Configuring Radioligand Receptor Binding Assays for HTS Using Scintillation Proximity Assay Technology


1. Introduction

Rapid progress in the fields of genomics, proteomics, and molecular biology has both increased the numbers of potential drug targets, and facilitated development of assays to screen these targets (1–5). In parallel with these changes, developments in robotics and combinatorial chemical synthesis have driven the production of very large numbers of compounds with potential for pharmacological activity (1–5). The need to screen these large libraries of drug candidates against multiple new targets has stimulated improvements in technology, instrumentation, and automation that have revolutionized the field of drug discovery, and evolved into the field of high throughput screening (HTS) (1,5–9). Radioligand binding assays have historically been the mainstay of drug discovery and drug development (6–8). In the era of HTS, incorporation of scintillation-proximity technology together with improved automation and radiometric-counting instrumentation have served to maintain radioligand receptor-binding as one of the premier tools of drug discovery (1,5,8,9). Radioligand binding assays are extremely versatile, easy to perform, can be automated to provide very high throughput (10–12). The quality of the data allows the determination of drug affinity, allosteric interactions, the existence of receptor subtypes, and estimates of receptor numbers (10–12). This chapter provides an overview of radioligand receptor-binding assays and discusses some of the issues associated with the conversion of traditional filtration assays.
to a homogeneous scintillating proximity assay (SPA) format that is more compatible with automation and HTS.

1.1. Basic Radioligand-Binding Theory

The classical definition of a receptor involves a functional response, and while receptor binding should be saturable, reversible, and stereo-selective, functional activity is not measured in binding experiments (10–12). Despite recent developments in nonradioactive techniques like fluorescence polarization (13,14), radioligand-receptor binding is the most commonly used technique for the biochemical identification and pharmacological characterization of receptors in drug discovery (10–12). There are a number of excellent reviews that provide an overview of the theory and methodology of traditional radioligand receptor-binding assays, together with a discussion of the potential problems and artifacts associated with the various formats available (10–12,15–18). SPA radioligand receptor binding assays share the same issues as more traditional formats; choice of radioligand, selection of receptor preparation, optimization of assay conditions, and appropriate analysis of the data (1,8–12).

There are two major categories of radioligand binding studies: kinetic studies and equilibrium studies (10–12). Kinetic binding experiments typically define the time-course of ligand association and dissociation with the receptor, and are generally used to optimize binding conditions and demonstrate the reversibility of the ligand-receptor interactions (10–12). For drug-discovery purposes, saturation and competition (displacement) binding experiments are the two types of equilibrium (steady-state) binding studies most commonly utilized to estimate the classical binding parameters; the dissociation constant \(K_d\), the binding capacity \(B_{max}\), and the affinity \(K_i\) of a competing drug for the receptor ligand (10–12). Specific binding is the proportion of total binding of a radioligand to a receptor preparation that can be displaced by an unlabeled compound known to bind to the receptor of interest (10–12). Competition for receptor-binding sites might involve the same unlabeled chemical species as the radioligand (homologous displacement), or a different chemical species (heterologous displacement) (10–12). Nonspecific binding includes binding of the radioligand to glass-fiber filters, adsorption to the tissue, and dissolution in the membrane lipids (10–12). To reduce the chances of the unlabeled ligand displacing radioligand from saturable nonreceptor sites such as uptake carriers or enzymes, it is generally recommended that the displacing ligand should be structurally dissimilar from the radioligand. Operationally, nonspecific binding is defined as the amount of radioligand bound in the presence of an appropriate excess of unlabeled drug. An assay is considered barely adequate if 50% of the total binding is specific; 70% is good and 90% is excellent (10–12).
1.2. Filtration-Format Radioligand Binding

The basic outline of most radioligand binding assays is very similar; a preparation containing the receptor is incubated with a radioligand for a period of time, the bound ligand is separated from the free ligand, and the amount of radioligand bound is quantified by liquid-scintillation counting \((10–12)\). It is important to prevent significant dissociation of the receptor-radioligand during separation, a problem typically addressed by performing the separation as rapidly as possible, or at reduced temperature to slow the rate of dissociation \((10–12,15–18)\). Although centrifugation, dialysis, and gel filtration are options, the separation technique most widely used with membrane preparations and whole cells is filtration, either on a vacuum manifold or by a cell harvester \((10–12,15–18)\). The bound ligand is retained on glass-fiber filters, and the free ligand passes through. After the initial filtration, filters are rinsed extensively with assay buffer to reduce the level of nonspecific binding. Filters may also be prerinsed or presoaked with solutions such as 0.1% polyethylenimine to decrease nonspecific binding \((10–12)\). Characteristically, nonspecific binding attains steady-state more rapidly than specific binding, and increases linearly with the radioligand concentration rather than reaching saturation.

