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USE OF FLUORESCENCE POLARIZATION DETECTION FOR THE MEASUREMENT OF FLUOPEPTIDE™ BINDING TO G PROTEIN-COUPLED RECEPTORS

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ABSTRACT

G protein-coupled receptors (GPCRs) represent the single largest molecular target of therapeutic drugs currently on the market, and are also the most common target in high throughput screening assays designed to identify potential new drug candidates. A large percentage of these assays are now formatted as radioligand binding assays. Fluorescence polarization ligand binding assays can offer a non-rad alternative to radioligand binding assays. In addition, fluorescence polarization assays are a homogenous format that is easy to automate for high throughput screening. We have developed a series of peptide ligands labeled with the fluorescent dye BODIPY® TMR whose binding to GPCRs can be detected using fluorescence polarization methodology. BODIPY® TMR has advantages over the more commonly used fluorescein dye in high throughput screening (HTS) assays due to the fact that its excitation and emission spectra are red-shifted approximately 50 nm relative to fluorescein. Assays based on BODIPY® TMR ligands are therefore less susceptible to interference from tissue auto-fluorescence in the assay matrix, or the effects of colored or fluorescent compounds in the screening libraries. A series of BODIPY® TMR labeled peptides have been prepared that bind to a range of GPCRs including melanin concentrating hormone, bradykinin, and

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melanocortin receptors. Conditions have been optimized in order to utilize a comparable amount of receptor membrane preparation as is used in a radioligand binding assay. The assays are formatted in 384-well microplates with a standard volume of 40 μL. We have compared the assays across the different fluorescence polarization (FP) readers available to determine the parameters for each instrument necessary to achieve the required precision.

Key Words: Fluorescence; Peptide; Receptor; Ligand; Binding

INTRODUCTION

Over the past decade, high throughput screening (HTS) has become an integral part of the drug discovery process. As the discipline has developed, most HTS programs have been required to perform more screens per year on increasingly larger compound libraries, which has been the driving force towards assay automation, miniaturization, simplification, and cost-reduction. Additionally, HTS assays must be highly sensitive and robust in order to accurately identify active compounds ("hits") and avoid generating "false positive" results.

Plasma membrane-bound G protein-coupled receptors (GPCRs) are the primary conduit through which cells sense and respond to events in their extracellular environment, and are one of the most common targets of current therapeutic drugs. G protein-coupled receptors are also currently the largest class of molecular targets in HTS assays.[1] Many HTS assays for screening at GPCRs have been formatted as radioligand binding assays. However, due to the increasing volume of HTS assays, the use of radioactivity-based assays has led to safety, handling, waste disposal, and cost concerns. Suitable non-radioactive formats to replace the use of radioactivity have become more and more attractive.

Fluorescence polarization (FP) detection is a homogenous, non-radioactive assay format that is potentially well suited for HTS assays.[2,3] Although FP has seen limited use as a detection methodology for enzyme-substrate type assays, until now the available instrumentation has lacked the necessary sensitivity and precision required to replace radioligand binding assays. Recently, several new FP plate readers have been introduced with improved specifications, so that it is now possible to utilize FP detection for GPCR-ligand binding assays in microplate formats.

In developing, optimizing, and validating HTS assays a number of statistical parameters are typically considered, including signal to background ratio, standard deviation (SD), and coefficient of variation (CV) as measures of assay precision and robustness. The "*Z* value" is a recently defined statistical parameter that has gained common acceptance in most HTS laboratories as a measure of the ability of the assay to accurately identify active compounds.[4] Both the assay window and measurement variability are factors in the calculation an assay's *Z* value. A *Z* value of 0.5 or greater indicates an assay is acceptable for HTS.

