for a given receptor protein one has to consider yield, functionality, post-translational modifications and the possibilities for scale up [162]. From a pharmacological point of view, insect and mammalian cells provide the most successful host organism in relation to receptor activity. However, for structural studies by NMR or X-ray crystallography, where much larger amounts are needed, it is important to have high levels of homogenous protein expression, which favors *E. coli* and yeast over insect and mammalian cells [158].

6. The quantitative use of receptor assays in biological matrices

Next to the role of receptor binding assays in drug discovery and the elucidation of structure–activity relationships, they are also applicable to the quantitative determination of receptor binding ligands in complex biological samples [9,27]. Quantification of biological levels using a receptor assay provides an overall concentration of all analytes that affect binding, which

is likely better correlated to the pharmacological effect of a drug or drug metabolite than the concentration of individual ligands measured with chromatographic or mass-spectrometric methods or the concentration of all analyte-related ligands using immunoassays. Most ligand-binding receptor assays applied to quantitative bioanalysis, are based on classical radioreceptor assay formats requiring separation of free from bound ligand.

In the development of quantitative receptor assays for bioanalysis of drugs and their metabolites, a few basic factors need to be considered [9,27]. First, the incubation medium should be closely related to the human physiological condition if meaningful quantification of total biological activity is the goal. The effect of incubation time, temperature, pH and ionic strength on the affinity of the ligand for its receptor and on receptor stability need to be assessed. Especially the presence of sodium can be detrimental for agonist binding affinity in some receptors [171–173]. The second aspect is the choice of a suitable receptor preparation. Animal tissue can be obtained with relative ease at low costs; however, due to receptor heterogeneity, low expression levels and non-human pharmacology, an increasing trend towards the use of recombinant receptors is seen, as already discussed above. The ligand that is used in the binding assay, either radioactively or non-radioactively labeled, is a third factor to consider. In quantitative receptor assays, the labeled ligand should bind reversibly with high affinity to the receptor of interest and non-specific binding should be minimal. The K_d should be less than 10 nM to prevent loss of binding during separation of bound from free ligand, due to a fast dissociation rate. If the ligand, on the other hand, displays a dissociation constant of less than 10 pM, it will be difficult to achieve equilibrium in a reasonable time, due the slow dissociation rate. Moreover, the ligand should be chemically stable and resistant to enzymatic degradation. It is preferred to choose the most potent enantiomer to set-up a bioanalytical receptor assay. For a radioligand, it is advisable to choose one with high specific activity and purity. A fluorescent ligand should have an excitation wavelength larger than 450 nm to avoid interference from autofluorescence from the matrix, a high fluorescence intensity in aqueous media and be photostable (see also Section 3 for more details).

A receptor assay can be performed directly in the matrix if the concentration of the drug is high enough and the biological matrix does not interfere with the determination. Otherwise, it is required to perform adequate sample pretreatment steps to eliminate matrix interferences and to enrich the analyte of interest. Quantitative receptor binding assays need to be validated according to guidelines, as for example outlined in the Washington Conference Report on Analytical Method Validation [174,175] to demonstrate its performance and the reliability of the analytical results, especially if the methods shall be used in hospitals or clinical chemistry laboratories.

Quantitative receptor assays have been used to determine plasma levels of benzodiazepines [176] and serum levels of morphine and its active metabolite, mainly morphine-6-glucuronide (M6G) [177]. To correlate loss of memory with the benzodiazepine concentration, plasma concentrations were determined in suicidal patients, who took a large dose of benzodiazepines at once. Due to high benzodiazepine concentrations, no correlation was demonstrated between the benzodiazepine concentration in plasma and the severity of memory impairment. Nevertheless, the data suggested that loss of memory already occurred at low concentrations of benzodiazepines, where sedative effects manifested at higher benzodiazepine concentrations [176,178].

A direct radioreceptor assay for the determination of morphine and its active metabolites, mainly M6G, was developed and used to measure the opioid activity in serum of neonates. Morphine is used as an analgesic in neonates to relieve pain and to prevent stress during painful and invasive procedures. Morphine causes, however, serious side effects, mainly through its active metabolite M6G. The radioreceptor assay proved useful as a screening tool in the assessment of opiate activity in serum of neonates treated with morphine [177].

7. Summary

This review gives an overview of various assay technologies and applications for the measurement of receptor–ligand interactions. The measuring principles, advantages and disadvantages of the different assay technologies are discussed and highlighted on some practical examples. Over the past years, a shift from radioactive to fluorescence-based detection of receptor–ligand interactions has been observed, with emphasis on the mix-and-read assays. Moreover, mix-and-read assays with the ability to miniaturize and multiplex have emerged, as they are of great interest in high-throughput screening (HTS) in order to reduce reagent consumption and costs. A major drawback of the current detection technologies is the need to label and/or immobilize one or both of the interacting partners, with the possibility of altered binding characteristics and specificity. It is therefore difficult to have a universal non-radioactive mix-and-read assay format for all receptors currently known, which makes the classic radioreceptor assay still the method of choice in drug screening. Genetic engineering of receptors and their expression in heterologous hosts open new possibilities for labeling and immobilization, which facilitate the development and implementation of mix-and-read assays in routine HTS.