The relative ease of radioligand-binding assays together with the availability of radioligands for many different receptor types and the variety of receptor preparations that can be attained have all contributed to the popularity of the technique \((5–12)\). However, the physical separation of bound from free radioligand involving reagent transfer, filtration, and multiple wash steps, is a time consuming and relatively labor-intensive process that can significantly limit throughput in HTS. Filtration-binding assays also expose personnel to the hazards of manipulating radioactive solutions, and generate a significant volume of radioactive waste that is costly to dispose of \((1,8,9)\). In recent years, the development of the Multiscreen® Assay System (Millipore) of micotiter filter plates, the MAP® filter plate aspirator (Titertek), and photomultiplier tube (PMT) microplate liquid-scintillation counters (Wallac Microbeta®, & Packard Topcount®) have significantly improved the ability to automate filtration-binding assays for HTS \((6,19,20)\). However filtration screens generate more radioactive waste and are sufficiently labor-intensive that throughput is limited. The demands of HTS make other screening formats more desirable.

1.3. Scintillation Proximity Radioligand Binding Format

SPA technology is a radioisotopic homogeneous-assay system that requires no separation step and allows the design of high-throughput receptor-binding assays that rely on pipetting in a “mix and measure” format \((1,8,9)\). SPA involves the use of fluoromicrosphere beads containing scintillant that are
coated with acceptor molecules to capture biologically active molecules such as receptors, which can in turn bind radioactive ligands \((1,8,9)\). When the radioligand binds to a receptor coupled to the bead, the radioisotope is brought in close proximity to the scintillant and effective energy transfer from the beta-particle will take place, resulting in the emission of light \((1,8,9)\). No light is detected from unbound radioligand in free solution because the beta-particle released has a minimum path length of decay that is too distant from the scintillant in the bead, and the energy is dissipated in the assay buffer \((1,8,9)\).

The homogeneous receptor binding format provides the ability to measure weak interactions without disturbing the equilibrium with a separation step, and makes it possible to monitor the rate of association or dissociation of a radioligand from its receptor \((1,8,9)\).

1.4. Conversion of a Filtration Radioligand Binding Assay to an SPA Format

While radioligand-binding assays may be directly configured in SPA format for HTS, it is preferable to first develop a filtration assay. The more traditional filtration assay may be used to compare and validate the SPA format, and many of the critical experimental variables defined in the filtration assay may be directly transferable to the SPA format, including; selection of radioligand, choice of receptor preparation, buffer composition, pH, temperature, and reaction time.

1.5. Selection of Radioligand

The important characteristics to be considered for the selection of the radioligand include the radioisotope, the extent of nonspecific binding, the selectivity and affinity for the receptor, and whether the radioligand is an agonist or an antagonist \((10–12)\). The radioligand must be soluble and stable in the incubation medium. Each radioligand has a unique pharmacological profile and the one utilized should bind selectively to the receptor type, or subtypes, of interest under the assay conditions used. Usually, high-affinity ligands are preferred because a lower concentration of radioligand can be used in the assay, resulting in lower levels of nonspecific binding, and a slower rate of dissociation \((10–12)\). Agonists may label only a subset of the total receptor population (high affinity state for G-protein coupled receptors [GPCR’s]), whereas antagonists generally label all available receptors \((10–12)\).