Most FP assays reported in the literature have utilized fluorescein as the fluorophore. As we have described previously, we have labeled a series of GPCR

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peptide ligands (FluoPeptides™) with BODIPY® TMR (Molecular Probes, Inc., Eugene, OR) as an alternative dye to fluorescein for FP assays.[5,6] BODIPY® TMR has spectral excitation and emission wavelength maxima that are red-shifted by approximately 40 nm relative to fluorescein. This red-shifted dye gives improved performance in HTS assays, since the signal is less susceptible to interference from receptor membrane autofluorescence or quenching by colored compounds in test libraries. In the present work, we describe the characterization of additional novel FluoPeptides™ in receptor binding assays, and compare the assay precision obtained on several FP readers currently available.

MATERIALS AND METHODS

Receptor Membrane Preparations

Melanocortin MC4 and MC5, and bradykinin B2 receptor membrane preparations were obtained from Receptor Biology, Inc. (Beltsville, MD). Melanin concentrating hormone (MCH) receptor membrane preparations were obtained from Euroscreen s.a. (Brussels, Belgium).

FluoPeptides™

In general, peptides were synthesized on an Applied Biosystems (Foster City, CA) Pioneer™ Peptide Synthesis System using standard Fmoc-protection chemistry. Following cleavage from the resin with trifluoroacetic acid (TFA), the peptides were reacted with BODIPY® TMR SE (Molecular Probes, Inc., Eugene, OR) in *N*-methylpyrrolidinone. It is necessary to perform the labeling step after cleaving the peptide from the resin, because BODIPY® dyes are not stable to the acidic (TFA) cleavage conditions. The resultant FluoPeptides™ were purified by standard reversed-phase HPLC using gradients of 0.1% aqueous TFA vs. 0.1% TFA in acetonitrile.

Instrumentation

Fluorescence polarization was measured on Wallac Victor^{2}V™ and Wallac ViewLux™ (PerkinElmer Life Sciences, Boston, MA), TECAN ULTRA™ (Durham, NC), and LJL Analyst™ (Molecular Devices Corporation, Sunnyvale, CA) instruments. Spectral filters and dichroic mirrors were either factory supplied, or obtained from Omega Optical, Inc., Brattleboro, VT. The recommended optimal instrument configurations are shown in Table 1.

The signal acquisition time can be adjusted on each instrument, with increased precision achieved by longer acquisition times. In order to validly judge whether an assay's *Z* value was suitable for HTS, each instrument was configured

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Table 1. Optimal Spectral Filter Sets and Dichroic Mirrors for Use with BODIPY® TMR-Labeled Receptor Ligands

Instrument	Excitation Filter	Emission Filter	Dichroic Mirror
Victor^{2}V	531(25)	595(60)	N/A
ViewLux	535(20)	598(25)	BODIPY dichroic
ULTRA	535(25)	580(30)	Type 1 560
Analyst	535(22)	580(30)	555DRLP

to give total plate read times considered acceptable in the HTS environment. Accordingly, the integration time for the Analyst and Victor^{2}V was set at 0.2 and 0.5 s/well, respectively. The corresponding settings on the ULTRA were 10 lamp flashes with a 40 μs integration time. The ViewLux is an ultra high throughput imaging system that was set to image the entire plate for 20 seconds per channel.

Receptor–Ligand Binding Assays

Assays were formatted using reagent quantities similar to those used for a corresponding [^{125}I]-ligand filtration assay in order to be competitive from a cost standpoint for HTS assays. Binding buffers and incubation conditions were essentially the same as recommended by the receptor membrane vendor with the exception of the replacement of bovine serum albumin (BSA) with a propriety FP assay buffer supplement supplied by PerkinElmer Life Sciences, Boston, MA. BODIPY® TMR-labeled peptides have been found to bind non-specifically to BSA.

Unless otherwise stated, assays were conducted in Corning Costar® 384-well black NBS plates (Model 3654; Corning Inc., Acton, MA). The total assay volume was 40 μL, obtained by the sequential addition of 20 μL of competitive displacing ligand, 10 μL of fluorescent tracer, and 10 μL of receptor membrane preparation.

Data Analysis

Binding data was analyzed using GraphPad Prism® software (GraphPad Software, Inc., San Diego, CA). Competitive binding data was fit to a one-site competitive binding equation using non-linear regression curve fitting. Data points shown are the mean ± 1SD ($n = 6$).

