An ECL Multiplexed Assay for GPCRs

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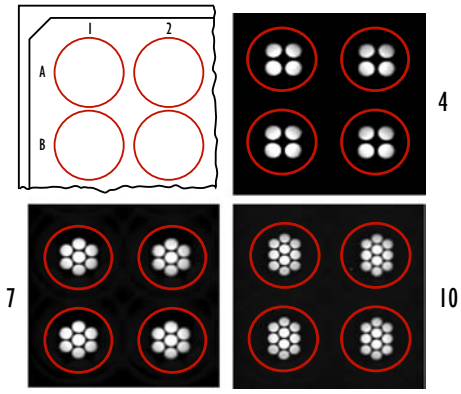
❶ Abstract

G Protein-Coupled Receptors (GPCRs) are integral membrane proteins that bind specific ligands and transmit changes in the external cellular environment to the interior of the cell. GPCRs constitute one of the largest families of proteins with the broadest span of therapeutic indications. They have proven to be highly tractable and selective drug targets with ~24% of the drug sales targeting GPCR-mediated mechanisms. Traditional screening of GPCRs has utilized the binding of a radioligand to a membrane receptor preparation, followed by separation of the unbound radioligand from the ligand-receptor complex. This gold standard method produces accurate and clean data, but is time-consuming and produces significant radioactive waste. Here, we present work done in conjunction with Meso Scale Discovery using their plate-based electrochemiluminescent technology to develop a 96-well multi-array, non-radioactive membrane receptor-ligand binding assay that simultaneously examines 2 related GPCRs within the same well (i.e. multiplexing).

❷ Meso Scale Discovery Multiplexing



96 wells: 4, 7, and 10 spots/well

Multi-Spot™ plates enable multiplexing.

Microfluidics allows "addressing" to immobilize distinct species on each electrode.

SECTOR™ Imager 6000 reader results ≤ 0.05% optical cross-talk.

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❸ Assay Protocol for Multiplex GPCRs

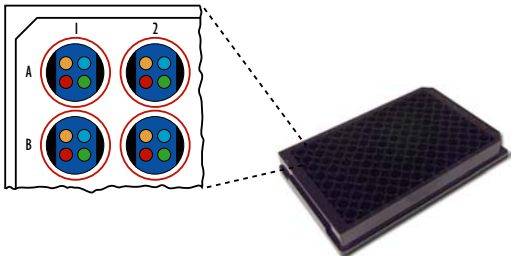
1. Cellular membranes are immobilized onto 4 spatially distinct electrodes within the same well in MSD Multi-Spot 4-Spot Custom Coated 96-well plates. Dispense 0.2 µL (0.5 µg per spot) of membranes in Binding Buffer (25mM HEPES-KOH, pH 7.3, 1mM $CaCl_2$, 5mM $MgCl_2$, and protease inhibitors (Roche, 1836170)) and incubate for 1h at room temperature.

2. Wash plates with Binding Buffer.

3. Dispense 3 µL of unlabeled compound, followed by 42 µL MSD TAG labeled ligand in Binding Buffer and MSD Blocker A (0.5% final) and incubate for 1hour at room temperature.

4. Wash plates with Binding Buffer.

5. Dispense 150 µL of MSD Read Buffer T (2X, surfactant free) and image plate on an MSD SECTOR Imager 6000 reader. Plate is read in ~70 seconds, regardless of plate type. Total time for the assay is 2.5 hours, including liquid handling time.