Although \(^{33}\text{P}\) and \(^{35}\text{S}\) are occasionally used, \(^{3}\text{H}\) and \(^{125}\text{I}\) are the isotopes most commonly used to label ligands for binding assays \((10–12)\). It is important that the radioligand should have sufficient specific activity to allow accurate detection of low levels of binding \((10–12)\). The selection of radioisotope is
especially critical for SPA, because the basis of the proximity effect is that an emitted $\beta$ particle will only travel a limited distance in an aqueous environment, and that path length is dependent on the energy of the emitted particle \((1,8,9)\). In order for the radioactive disintegration to be detected, the $\beta$ particle must interact with the scintillant in the bead, resulting in energy transfer and emission of light. Electrons from $^3$H have a range of energies leading to an average path length of 1.5 $\mu$m, and the two monoenergetic internal-conversion electrons emitted by $^{125}$I have path lengths of 1 $\mu$m and 17.5 $\mu$m, respectively. $^3$H and $^{125}$I are ideally suited to SPA in that only bound ligands brought in close proximity to the scintillant will generate a signal. In contrast, $^{14}$C, $^{35}$S, and $^{33}$P have path lengths with mean ranges of 58, 66, and 126 $\mu$m, respectively, that are less suited to the proximity principle due to the higher signals produced by unbound radioactive ligand \((1,8,9)\).

### 1.6. Selection of Receptor Preparation

Radioligand binding is an extremely versatile technique that can be applied to a wide variety of receptor preparations including purified and solubilized receptors, membrane preparations, whole cells, and tissue slices \((10–12)\). Membrane preparations are the most widely utilized receptor source, but access to the receptor of interest, especially human receptors, remains a critical issue. The advent of molecular- and cell-biology techniques to clone and express human receptors have been enabling technologies for HTS that have provided access to cell lines with high receptor-expression levels \((2,3,5)\). Stable cell lines can be expanded in cell culture to high density and crude membrane fractions may be easily generated by the differential centrifugation of cells homogenized in a hypotonic buffer \((8)\) (see below). A more pragmatic solution may be to purchase the receptor sample of interest from a number of commercial sources that provide a quality controlled and validated preparation that comes unencumbered with intellectual property issues. Although these reagents may appear expensive, this may be offset by reduced in house development costs.

### 1.7. Selection of SPA Bead

SPA beads are available in two types, Yttrium silicate (Ysi) or Polyvinyltoluene (PVT). PVT beads containing diphenylanthracine (DPA) have an average diameter of 5 $\mu$m, a density of 1.05 g/cm$^3$ and a typical counting efficiency of 40% compared to liquid scintillation counting. Ysi beads have scintillant properties by virtue of cerium ions within the crystal lattice, have an average diameter of 2.5 $\mu$m, a density of ~4.0 g/cm$^3$ and a typical counting efficiency of 60% compared to liquid-scintillation counting. Although Ysi is one of the most efficient solid scintillators known and provides a higher output
than PVT beads in SPA assays, PVT beads are less dense and more compatible with automation. A variety of coupling molecules are available for binding receptor preparations to the surface of SPA beads. These include wheat germ agglutinin (WGA), streptavidin, poly-L-lysine, protein A, glutathione, copper his-tag, antirabbit, antimouse, antisheep, and antiguinea pig antibodies. The binding capacity of the beads for the receptor preparation and the level of nonspecific interaction with the radioligand, are both important criteria to be evaluated before selection of bead type and coupling molecule.

1.8. Buffer Composition

Radioligand binding to membrane preparations can often be achieved in relatively simple buffer solutions such as HEPES (10–20 mM), Tris-HCl (10–170 mM), or phosphate buffers (30 mM), generally in the physiological range of pH 7.0 to 8.0 (10–12). Ionic composition may be important, and cations such as Na⁺, Mg²⁺ and Ca²⁺ are frequently included in buffers to either enhance specific binding, or inhibit nonspecific binding (10–12). GTP is sometimes included in the buffers for GPCR binding assays because it can modulate agonist affinity for the receptor and convert a complex inhibition curve (biphasic) to a simple (single-site) inhibition curve (10). It is important that the radioligand and receptor should be stable throughout the incubation period. It is not uncommon to include antioxidants such as ascorbic acid, or enzyme inhibitors such as pargyline, a monoamine oxidase inhibitor, in the assay buffer to control chemical and enzymatic stability (10–12). Similarly, a variety of protease inhibitors and chelating agents such as ethyleneglycoltetraacetic acid (EGTA) or ethylenediamine tetraacetic acid (EDTA) may be included to preserve receptors and ligands from proteolytic degradation (10–12). Typically, the conversion of a filtration binding assay to an SPA format does not require a change in the assay buffer (8).