Assay precision was assessed by calculating the Z value using the equation below, where σ_b = SD of the bound signal, σ_d = SD of the displaced signal, μ_b = mean bound signal, and μ_d = mean displaced signal.[4]

$$Z = 1 - \left[\frac{(3\sigma_b + 3\sigma_d)}{|\mu_b - \mu_d|}\right]$$

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RESULTS

Melanocortin MC4 Receptor Binding Assay

The melanocortin MC4 receptor has been recognized as an important component in the signaling pathways involved in the regulation of food intake and energy balance, and is a potential target for developing anti-obesity drugs.[7] The standard radioligand used for MC4 receptor binding assays is [^{125}I]-[Nle^4,D-Phe^7]-α-MSH (NDP-α-MSH). We therefore prepared the analogous BODIPY® TMR NDP-α-MSH as a tracer for fluorescence polarization detection.

BODIPY® TMR NDP-α-MSH at a concentration of 1 nM per well in 50 mM HEPES, pH 7.4 containing 2.5 mM $CaCl_2$ and buffer supplement was incubated with 6.4 μg/well of human MC4 receptor membranes ($B_{max} = 2.58$ pmol/mg protein). The assay was incubated for one hour at room temperature, and the plate then imaged for 20 seconds per channel on the Wallac ViewLux. Generating competitive displacement curves using four MSH derivatives (Fig. 1) yielded the same rank order of potency as obtained in a corresponding radioligand binding assay (Table 2). Pooling the data from the four curves yielded a Z value of 0.68.

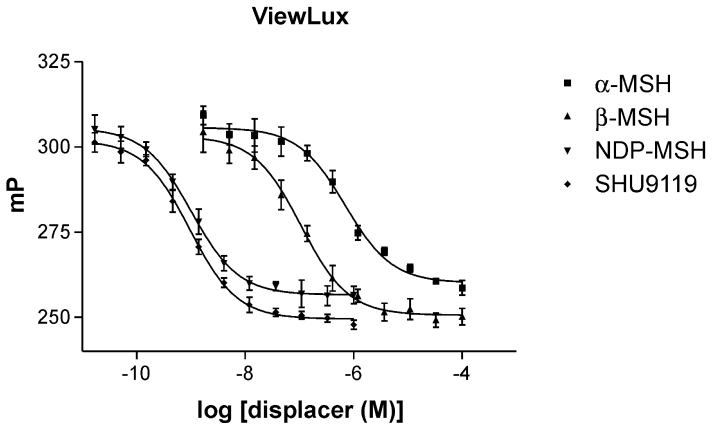

Figure 1. Displacement of BODIPY TMR NDP-α-MSH from MC4 receptors using a series of MSH analogs measured on the Wallac ViewLux.

Table 2. Potency of Competing Ligands in Displacing BODIPY® TMR NDP-α-MSH or [^{125}I] NDP-α-MSH from MC4 Receptors

Displacer	K_i Fluorescence Polarization (nM)	K_i Radioligand (nM)
SHU 9119	0.63	0.38
NDP-α-MSH	0.68	1.1
α-MSH	458	690
β-MSH	90	300