Receptor-containing membranes

↓

Compounds and MSD Labeled Ligand

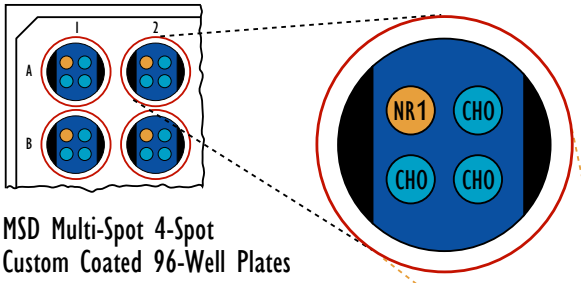
↓

MSD Read Buffer

↓

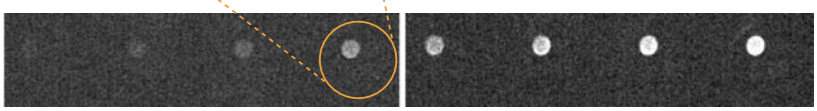
An ECL Multiplexed Assay for GPCRs

❹ Neuropetide Receptor 1: Ligand & Membrane Titration



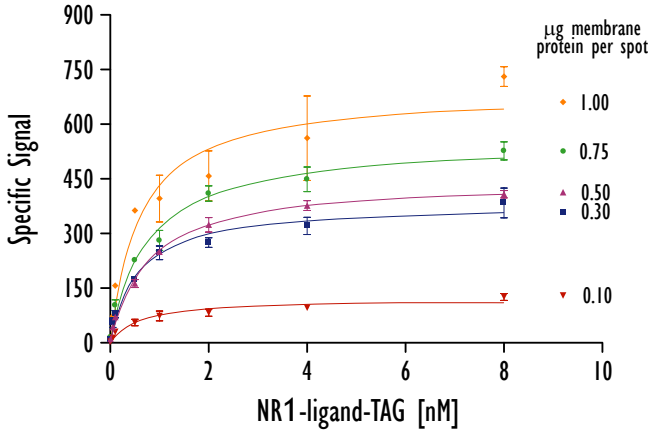
MSD Multi-Spot 4-Spot
Custom Coated 96-Well Plates

Neuropeptide Receptor 1(NR1)-containing membranes were immobilized on one of the four spatially distinct electrodes, while CHO-K1 membranes prepared from the parent cell line (background) were immobilized on the remaining three electrodes within the same well. Titrations of membrane were assayed in ligand-binding isotherms. K_d values remain constant for each membrane concentration and agree with published data. Use of as little as 0.1 µg of membrane protein per spot demonstrates the sensitivity of MSD Multi-Spot technology.



images shown were obtained with the SECTOR Imager 6000 and represent 0.75 µg membrane protein per spot

NR1-ligand-TAG [nM] → 0.01 0.05 0.10 0.50 1.00 2.00 4.00 8.00



µg Membrane protein per spot	Signal/Background	K_d, nM(95% C.I.)
0.10	7.6	0.59 (0.14 - 1.00)
0.30	27.8	0.56 (0.28 - 0.85)
0.50	23.9	0.77 (0.58 - 0.97)
.075	34.4	0.76 (0.41 - 1.11)
1.00	42.1	0.60 (0.10 - 1.09)

Background signal was obtained with membranes prepared from the parent cell line, CHO-K1.

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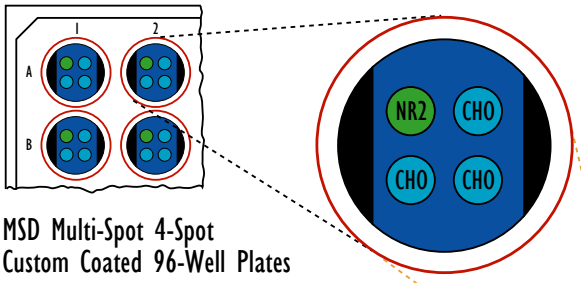
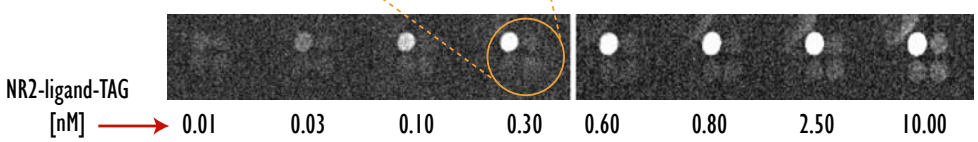
❺ Neuropeptide Receptor 2: Ligand & Membrane Titration

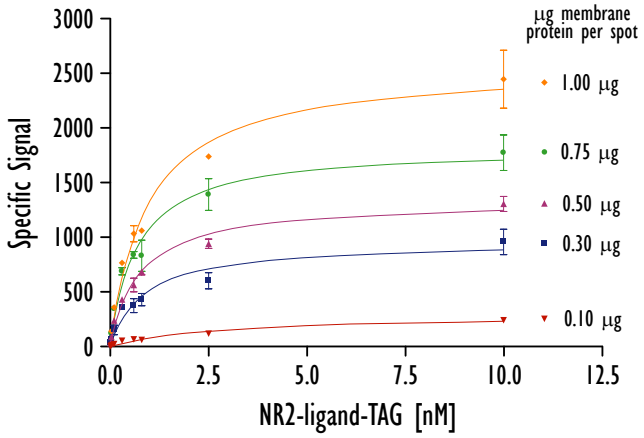


MSD Multi-Spot 4-Spot
Custom Coated 96-Well Plates

Neuropeptide Receptor 2 (NR2)-containing membranes were immobilized on one of the four spatially distinct electrodes, while CHO-K1 membranes prepared from the parent cell line (background) were immobilized on the remaining three electrodes within the same well. The images show results obtained from a titration of the NR2 ligand labeled with MSD TAG, and agree with published data.