2. Materials

2.1. Cell Culture

1. AV12 cells (Syrian Hamster fibroblasts, ATCC # CRL 9595).
2. Methotrexate or hygromycin.

2.2. Membrane Preparation

1. 50 mM Tris-HCl, pH 7.4.
2. Bicinchoninic acid (BCA) kit; Micro BCA™ Protein Assay Reagent (Pierce, Rockford, IL).

2.3. Filtration Format Receptor Binding Assay

1. Binding assay buffer: 50 mM Tris-HCl, 0.5 mM EDTA, 10 mM MgSO₄, 0.1%. Ascorbic acid, 10 µM Pargyline, pH 7.75 at 25°C.
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2. 5-Hydroxy(3H)tryptamine trifluoroacetate (Code TRK1006 Amersham, Piscataway, NJ) at a final concentration of 5 nM per well.
3. Beta Plate scintillation counter (Perkin Elmer Wallac Inc., Gaithersburg, MD).

2.4. SPA Format Receptor Binding Assay

1. Binding Assay Buffer: 50 mM Tris-HCl, 0.5 mM EDTA, 10 mM MgSO₄, 0.1% Ascorbic Acid, 10 µM Pargyline, pH 7.75 at 25°C.
2. 5-Hydroxy(3H)tryptamine trifluoroacetate (Code TRK1006 Amersham) at a final concentration of 5 nM/well.
3. Wheat-germ agglutinin (WGA) SPA beads were obtained from Amersham Pharmacia Biotech.

2.5. Automation used for High Throughput SPA Assay

1. Multidrop (TiterTek Instruments, Huntsville, AL).
3. SLT Dispenser (Tecan US).
4. ORCA arm robotic system (Beckman Coulter, Fullerton, CA).
5. Microbeta Scintillation Counter (Perkin Elmer Wallac).

3. Methods

3.1. Cell Culture

The generation of AV12 cells stably transfected with the eukaryotic expression vector phd containing the coding region for the human 5HT₂C receptor was described previously (3). AV12 cell lines were grown in suspension with selection for resistance to methotrexate or hygromycin (3).

3.2. Membrane Preparation

The preparation of membranes has been described previously (8). Briefly:

1. Suspension cells are grown in a stirred 30-L fermenter (37°C, 5% CO₂) to a cell density of 2 – 3 × 10⁶ cells/mL, and 15 L are harvested on a daily basis by centrifugation, washed in phosphate-buffered saline (PBS), and stored as frozen cell pastes at –80°C.
2. To loosen the frozen cell paste, 30 mL of 50 mM Tris-HCl, pH 7.4, at ambient temperature are added to 7.5 grams of pellet.
3. The cell slurry is homogenized on ice in a 55-mL glass/teflon dounce, transferred to a 250-mL conical tube that is then filled to the neck with buffer, mixed, and centrifuged in a table top centrifuge at 200g (1060 RPM, GH-3.7 rotor) at 4°C for 15 min.
4. The supernatant is collected and saved on ice.
5. The pellet is resuspended and subjected to the homogenization and centrifugation procedure just described.
6. The 200g supernatant is again collected and combined with the first supernatant stored on ice.
7. The combined supernatants are then centrifuged at 14,250 rpm in a Sorvall RC5 centrifuge (GSA SLA-1500 rotor) for 50 min at 4°C.
8. The supernatant is gently removed and discarded, and the remaining membrane pellet is resuspended using the dounce homogenizer.
9. The membrane protein concentration is determined (BCA kit) and aliquots of the membrane preparation are quick frozen in liquid nitrogen and stored at −80°C. The average yield is 1.2% of starting weight.