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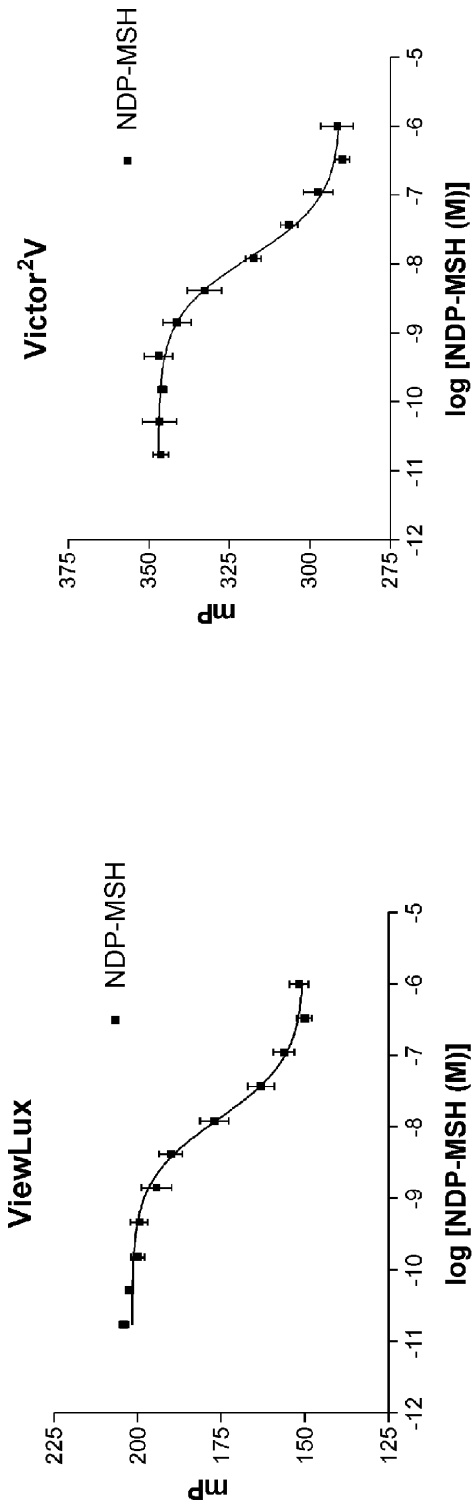

Figure 2. Comparison of precision obtained reading the same plate on the ViewLux and Victor²V instruments.

The Wallac Victor^{2}V multi-label reader also demonstrates the sensitivity required for FP GPCR-ligand binding assays. The assay precision achieved using the ViewLux was compared to the precision with the Victor^{2}V. The same plate was imaged on the ViewLux for 20 seconds per channel and also read on the Victor^{2}V with an integration time of 0.5 s/well. The precision obtained with the ViewLux ($Z = 0.67$) was significantly higher than with the Victor^{2}V ($Z = 0.56$) as shown below (Fig. 2). Although the precision with theVictor^{2}V was lower, it does satisfy the criteria for an acceptable HTS assay ($Z > 0.5$). However, under these conditions a 384-well plate can be read on the ViewLux in approximately 1 min, where it would require approximately 10 min on the Victor^{2}V.

Melanocortin MC5 Receptor Binding Assay

The melanocortin MC5 receptor subtype has also been heretofore characterized using the radioligand [^{125}I] NDP-α-MSH. The MC5 receptor binding assay was also carried out using BODIPY TMR NDP-α-MSH as tracer under incubation conditions described above with 2.1 μg/well of human MC5 receptor membranes ($B_{max} = 2.58$ pmol/mg protein). The plate was read on the LJL Analyst with an integration time of 0.2 seconds per well. A series of competition curves using MSH analogs (Fig. 3) yielded a rank order of potency consistent with rad filtration data although the low-potency agonists have different orders (Table 3).

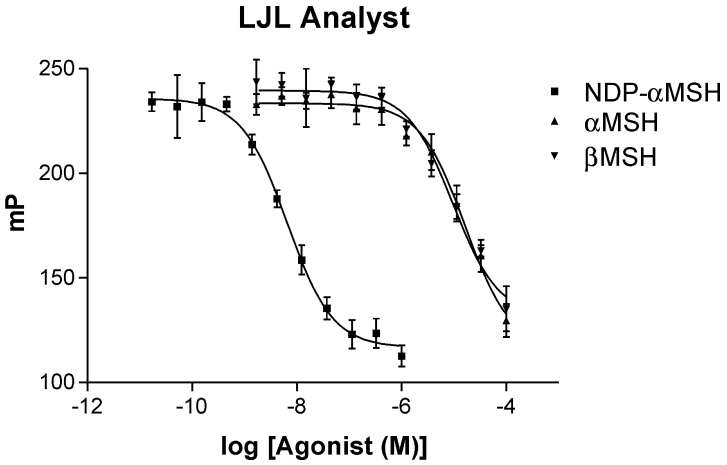

Figure 3. Displacement of BODIPY TMR NDP-α-MSH from MC5 receptors using a series of MSH analogs measured on the LJL Analyst.

