Optimization of SPA receptor binding assays.

Review

Receptor-ligand interactions have been extensively used as targets for drug screening. Scintillation Proximity Assay (SPA) is a well-established homogeneous screening technology. In this article, the key issues about the optimization of SPA receptor assays are discussed.

Scintillation proximity assay has been successfully applied to receptor binding assays by immobilizing receptors directly on to SPA beads via several coupling methods. Once immobilized, the receptor is close enough to the bead so that, should a suitably labeled radiolabeled ligand binds to the receptor, it will be in close enough proximity to stimulate the bead to emit light (See Figure 1). Any unbound radioligand is too distant from the bead to transfer energy and goes undetected. The bead therefore only detects the population of ligand molecules which are receptor-bound. The discrimination of binding by proximity means that no separation of bound and free ligand is required, as in traditional methods.

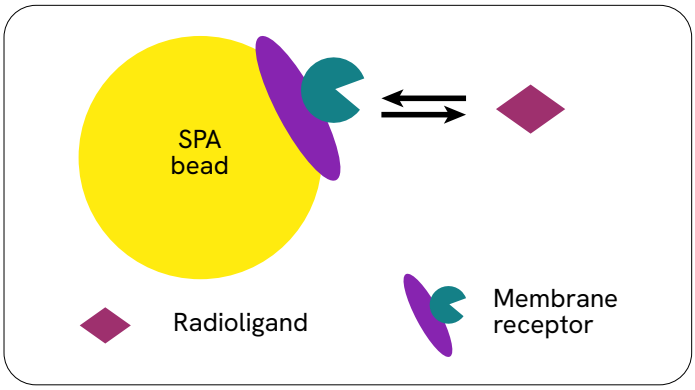
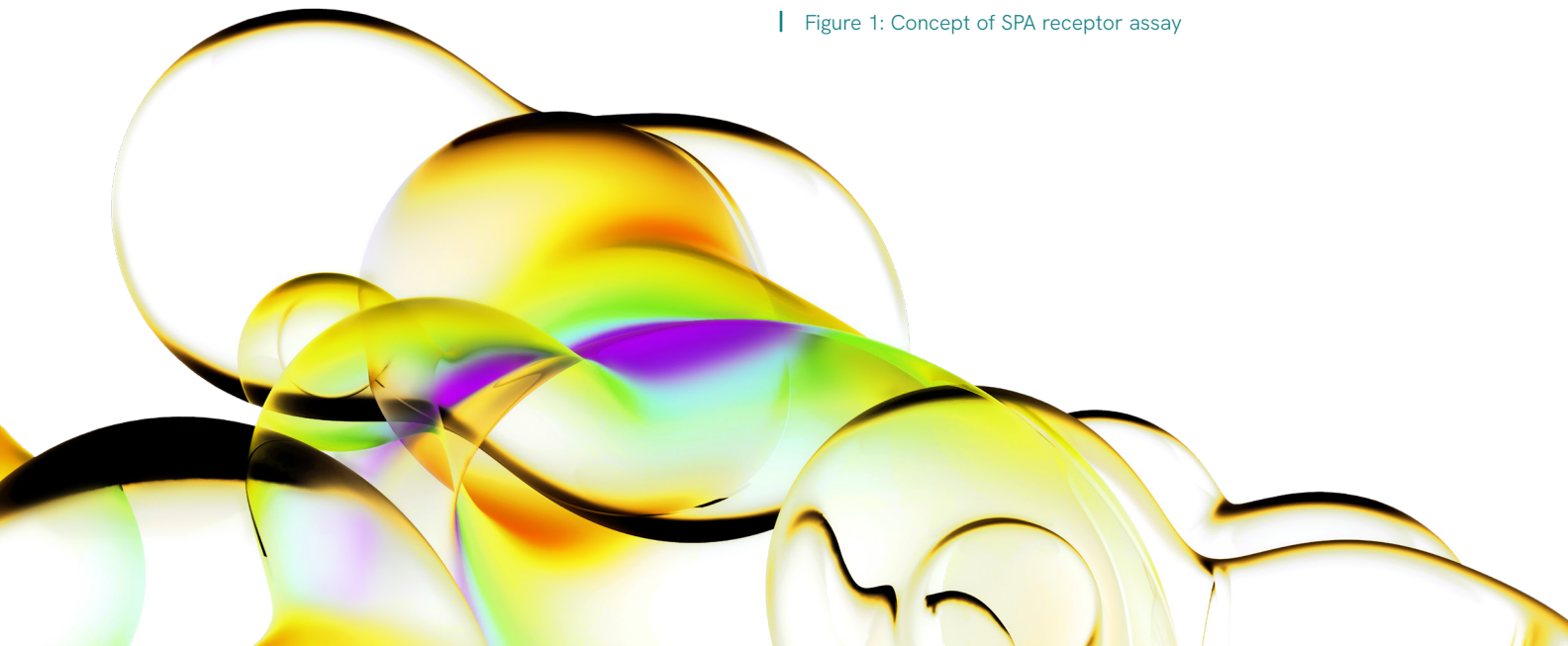