3.3. Filtration-Format Receptor-Binding Assay
1. Fifty microliter of compound, unlabeled 5HT or binding buffer are added to each well of a 96-well microtiter plate, followed by 50 µL of 20 nM ³H-5HT, and 100 µL (20 µg) of the 5HT₂C membrane preparation.
2. Plates are sealed, placed on an orbital shaker for 2 min at setting 6, and then incubated for 30 min at 37°C.
3. The film is removed, 50 µL of 25% TCA is added to terminate the reaction, and the well contents are aspirated and transferred to a glass-fiber filter mat with a TomTec® cell harvester. After three wash cycles with ice-cold binding buffer, the filter mats are removed and dried in the microwave oven for 1 min.
4. Filter mats are then placed in a plastic bag, scintillation fluid is added, the fluid is spread out to cover the whole filter and air bubbles are removed with a rolling pin. The plastic bag is sealed with a heat-seal apparatus, mounted in a rack, and counted in a Beta Plate counter.

3.4. SPA-Format Receptor-Binding Assay
1. Twenty microliter of compound, unlabeled 5-HT, or assay buffer is added to each well of a 96-well microtiter plate.
2. Fifty microliter of 15-nM [³H]-5HT ligand is then added to the wells followed by 80 µL of 5HT₂C membranes (20 µg; see Notes 5 and 6) and the plates are shaken for 1 min.
3. After a 30-min incubation at room temperature, 0.5 mg of WGA-SPA beads are added (see Notes 3, 4, and 7), plates are mixed by shaking every 30 min for 2 h and then counted in a MicroBeta counter (see Notes 9 and 19).

3.5. Automation Used for High Throughput SPA Assay
1. The assay is as described in Subheading 3.4.
2. Compounds delivered as dimethyl sulfoxide (DMSO) stocks in 96-well plates are diluted in binding buffer added by Multidrop.
3. Diluted compounds and controls are transferred and added to assay plates using a Megaflex.
4. Membranes and radioligands are added to assay plates with an SLT Dispenser.
5. WGA-SPA beads are kept in suspension in binding buffer by a magnetic stir bar and added to assay plates by a Megaflex.
6. Plates are shaken on a Hotel Shaker and an ORCA arm robotic system is used to move plates between and load them into workstations.
7. Plates are counted in a Wallac Microbeta (6-Detector counter).

4. Notes
1. We will now describe the conversion of a radioligand receptor binding assay from a filtration to a SPA format, compatible with HTS. To illustrate this process and provide an example for discussion, we have selected a member of the 5-hydroxytryptamine (5-HT, Serotonin) receptor family that have been implicated in a variety of pathological conditions including anxiety, depression, aggressiveness, obsessive-compulsive behavior, schizophrenia, eating disorders, and alcoholism (2,3,8). With the exception of 5-HT₃, which is a ligand gated ion channel, 5-HT receptors belong to the superfamily of GPCRs. There are seven receptor subtypes 5-HT₁–₇, based on radioligand-binding properties, signal transduction mechanisms, and deduced amino acid sequences (2,3,8).

4.1. Filtration Format Assay
2. A filtration format radioligand-binding assay (described in Subheading 3.3.), using ³H-5HT as the ligand and membranes prepared from AV12 cells expressing the human 5HT₂C receptor, had been developed and is to be converted to SPA format for HTS (Fig. 1). ³H-5HT exhibited saturation binding to 20 µg of 5-HT₂C membranes in the filtration assay, and the Kᵋ of 4.5 nM for 5-HT binding is consistent with published data (2,3,8). At 3 nM ³H-5HT, specific binding is 82% of the total binding observed, indicating a very good assay.

4.2. Optimization of SPA-Format Assay
3. Based on previous experience (8), WGA-PVT beads were selected for an initial evaluation of the SPA format (Fig. 2).
4. Three variations on the SPA format are possible; membranes may be precoupled to the beads prior to the addition of ligand, all components of the assay can be added together at T₀, or beads can be added after the receptors and ligand have been incubated together. There are advantages and disadvantages to each assay format, depending on the ligand and receptor preparation of interest. Although it adds an extra step, one possible advantage of the precoupled format is that excess uncoupled membranes can be removed prior to the assay thereby ensuring that only binding sites coupled to beads are available. We selected the delayed format of bead addition for the example we will discuss.
5. Five nanomolar [³H]-5HT ligand and the indicated amounts of 5HT₂C membranes were added to wells as described in Subheading 3.4. Total binding increased with the amount of 5HT₂C membranes added in a dose dependent manner up to ~20 µg/well, then reached a plateau such that no significant increase in signal was achieved with addition of more membranes. The data are consistent with saturation of the binding capacity of the WGA-SPA beads at 20–30 µg of membrane protein.
Fig. 1. Saturation binding of $^3$H-5HT to 5-HT$_{2C}$ membranes filtration format. Compound, unlabeled 5-HT or binding buffer were incubated for 30 min at 37°C with the indicated amounts of $^3$H-5HT and 20 µg of 5HT$_{2C}$ membranes. The contents of the wells were transferred onto a filter mat and washed using a TomTec® cell harvester, and filters were counted in a Beta Plate counter as described in Materials and Methods. Each point represents the mean +/- SDM of quadruplicate determinations.