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Table 3. Potency of Competing Ligands in Displacing BODIPY® TMR NDP-α-MSH or [^{125}I] NDP-α-MSH from MC5 Receptors

Displacer	K_i Fluorescence Polarization (nM)	K_i Radioligand (nM)
NDP-α-MSH	2.4	1.8
α-MSH	5900	470
β-MSH	3400	810

Bradykinin B2 Receptor Binding Assay

Bradykinin and its three receptor subtypes mediate multiple physiological responses to injury and trauma.[8] The human bradykinin B2 receptor has been cloned, and is a current target in HTS assays.[9] A large number of bradykinin analogs have previously been described, including to potent antagonist HOE 140.[10] We have prepared BODIPY® TMR HOE140 for FP receptor binding assays using the B2 receptor.

BOPIPY® TMR HOE140 tracer at a well concentration of 2 nM in 50 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 0.1% 1–10 phenanthroline and 2% buffer supplement was incubated with 8.0 µg of human B2 receptor membranes ($B_{max} = 4.1$ pmol/mg protein) in a total volume of 40 µL per well. The amount of receptor preparation used was equivalent to the amount recommended by the manufacturer for a radioligand filtration assay. Following a one hour incubation at room temperature, the 384-well plate was imaged for 20 seconds per channel on the Wallac ViewLux. Competitive displacement curves were generated using three competing peptide ligands (Fig. 4). The rank order of potency of the competing

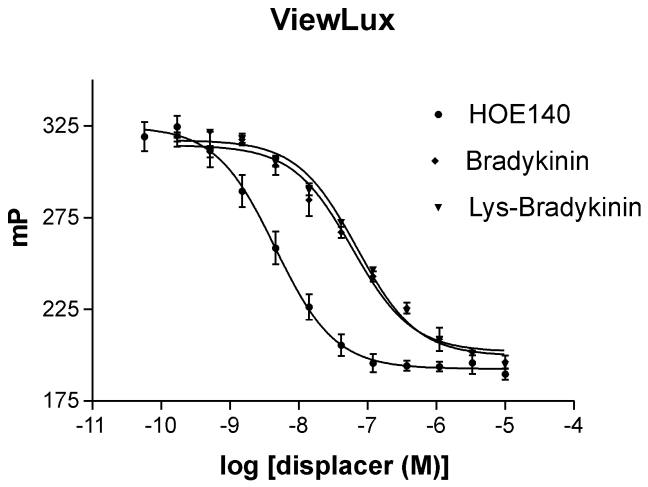

Figure 4. Displacement of BODIPY TMR HOE140 from bradykinin B2 receptors using known displacers.

Table 4. Potency of Competing Ligands in Displacing BODIPY® TMR HOE 140 or [^{3}H]-Bradykinin from B2 Receptors

Displacer	K_i Fluorescence Polarization (nM)	K_i Radioligand (nM)
HOE140	1.36	0.39
Bradykinin	8.65	0.79
Lys-Bradykinin	28	1.5

ligands as measured by K_I values was the same as measured in a filtration radioligand binding assay (Table 4). A Z value of 0.75 was calculated by using the average standard deviation on the three curves.

Melanin Concentrating Hormone Receptor Binding Assay

The neuropeptide melanin concentrating hormone (MCH) has also been implicated in the control of feeding behavior through its receptor located in the hypothalamus.[11] The MCH receptor is therefore a current target in HTS assays to identify potential anti-obesity drugs. The development of suitable radioligands for labeling the MCH receptor has been difficult due to the chemical instability and hydrophobicity of the peptide. Currently, the radioligands of choice for MCH receptor binding assays are either [^{125}I]-MCH or [^{125}I]-[Phe^{13},Tyr^{19}]-MCH. We synthesized BOPIPY® TMR [Phe^{13},Tyr^{19}]-MCH and have found it suitable for an FP MCH receptor binding assay.