Figure 1: Concept of SPA receptor assay



SPA receptor binding assays have been developed using receptors prepared from several different sources ranging from crude tissue preparations to soluble purified proteins (see Table 1 preparations). The method is generally applicable to both [^{3}H] and [^{125}I] labeled ligands provided a number of factors are carefully optimized in the assay design and development.

Table 1: Examples of SPA receptor assays.

Transforming Growth Factor α[1]
Epidermal Growth Factor[2]
Somatostatin, GHRH[3]
4F2hc-LAT1[4]
Calcium-sensing receptor (CaSR)[5]

The following comments on assay development apply generally to the assay of receptors in membranes, which are derived from tissues or cultured cell-lines and employ either wheat germ agglutinin polyvinyl toluene (WGA-PVT) beads or poly-lysine coated yttrium silicate (PL-YSi) beads. Approaches involving antibodies and biotinylation can be used for soluble receptors[4], but these will not be discussed further in this article.

Development of SPA receptor binding assays requires much of the same experimentation as for the development of assays by traditional methods. However, some key issues will be highlighted prior to a discussion of the assay development process.

SPA is a homogeneous assay technology. This means that the binding event is not disturbed by a separation step. It is therefore possible to monitor the rate of association and/or dissociation of a labeled radiolabeled ligand from its receptor. The lack of a separation step is also an advantage when assaying receptors of weak affinity (e.g., soluble epidermal growth factor receptor[6]), where filtration or washing steps may shift the apparent equilibrium of the binding event under study. However, the lack of a separation step introduces some phenomena which are not normally apparent with traditional methods. These issues are outlined below.

SPA is an extremely powerful technique when large numbers of samples are required to be assayed simultaneously. The removal of the separation step means that SPA assays are fast, simple, and easy to automate. However, to measure many samples the assay must be stable. As SPA is a homogeneous assay technique, the signal output varies until the assay has reached equilibrium. In traditional methods, the assay may be terminated prior to the attainment of equilibrium and the samples counted. In SPA, however, it is important that the assay is allowed to reach equilibrium before counting is performed. This may require a more prolonged incubation period. During the assay incubation period, it is therefore essential to ensure that the assay components are themselves stable. For example, this may involve the addition of protease inhibitors to protect the ligand and/or receptor from the effect of degrading enzymes present in the receptor preparation.

Another aspect of homogeneous assays which must be addressed, is the measurement of light in colored samples. As SPA assays do not have a separation step, all the components are still present when the sample is counted. Samples which are colored absorb a proportion of the light emitted from the bead giving a reduced signal, which may appear as an inhibition of binding. This can be overcome by color quench correction programs applied automatically by the appropriate scintillation counter.

SPA is a solid phase technology; therefore, it is important to optimize the amount of receptor with respect to the immobilizing surface. The surface area of the beads is finite and the signal attainable therefore depends on the number of receptors bound to the beads, affinity of the ligand for the receptor and the specific activity of the ligand employed. As with all assay techniques this invariably involves 'trade-offs' between signal, noise, and sensitivity, which are particular to each assay.