Fig. 2. Specific $^3$H-5HT binding to 5HT$_{2C}$ membranes coupled to WGA-PVT SPA beads. Unlabeled 5-HT (10 µM), assay buffer, $[^3]$H-5-HT ligand and the indicated amounts of 5HT$_{2C}$ membranes were incubated for 30 min at room temperature. One milligram of WGA-PVT SPA beads were added, plates were mixed by shaking every 30 min for 2 h and then counted in a MicroBeta (Wallac) counter as described in Materials and Methods. Each point represents the mean +/- SDM of quadruplicate determinations.
For all of the membrane concentrations tested, specific binding exceeded 80% of the total binding observed.

Since SPA beads are one of the more expensive components of the assay, we also performed a membrane-titration experiment with half the amount of beads (Fig. 3), and found that in the range tested, 0.5 mg was as effective as 1.0 mg of beads.

On the basis of these data, 20 µg/well of membranes and 0.5 mg/well of WGA-PVT beads were selected for an evaluation of saturation binding (Fig. 4). [3H]-5HT exhibits saturation binding to 20 µg of 5HT₂C membranes in the SPA assay, and the Kᵩ of 3.0 nM for 5-HT binding is consistent with both the filtration assay (Fig. 1) and published data (2,3,8). At 2.5 nM [3H]-5HT, specific binding was 94.6% of the total binding observed, indicating a very good assay.

Consistent with the lower counting efficiency of the SPA PVT-beads, the B_max for the SPA format was 3279 CPMs compared to 4123 CPMs for the filtration assay.

To further validate the assay format we analyzed four known serotonergic compounds and unlabeled 5-HT in heterologous and homologous displacement binding assays (Fig. 5). The rank potency of the compounds tested is in agreement with the published pharmacology: 5-HT > 1-(m-Chlorophenyl-) Piperazine hydrochloride > 1-(1-Napthyl) Piperazine hydrochloride > Ketanserin tartarate = Clozapine (2,3,8).

Fig. 3. Effect of bead amount on the specific [3H]-5HT binding to 5HT₂C Membranes Coupled to WGA-PVT SPA Beads. Unlabeled 5-HT (10 µM), assay buffer, 5 nM [3H]-5HT ligand and the indicated amounts of 5HT₂C membranes, were incubated for 30 min at room temperature. Either 0.5 mg or 1.0 mg of WGA-PVT SPA beads were added, plates were mixed by shaking every 30 min for 2 h and then counted in a MicroBeta (Wallac) counter as described in Materials and Methods. Each point represents the mean ± SDM of quadruplicate determinations.
4.3. HTS Issues

11. In a typical HTS operation compounds are delivered already dissolved in DMSO, typically in the 1–10 mM range, and are then diluted into aqueous buffers to achieve the desired concentration for the screen. It is therefore important to define the DMSO tolerance of the assay (Fig. 6). The level of $^3$H-5HT binding to 5-HT$_{2C}$ membranes is unaffected by concentrations of DMSO less than 1.67%, but decreased significantly as the concentration of DMSO increased further (Fig. 6).

12. To generate sufficient amounts of membrane preparation for the HTS screen, a number of separate membrane lots were made from cell pastes harvested on different days. To ensure that these distinct membrane lots were of a consistent quality suitable for the HTS, displacement binding curves were run for each lot (Fig. 7A). Each of the 10 membrane lots tested exhibited overlapping homologous displacement binding curves (Fig. 7A), and therefore were judged acceptable.