images shown were obtained with the SECTOR Imager 6000 and represent 0.50 µg membrane protein per spot



µg Membrane protein per spot	Signal/Background	K_d, nM(95% C.I.)
0.10	2.9	2.89 (0.87 - 4.91)
0.30	11.6	0.87 (0.28 - 1.47)
0.50	14.9	0.75 (0.46 - 1.04)
.075	21.3	0.69 (0.37 - 1.01)
1.00	29.1	0.95 (0.58 - 1.32)

Background signal was obtained with membranes prepared from the parent cell line, CHO-K1.

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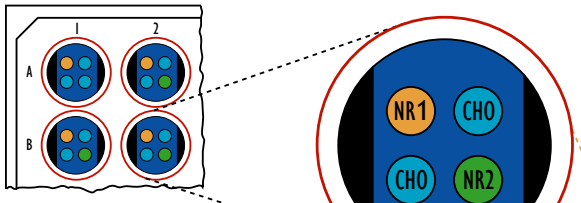
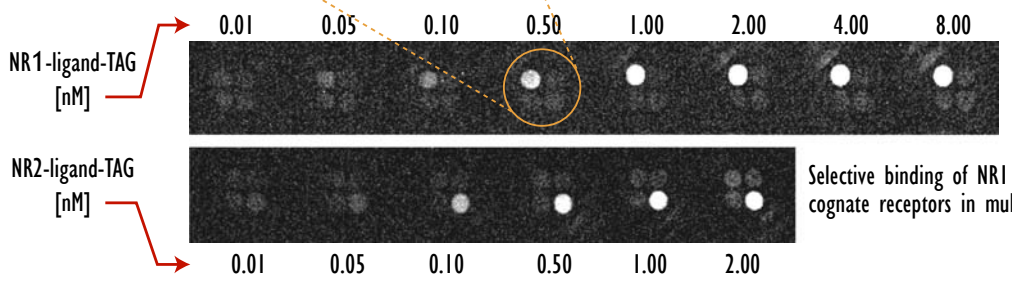
6 MSD Multiplex Assay with Neuropeptide Receptors 1 and 2

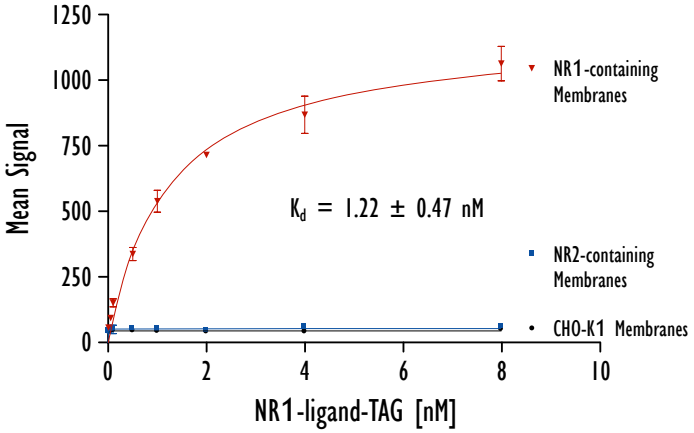


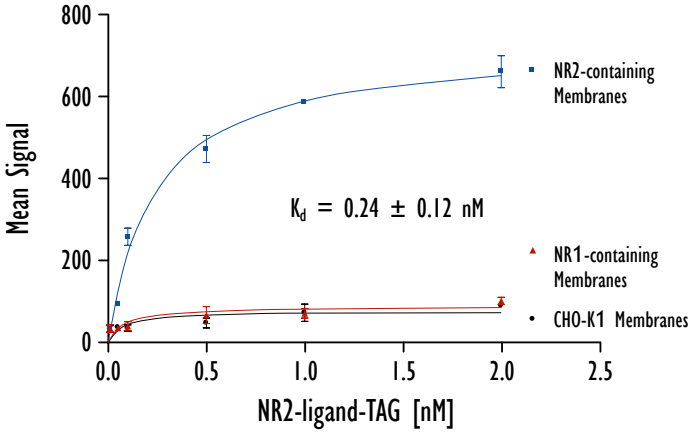
MSD Multi-Spot 4-Spot
Custom Coated 96-Well Plates

Neuropeptide Receptors 1 (NR1) and 2 (NR2) were immobilized on spatially distinct electrodes in the same well, 0.5 µg per spot, in MSD Multi-Spot 4-Spot 96-well plates. Membranes isolated from the parent cell line, CHO-K1, were immobilized on the remaining two electrodes in each well. The K_d values obtained from these ligand-binding isotherms demonstrate that the binding specificity of individual labeled ligands was conserved in this multiplex assay.