Because SPA is an enabling technology, the fundamental aspects of SPA receptor assays are similar to those of the more traditional ligand binding assays using filtration as a separation technique. Consequently, it is advisable to consult the literature available for the receptor of interest before designing the SPA assay. Current publications may suggest a suitable ligand as well as a source for the receptor, either from a tissue or cell line, and will often describe a method for the preparation of membranes from the starting material.

A detailed flow chart for the development of a receptor binding SPA is given in Figure 2.

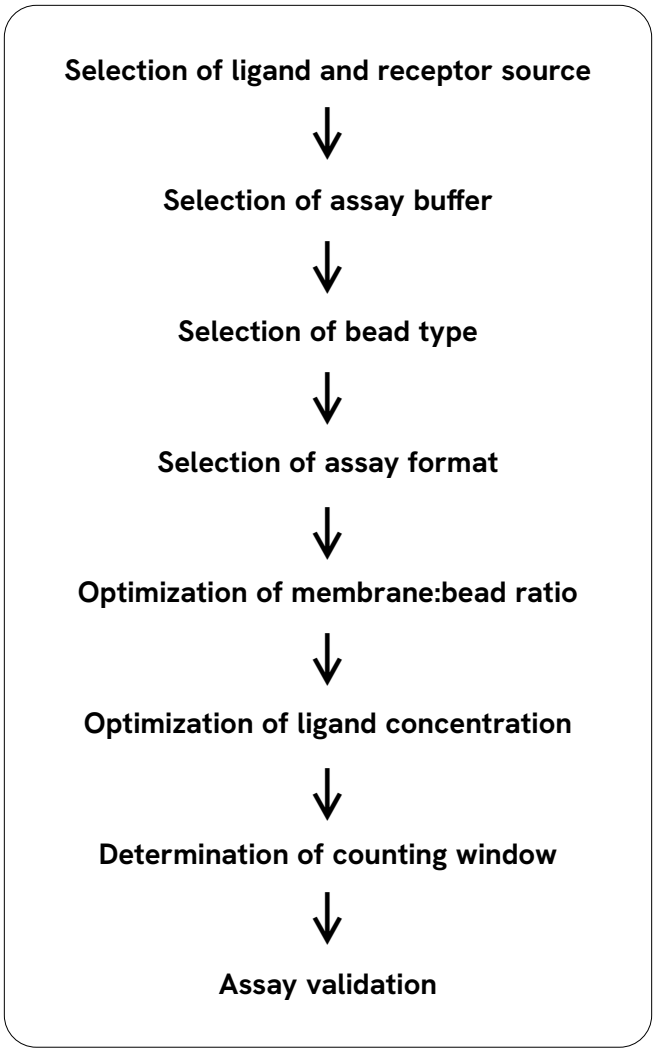


Figure 2: Flowchart for the development of a SPA receptor binding assay

Selection of ligand and receptor source

Consulting the literature will enable a suitable ligand to be selected. There may be a choice of ligands for the receptor of interest, labeled with different radioisotopes, and/or at different positions, or with different affinities for the receptor. Related to the issue of ligand selection, is the receptor density of the receptor source. Ideally, the receptor density should be as high as possible.

Several factors should be considered when selecting a particular starting material as the receptor source for SPA ligand binding assays. If a tissue is to be used, it should be a relatively rich source of the receptor of interest due to the finite surface area of the SPA beads, and hence capacity for receptor membranes. Tissue sources of receptors may contain more than one receptor subtype, and this may cause problems. If necessary, membrane preparations may be further purified by techniques such as differential centrifugation to yield enriched membrane fractions; low yields may be a problem. Alternatively, membranes can be prepared from a particular region of a given tissue that is enriched in the receptor protein.

Over the years, cell lines have been widely used for receptor screening assays. To establish screening assays for specific receptor subtypes and with receptors of human origin, transfected cell lines are being increasingly employed. If a cell line is selected as the receptor source, again the density of receptors is important, particularly if the ligand is of low specific activity. Typical expression levels in the region of 50,000 receptors per cell are required for ^{125}I-ligands (~2000 Ci/mmol). This corresponds to approximately 200 fmol/mg membrane protein. Higher expression levels of the order of 500,000 receptors per cell are often required for ^{3}H-ligands (20-80 Ci/mmol), corresponding to densities greater than 2 pmol/mg membrane protein.