13. In addition, since the HTS may be run over the period of several weeks and each membrane preparation may be used on several different days, stability to freeze-
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**Fig. 5.** Displacement binding of $^3$H-5HT to 5HT$_{2C}$ membranes. Unlabeled 5-HT, assay buffer, or competing compounds at the indicated concentrations, were incubated for 30 min at room temperature with 5 nM $[^3]$H-5HT and 20 µg of 5HT$_{2C}$ membranes. 0.5 mg of WGA-PVT SPA beads were added, plates were mixed by shaking every 30 min for 2 h and then counted in a MicroBeta (Wallac) counter as described in Materials and Methods. Each point represents the mean +/- SDM of quadruplicate determinations.

**Fig. 6.** DMSO tolerance. Unlabeled 5-HT (10 µM), or assay buffer with the indicated concentrations of DMSO were incubated for 30 min at room temperature with 5 nM $[^3]$H-5HT and 20 µg of 5HT$_{2C}$ membranes. 0.5 mg of WGA-PVT SPA beads were added, plates were mixed by shaking every 30 min for 2 h and then counted in a MicroBeta (Wallac) counter as described in Materials and Methods. Each point represents the mean +/- SDM of quadruplicate determinations.
Fig. 7. Membrane quality control. (A) Comparison of membrane lots. Unlabeled 5-HT at the indicated concentrations or assay buffer were incubated for 30 min at room temperature with 5 nM [3H]-5HT and 20 µg of the indicated lots of 5HT2C membranes. 0.5 mg of WGA-PVT SPA beads were added, plates were mixed by shaking every 30 min for 2 h, and then counted in a MicroBeta (Wallac) counter as described in Materials and Methods. Each point represents the mean +/- SDM of quadruplicate determinations. (B) Stability to freeze-thaw. The assays were performed as described in 7A save that the 5HT2C membranes used had been subjected to between 1 and 3 cycles of freeze-thaw, as indicated. Each point represents the mean +/- SDM of quadruplicate determinations.
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thaw is essential (Fig. 7B). The 5-HT<sub>2C</sub> membranes used in the HTS were found to be stable to at least three cycles of freeze-thaw, using a dry ice/ethanol bath to snap-freeze membranes (Fig. 7B).

14. It is also important to test reagent stability throughout a typical screening shift (8). Typically all assay components are either maintained on ice or at ambient temperature, so the levels of total, specific, and nonspecific binding were determined over a 12-h period. For the 5-HT<sub>2C</sub> SPA assay, there did not appear to be any significant reagent stability issues under any of the conditions (data not shown), but because there appeared to be less variability in the data generated with reagents kept on ice, this procedure was adopted for the HTS process.

15. In the HTS process, competition for counter time from other assays or assays with sufficiently high throughput, may cause delays before counting. To assess the stability of the SPA-assay signal after incubation, plates may be counted at various times after the assay has been terminated. In general, SPA assays exhibit good signal stability for up to 24 h, and often beyond (data not shown).

4.4. Assay Window and Reproducibility

16. In order to rapidly identify the active compounds from the hundreds of thousands of compounds run in HTS, it is important that the assay be optimized to discriminate between active and inactive compounds (4,21,22). The assay window, dynamic range, or the degree of separation between the background and maximum signals, should be both robust and reproducible (21,22). Due to the nature of the procedure and the perturbation introduced by automation and human-associated random error all assay measurements contain a degree of variability, and it is in the context of this assay variability that active compounds must be distinguished from inactive compounds (21,22). The lower the assay variability the higher the degree of confidence that an activity is “real” and will confirm upon re-testing (21,22).

17. To address these assay window and reproducibility issues for the 5-HT<sub>2C</sub> binding SPA, two full 96-well plates for maximum binding and nonspecific binding were run on three separate days (Fig. 8). There is a robust separation of the total and nonspecific binding responses on all three days, and the variability associated with these signals produced signal windows of between 5 and 10 standard deviations on all three days, indicating that the 5-HT<sub>2C</sub> binding SPA is very good for HTS.

4.5. HTS Data

18. In the primary screen 150,000 compounds were tested in singlet wells at a final concentration of 10 µM and 0.5% DMSO (Table 1). A total of 2,752 compounds were identified as active because they inhibited <sup>3</sup>H-5HT binding by ≥ 50%, producing an overall active rate of 1.83% (Table 1).