BOPIPY® TMR [Phe^{13},Tyr^{19}]-MCH at a concentration of 1 nM in 25 mM HEPES, pH 7.4 containing 2 mM $MgCl_2$ and buffer supplement was incubated with

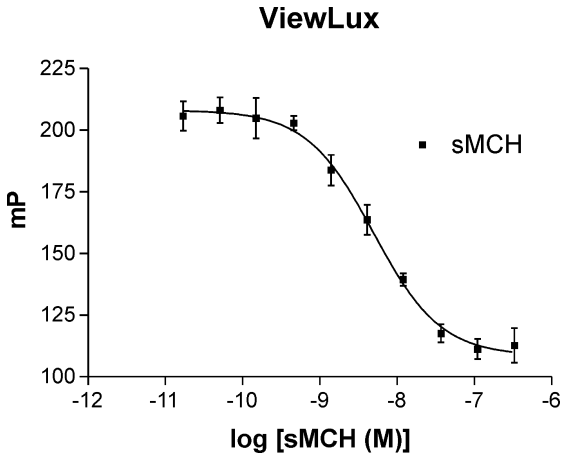

Figure 5. Displacement of BODIPY® TMR [Phe^{13},Tyr^{19}]-MCH from MCH receptors using salmon MCH.

3.4 μg of human MCH receptor membranes ($B_{max} = 1.8$ pmol/mg protein) for 3 hours, and then imaged on the Wallac ViewLux for 30 seconds per channel. A competitive displacement curve using salmon MCH as the competitor is show in Fig. 5. Care must be taken in handling BOPIPY TMR [Phe^{13},Tyr^{19}]-MCH, since analogous to its radiolabeled counterpart, it is susceptible to oxidation and may stick to plastic surfaces. When preparing solutions of tracer or competing ligands, low-retention pipette tips and non-binding surface plasticware should be used.

DISCUSSION

Radioligand binding to GPCR is one of the most common assay formats currently utilized in HTS. Despite its popularity, the pressure to reduce the use of radioactivity in HTS laboratories has led to considerable effort to develop non-rad alternatives, both direct binding assays as well as second messenger functional assays. We have prepared a series of peptide ligands labeled with the dye BOPIPY® TMR, whose binding to GPCRs can be monitored using FP detection. As described herein, suitable ligands have been developed for binding to the melanocortin MC4 and MC5 receptors, the bradykinin B2, and the MCH receptor. In addition to avoiding the use of radioactivity, FP assays are homogeneous "mix and read" assays that can be easily formatted for HTS.

Most FP binding assays can be formatted using similar buffers, incubation conditions, and receptor membrane quantity as would be used for a corresponding radioligand binding assay. A notable difference is the need to avoid using BSA as a blocking agent in the assay buffer, since BOPIPY® TMR-labeled ligands have been found to bind non-specifically to BSA. As an alternative, a proprietary assay buffer supplement is available from PerkinElmer Life Sciences. BOPIPY® TMR-labeled ligands are also typically more hydrophilic than their radioligand counterparts, so care must be taken in their handling to avoid losses due to sticking to glass or plastic vessels.

Until recently, the main barrier to the development of receptor–ligand binding assays using FP has been the lack for precision of available FP readers when using tracers at low nanomolar concentration. Several instruments now available do have the required precision for these assays. However, instrument precision is still a major consideration, and limits the range of assays that can be developed. Based on the results described in this paper and those previously published,[5,6] we are able to make some general predictions as to assay parameters necessary for a successful FP assay. The most important factors in our experience are receptor expression level, ligand affinity, and ligand fluorescence intensity. In order to obtain the precision required for HTS assays the receptor expression level should be at least 1 pmol/mg protein; ligand affinity must be below 5 nM; and the fluorescence intensity of the tracer at least 40% of the intensity of free BOPIPY® TMR dye.

Future extension of this technology can be expected with improvements in FP reader sensitivity and precision, and the development of higher intensity, and more hydrophilic dyes.

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