The affinity of the radioligand for the receptor is another factor that should be considered. In practice this means that for systems where the receptor density is low, ^{125}I-labeled ligands are usually employed. Also, if the affinity of ligand for the receptor is low (i.e., >10 nM) the signal obtained may well be quite small. For systems where the receptor density is high, either ^{3}H- or ^{125}I-radioligands may be used if the affinity for the receptor is also high (i.e., <10 mM); if the affinity of the ligand for the receptor is low (i.e., >10 nM), ^{125}I is probably the radioisotope of choice for labelling. All the figures quoted are rough guides and take account of the absolute level of signal that the user requires in the assay.

Selection of assay buffer

This is usually very similar to the standard buffer quoted in the literature for the receptor system; it will include any cofactors required for ligand binding and any essential protease inhibitors that are quoted in the literature. The addition of protease inhibitors is of particular importance in homogeneous assays such as SPA. Protease inhibitors help prevent the degradation of the receptor or the ligand during the longer equilibration periods often required. This effect has been demonstrated in a SPA assay for binding of [^{125}I] Substance P to its receptor[8].

Selection of bead type

Two bead types are available; they are the WGA-PVT bead (Revvity product number RPNQ0001) and PL- YSi bead (Revvity product number RPNQ0010). The WGA-PVT bead has been widely used for receptor assays; it is the bead of choice because the density of the bead is 1.05 g/cm^3 and is therefore very amenable to automation. WGA-PVT beads bind to N-acetyl-β-D-glucosamine residues in membranes and selects for membrane glycolipids and glycoproteins[5]. The binding capacity of WGA-PVT beads is typically 10-30 µg membrane protein per mg of bead. However, glycosylated ligands will bind non- specifically to WGA-PVT beads. Also, some ligands may interact with the PVT core, in which case PL-YSi beads may be more appropriate. In the case of PL-YSi beads, there is an electrostatic interaction between negatively charged membranes and the positively charged beads[6]. The interaction is non-selective and therefore negatively charged ligands will bind to these beads. The typical capacity of these beads is 10 µg membrane protein per mg of bead. With both bead types, it is important to confirm that there is little or no non-specific interaction of the radioligand with the bead. These controls can take the form of a no membrane control, or alternatively, the use for example of a membrane preparation from the parental cell line in the case of transfected cell lines. Sometimes it is possible to reduce non-specific interactions by the addition of various reagents e.g., BSA, detergent or by variation of the ionic strength of the buffer.

Selection of assay format

There are three possible formats for the assay that can be selected: - pre-coupled bead, T_0 addition and delayed addition.

The pre-coupled bead format involves coupling the membranes to the beads prior to assay. The membrane to bead ratio is carefully optimized and hence lowers Non-specific Binding (NSB) and better signal to noise ratios may be obtained. Pre-coupling the membrane to beads means that one pipetting step is eliminated, an advantage for high throughput screening (HTS). Although SPA technology has been designed for HTS applications, it can also be applied to secondary screening applications such as the measurement of 'on' and 'off' rates. For these studies, the use of pre-coupled bead format is necessary to avoid interference of association of membrane and bead.[7]

The T_0 addition format involves the sequential addition of test samples, radioligand, membrane, and bead as separate additions. The coupling of membrane to beads occurs simultaneously with ligand-receptor binding. This is the most widely used format for screening assays. This format is easy to automate, but excess bead is required to capture all the membranes.

For the delayed addition format test samples, radioligand and membrane are allowed to equilibrate prior to addition of beads. One disadvantage of this approach is that the addition of the SPA bead, after pre-equilibration of ligand and receptor preparation, causes an increase in volume and hence a reduction in the concentrations of the other assay components. This necessarily causes a shift in the assay equilibrium. The extent to which this effect is observed will depend on the rates of association and dissociation of the labeled radiolabeled ligand to and from the receptor.

Some users may have a strong preference for one format over the others and proceed with assay development using their preferred format. Other users may wish to evaluate all formats before selecting one which best suits their requirements.