19. Colored compounds with absorption maxima in the 400–450 nm range (red, yellow, or orange) will absorb or quench the emitted light from the SPA bead and
Fig. 8. Assay window and reproducibility. Unlabeled 5-HT (10 μM), or assay buffer, were incubated for 30 min at room temperature with 5 nM [³H]-5HT and 20 μg of 5HT₂C membranes. 0.5 mg of WGA-PVT SPA beads were added, plates were mixed by shaking every 30 min for 2 h and then counted in a MicroBeta (Wallac) counter as described in Materials and Methods. Two full 96-well plates for maximum binding and nonspecific binding (+10 μM unlabeled 5-HT) were run on three separate days. The data are represented on a separate scattergram (2 plates of max and min signals) for each of the 3 d, together with a combined scattergram (6 plates of max and min signals) for all 3 d.
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20. In 5-point 10-fold dilution IC$_{50}$ confirmation assays, 2,164 of these compounds were confirmed to inhibit 3H-5HT binding by $\geq$50%, producing an overall confirmation rate of 78.6% (Table 1), indicating that the SPA format was highly reproducible.

21. The primary screen of 150,000 compounds, and IC$_{50}$ assays to confirm active compounds were completed in a period of 12 wk, indicating that the SPA format supports HTS.

22. A number of structural platforms were identified with sufficient potency that they were further evaluated in a variety of secondary assays, indicating that the SPA format HTS was successful.

4.6. Conclusions

The SPA methodology for receptor-binding assays provides a number of benefits for HTS. The homogeneous format eliminates the need for a separation step, can be completely automated using robotic liquid handlers, reduces the number of operators required to run the assay, and thereby increases throughput. The homogeneous SPA format eliminates the need for addition of liquid scintillant and minimizes both the exposure of personnel to radioactive liquids and the amount of radioactive waste generated. The recent trends in HTS are to reduce costs and increase throughput by assay miniaturization, in which assays are carried out in smaller volumes using high-density well arrays of 384-, 864- and 1536-well plates. 384 well format assays have been developed using SPA technology and conventional PMT-based scintillation counters, but the time required to count plates becomes a rate-limiting step. Recent developments with new fluor-containing particles and charged coupled

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<th>Compound source</th>
<th># Tested</th>
<th>Rapid actives*</th>
<th>IC$_{50}$ actives*</th>
<th>% Confirmed actives</th>
</tr>
</thead>
<tbody>
<tr>
<td>Combinatorial chemistry</td>
<td>115,311</td>
<td>1465</td>
<td>1176</td>
<td>1.02</td>
</tr>
<tr>
<td>Organic file</td>
<td>35,391</td>
<td>1287</td>
<td>988</td>
<td>2.79</td>
</tr>
<tr>
<td>Total</td>
<td>150,702</td>
<td>2752</td>
<td>2164</td>
<td>1.44</td>
</tr>
</tbody>
</table>

*Active compounds inhibited 5-HT$_{2C}$ binding by $\geq$%, and were confirmed to inhibit 5-HT$_{2C}$ binding $\geq$ 50% in a 5-point 10-fold dilution IC$_{50}$ assay.
device (CCD) camera-based imaging systems should provide a means to config-
figure radioligand receptor-binding assays compatible with 96-well and higher-
density arrays to provide higher throughputs, and potentially reduce costs. In
addition to the SPA bead format discussed here there are also microtiter plate
proximity-based options (FlashPlate® [New England Nuclear] and Cytostar T™
[Amersham]) available for radioligand binding assays. The basic principles
are similar to the fluomicrosphere technology, in that receptors of interest can
be attached to the scintillant coated or impregnated plates by a variety of
passive coating or coupling procedures, and the assay is a simple matter of
adding radioligand with test compound, incubation, and measurement.
Continued innovation in the fields of SPA technology, signal-capture instru-
mentation, and automation will ensure that radioligand-binding assays will
continue to be the mainstay of HTS, drug discovery, and drug development.

References
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7. Froidevaux, S, Meier, M., Häusler, M., Macke, H., Beglinger, C., and Eberle, A.
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167–168.