Nomenclature

α-AR	α-adrenergic receptor
β-AR	β-adrenergic receptor
δ-OR	δ-opioid receptor
ε	molar extinction coefficient
τ	correlation time/diffusion time constant
μTAS	micro total analysis system
5-HT	5-hydroxytryptamine
A	analyte
A^+	agonist
A^-	antagonist
Ab	antibody
Abs	absorbance
Alpha	amplified luminescence proximity homogeneous assay

AP alkaline phosphatase
AR analyte–receptor complex
Ar Argon
Ar–Kr Argon–Krypton
BDZ-R benzodiazepine receptor
B_{max} maximal binding sites
Bodipy borondipyrromethine
BRET bioluminescence resonance energy transfer
BSA bovine serum albumin
CCD charge-coupled device
CHO Chinese hamster ovary
COOH carboxylic acid
COS African monkey cells
D diffusion coefficient
DHA dihydroalprenolol
DMSO dimethylsulfoxide
DNA deoxyribonucleic acid
EGF epidermal growth factor
EGFP enhanced green fluorescent protein
E_m/λ_{em} emission wavelength
ER estrogen receptor
ESI electrospray ionization
E_x/λ_{ex} exitation wavelength
FCS fluorescence correlation spectroscopy
FMAT fluorometric microvolume assay technology
FP fluorescence polarization
FRET fluorescence resonance energy transfer
FT freeze-thawing
$GABA_A$ gamma-aminobutyric acid
GFP green fluorescent protein
GLUT1 glucose transporter
GPCR G-protein coupled receptor
GRF growth hormone releasing factor
GTP guanine triphosphate
HEFP high efficient fluorescence polarization
HEK human embryonic kidney
He–Ne Helium–Neon
hIL-5 human interleukin-5
hM1-R human muscarinic-1 receptor
HRP horseradish peroxidase
HTS high-throughput screening
HuMOR human mu-opioid receptor
I.D. internal diameter
IAM immobilized artificial membranes
IC_{50} concentration inhibitor displacing 50% bound labeled ligand
I_p parallel intensity
I_s perpendicular intensity
k_{+1} association rate constant
k_{-1} dissociation rate constant
K_{AR} association constant A-R
K_d dissociation constant
K_i affinity constant
K_{LR} association constant L-R
L^* labeled ligand
LC liquid chromatography
L^*R labeled ligand–receptor complex
M6G morphine-6-glucuronide
MALDI matrix assisted laser desorption ionization
MeOH methanol
MS mass spectrometry
Mu-Alexa muscimol labeled with Alexa 532
MVF microvolume fluorometry
Mw molecular weight
nACh-R nicotinic acetylcholine receptor
NBD 7-nitrobenz-2-oxa-1,3-diazole
Ni-NTA nickel(II) nitriloacetic acid
NK1 neurokinin-1
NMR nuclear magnetic resonance
NT nucleoside transporter
OR opioid receptor
PA phosphatic acid
PC phosphatidylcholine
PDGF-R platelet-derived growth factor receptor
PE phosphatidylethanolamine
PEI polyethylene imine
PE-SH phosphatidylethanolamine-SH
PG phosphatidylglycerol
Pgp P-glycoprotein
PL phospholipid
PS phosphatidylglycerol
PUF pulsed ultrafiltration
QAC quantitative affinity chromatography
QY quantum yield
R receptor
RAD receptor affinity detection
RAM restricted-access material
Rho rhodamine
Ro-1986 didesethylflurazepam
RP-HPLC reversed-phase high-performance liquid chromatography
RRA radioreceptor assay
SAR structure–activity relationship
SE succinimidyl ester
SEC size-exclusion chromatography
Sf9 Spodoptera frugiperda
SPA scintillation proximity assay
SPR surface plasmon resonance
SUS suction upon swelling
TIRF total internal reflection fluorescence
TMB tetramethylbenzidine
TOF time of flight
TRF time-resolved fluorescence
TR-FRET time-resolved fluorescence resonance energy transfer
UV ultraviolet
V elution volume of L
V_i elution volume L in the presence of A
V_{max} total elution volume of L
V_{min} non-specific elution volume L
V_{spec} specific elution volume of L
WGA wheat germ agglutinin
x the ligand to be measured
Xe xenon

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