Optimization of membrane: bead ratio

The key part of the assay development process is the optimization of both the membrane bead ratio and the actual amounts of these components required to achieve the desired signal to noise ratio. The objective is to obtain a signal for further optimization.

For the pre-coupled bead format this involves incubating varying concentrations of membrane with a fixed amount of bead; aliquots are taken and B_o/NSB values are measured. In the case of the T_0 and delayed addition formats, this optimization process involves setting up a matrix varying the quantities of membrane and bead; B_o/NSB values are measured. In these experiments a fixed quantity of radioligand is added; this quantity will depend on the affinity of the radioligand for its receptor and should be a concentration at or around K_d. For ^{125}I ligands, typically quantities of membrane protein between 1 and 100 µg with bead weights between 0.5 and 2.0 mg per well should be investigated. The corresponding figures for ^{3}H ligands are 10 to 100 µg membrane protein and up to 4.0 mg of bead per well. These differences essentially reflect the relative specific activities of radioligands labeled with these isotopes.

Figure 3 shows the results of matrix experiment for the binding of *([125I]-SAR1, Ile8]-angiotensin II to* (Revvity product number NEX248) to bovine adrenal cortex membranes using the T_0 addition format. The data shows that small quantities of bead were insufficient to capture all the membrane and hence all the signal.

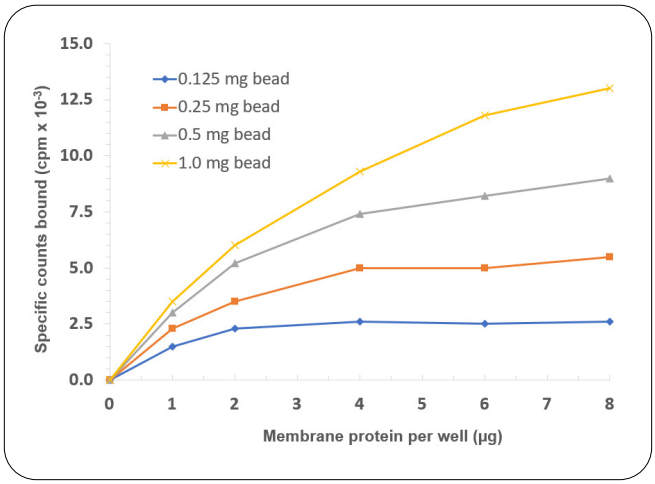


Figure 3: Matrix experiment for optimization of the bead to membrane ratio for the binding of [125I]-SAR1, Ile8]-angiotensin II to bovine adrenal cortex membranes. Assays were performed in 200 µL assay buffer (phosphate buffered saline, pH 7.3, containing 0.1% (w/v) BSA and 5 mM EDTA) containing 169 pM [125I]-SAR1, Ile8]-angiotensin II to, varying concentrations of bovine adrenal cortex membranes and different weights of WGA-PVT beads. NSB was defined using 50 µL of 2 µM [sar1,ile8] angiotensin II. Assays were counted using a TopCount scintillation counter.

Optimization of ligand concentration

The volume of the assay buffer and concentration of ligand is varied to optimize the signal and can easily be performed in a matrix format. The objectives of varying these parameters are:

(i) to maximize the signal to noise ratio

(ii) to increase the signal in systems where the affinity of the ligand for the receptor is relatively low.

This can be achieved by either using increased quantities of labeled ligand and/or decreasing the assay volume, but a consequence is that the non-proximity effect (NPE) component of the NSB may be increased; also sensitivity may be affected.