Selective binding of NR1 and NR2 ligands to their cognate receptors in multiplex format.





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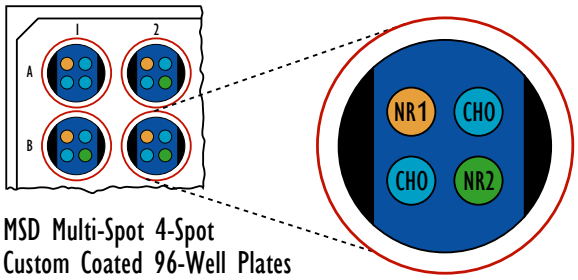
❼ Multiplexed Neuropeptide Receptors: Selective Inhibition of NR1

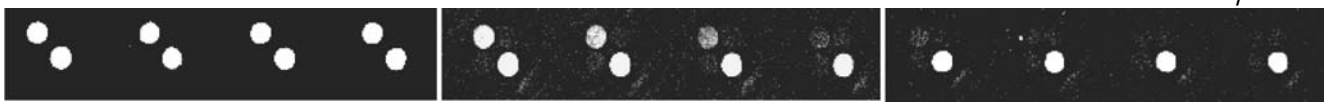


MSD Multi-Spot 4-Spot
Custom Coated 96-Well Plates

0.5 µg membrane per spot

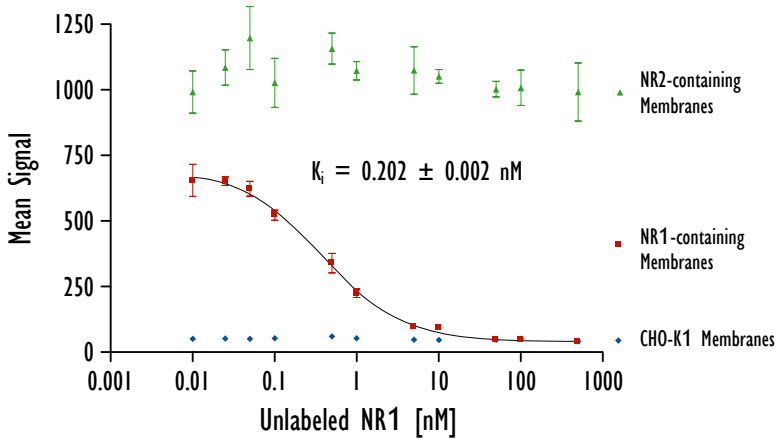
Neuropeptide Receptors 1 (NR1) and 2 (NR2), and CHO-K1 membranes prepared from the parent cell line, were immobilized on spatially distinct electrodes in the same well, 0.5 µg per spot, in MSD Multi-Spot 4-Spot 96-well plates. Unlabeled NR1 ligand was titrated against 1nM of MSD TAG-labeled NR1 ligand and 1nM MSD TAG-labeled NR2 ligand. These results show that selective inhibition of neutopeptide receptor 1 can be achieved the the multiplex format.

NR1 is selectively inhibited



Increasing concentration of Unlabeled NR1 ligand

1nM Labeled NR1 Ligand and 1nM Labeled NR2 Ligand
Challenged with Unlabeled NR1 Ligand



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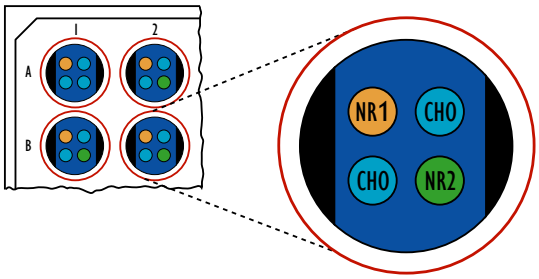
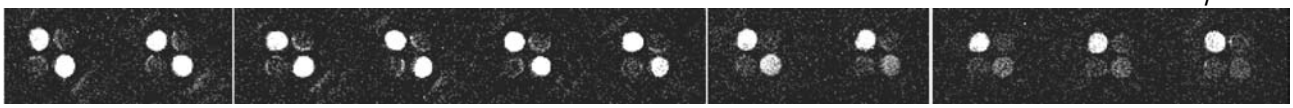
❽ Multiplexed Neuropeptide Receptors: Selective Inhibition of NR2