Determination of counting window

Time-course experiments should be performed to establish both the incubation time required for the attainment of equilibrium and the stability of the SPA counts at equilibrium. This should determine the 'time window' for the counting of a particular SPA receptor assay over which the equilibrated counts are stable. The time required for equilibrium to be established in an SPA receptor assay can often be reduced by the agitation of the assay tubes or plates on an orbital or vibrational shaker. The effect of agitation on the assay equilibrium can depend upon both the SPA bead type, the assay format and type of vial or plate employed. Agitation is desirable for assays employing WGA-PVT beads but is essential for assays employing PL-YSi beads because of the higher density of these latter beads and hence much more rapid rate of settling. Consequently, the effect of agitation on the time required to reach stable equilibrium should be investigated for each receptor studied. In T_0 addition assays, the equilibrium rate is determined by the rates of association of both the membrane preparation with the SPA bead and of the labeled ligand with the membrane bound receptor. The rate of association of membranes with WGA-PVT and PL-YSi beads is relatively rapid (20-60 minutes). Therefore, in many cases, the ligand binds to membranes already coupled to beads and the equilibration rate is mainly determined by the rate of association of the ligand with the receptor. Because SPA receptor assays are equilibrium assays, the stability of all assay components should be considered. Figure 4 shows a time course for the binding ^{125}I Bolton-Hunter labeled Substance P to the human neurokinin-1 (NK-1) receptor expressed in a CHO cell line[12] (hNK$_1$R CHO); the counts are stable for several hours when the membranes are prepared in the presence of a cocktail of protease inhibitors. In the absence of these inhibitors the signal increases initially but is not stable and decreases steadily over a period of several hours.

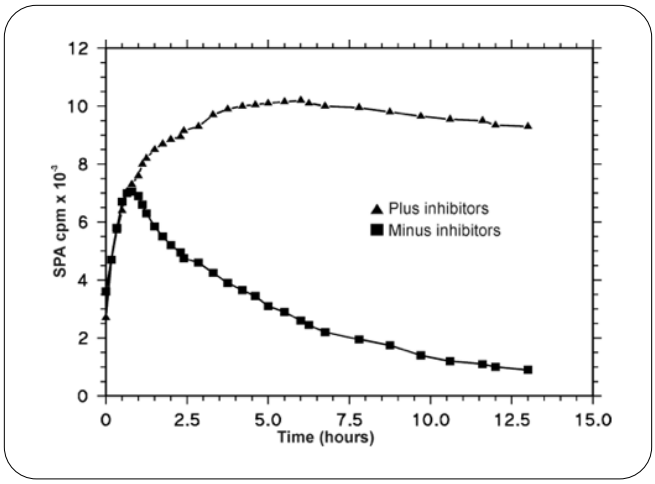


Figure 4: Time course of binding of [^{125}I] Substance P to hNK$_1$ R CHO membranes by SPA. Assays were performed in 200 µL assay buffer (50 mM Tris/HCl, pH 7.4, 0.1% (w/v) BSA, 1 mM EDTA, 2 mM $MnCl_2$) in the presence or absence of 400 µg/ml bacitracin, 20 µg/ml leupeptin, 40 µg/ml chymostatin and 1mM Pefabloc™(P) SC) containing 141 pM [^{125}I] Substance P, 6 µg hNK1RCHO cell protein and 0.5 mg WGA SPA beads. Non-specific binding was determined in the presence of 50 µM Substrate P. Assays were counted using a MicroBeta™ scintillation counter.

Assay validation

Once optimization of the signal in the assay is complete, it is necessary to validate the assay. The criteria are like those that the researcher would apply to a filtration assay.

Perform control experiments using a cell line or tissue which is known not to express the receptor of interest.

Perform competitive binding curves with known drugs (if available) for the receptor of interest and compare IC_{50}/K_i values.

Perform saturation binding experiments and compare K_d values. To obtain K_d values it is necessary to convert SPA counts to dpms. This can be achieved by measuring a sample which has radioisotope bound to the beads but no unbound radioactivity present, firstly as SPA counts and then in another counter standardized to give dpms for that radioisotope.

It will not always be possible to apply all these criteria to any particular receptor assay.

Finally, before analyzing colored samples, color quench correction curves should be installed.

The results of the development process should be an assay:

a. with an acceptable level of signal

b. with an adequate signal to noise ratio

c. with the required sensitivity

SPA technology overcomes the disadvantages of traditional filtration techniques for receptor assays. There is no separation step required and so radioactive handling and waste generated is minimized. SPA provides a method which is simple and rapid, and helps to automate HTS. This article has highlighted the key issues for the development of SPA receptor assays.

Professor Shigetada Nakanishi's group at the Institute for Immunology, Kyoto University, provided the hNK$_1$R CHO cell line used in this study.

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