MSD Multi-Spot
4-SpotCustom Coated
96-Well Plates



0.5 µg membrane per spot

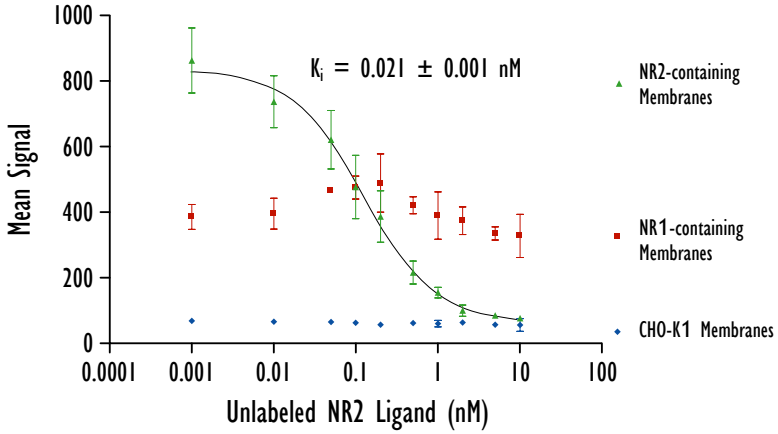
NR2 is selectively inhibited



Increasing concentration of Unlabeled NR2 ligand →

Neuropeptide Receptors 1 (NR1) and 2 (NR2), and CHO-K1 membranes prepared from the parent cell line, were immobilized on spatially distinct electrodes in the same well, 0.5 µg per spot, in MSD Multi-Spot 4-Spot 96-well plates. Unlabeled NR2 ligand was titrated against 1nM of MSD TAG labeled NR1 ligand and 1nM MSD TAG labeled NR2 ligand. These results show that neutopeptide receptor 2 can be achieved the the multiplex format.

1nM Labeled NR1 Ligand and 1nM Labeled NR2 Ligand
Challenged with Unlabeled NR2 Ligand



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❾ Validation of Pharmacology: Comparison of MSD with Filter Binding Assays

Neuropeptide 1 (NR1)-containing membranes, 1.0 µg per well, were passively immobilized onto MSD Multi-Array Custom Coated 384-well plates. Competition studies were conducted in which 6 ligands or compounds were titrated against 1nM MSD TAG-labeled NR1 ligand. Compounds were ranked based on potency and compared to analogous studies using Filter Binding, SPA, and LEADseeker™ technologies. Comparison of the results obtained with the MSD TAG-labeled ligand reveal equivalent performance to filter binding studies using radiolabeled ligand.

	MSD 384-well		Filter Binding 96-well		SPA 384-well		LEADseeker 384-well**	
	IC_{50} (nM)	Rank Order	IC_{50} (nM)	Rank Order	IC_{50} (nM)	Rank Order	IC_{50} (nM)	Rank Order
NR1 ligand	0.7	1	0.5	1	0.3	1	0.6	1
Synthetic Ligand 1	5	2	Not determined		Not determined		37	3
NR1 ligand-YG5*	3	2	2	2	2	2	4	2
Compound AMG 1	6	2	23	3	4	2	Not determined	
Synthetic Ligand 2	943	3	580	4	400	3	Not determined	
NR2 Ligand	$11x10^3$	4	$18x10^3$	5	$75x10^3$	3	$25x10^3$	4

*NR1 ligand with the addition of 5 glycines and one tyrosine
* * Maximum inhibition observed with LEADseeker was 80% vs. 100% for others

❿ Conclusions

Multiplex GPCR assays were achieved with Meso Scale Discovery's Multi-Spot 4-Spot Custom Coated 96-well plates and MSD TAG-labeled ligands. MSD TAG-labeled neuropeptide ligands exhibited pharmacological properties indistinguishable from radiolabeled ligands

Neuropeptide Receptors 1 and 2 immobilized in the same well (0.5 µg per spot) exhibited conserved binding specificity with respect to MSD TAG-labeled ligands, and were selectively inhibited with the appropriate unlabeled ligands.

The assay is fast (complete in 2.5 hours) and sensitive, using as little as 0.1 µg membrane per spot.

This assay is completely automated and affords a multiplex high-throughput workflow with the ability to screen 15,000 compounds per shift in workstation mode.

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