A Membrane Receptor Binding Assay Using MesoScale Electrochemiluminescence Technology

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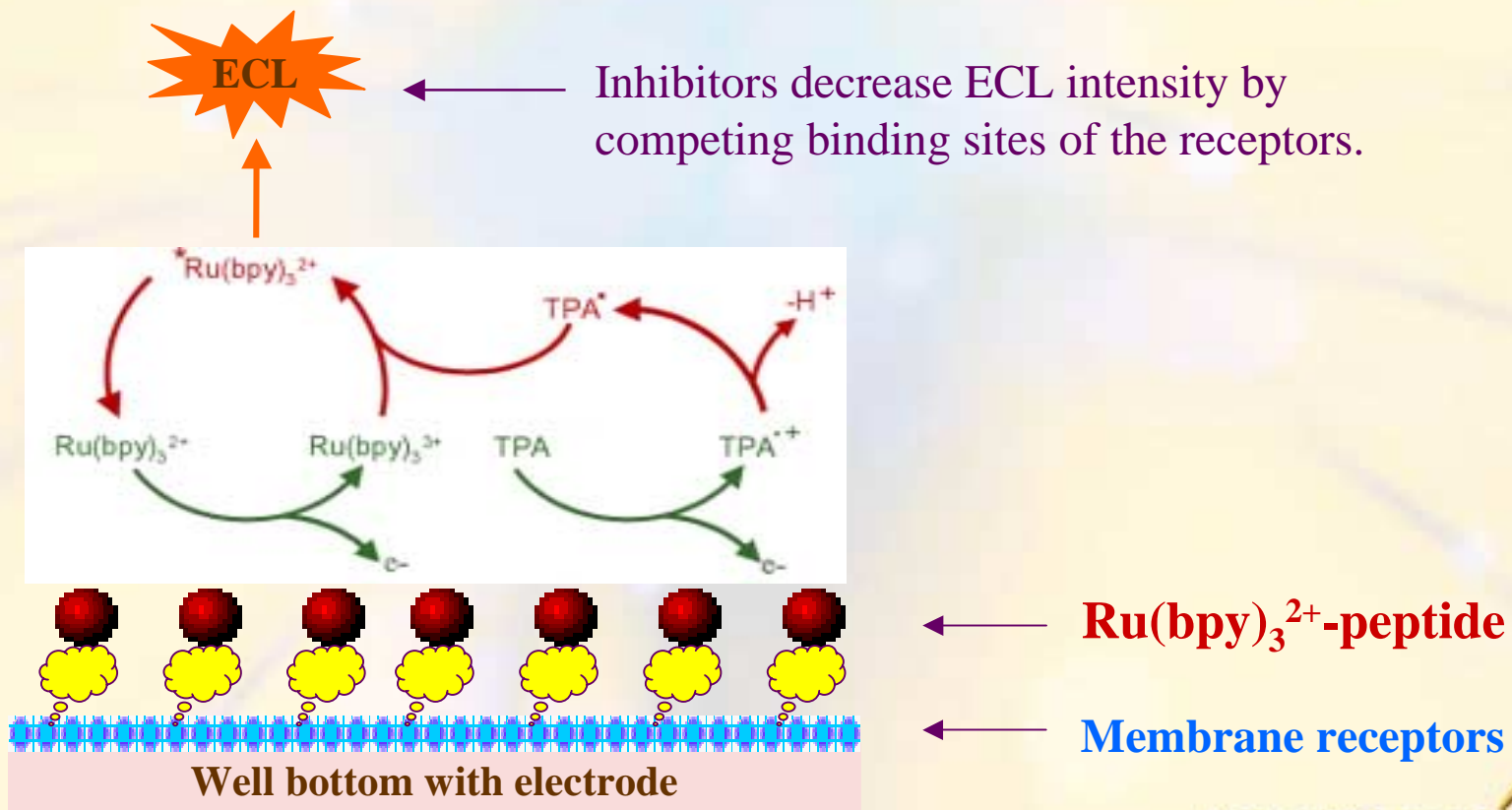
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Background

- **The receptor**
    - A GPCR stably expressed in HEK293 cells
    - Membrane

- **The ligand**
    - A neuropeptide with Ruthenium labeled at the N-terminus

- **The goal**
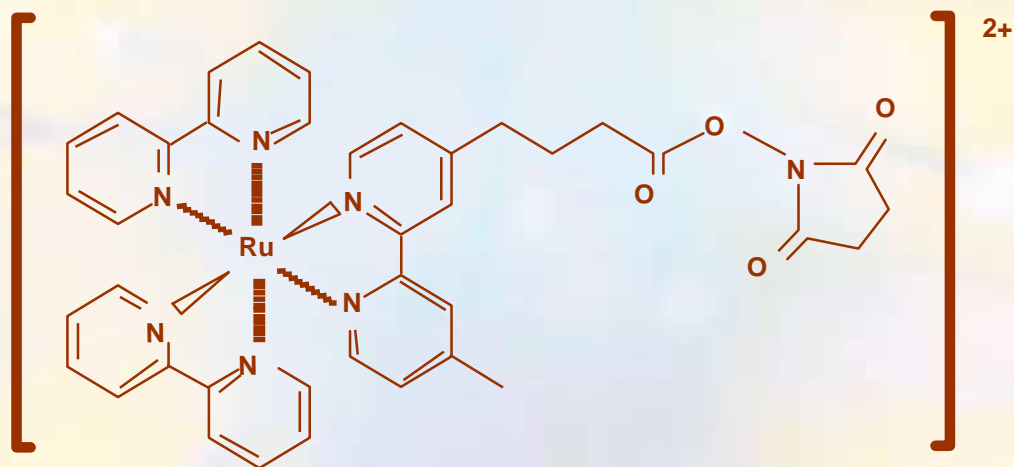   - To identify small molecules that bind to the target receptor

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Meso Scale Technology – Assay Mechanism

- Electrochemiluminescence (ECL) signal (image by CCD camera)
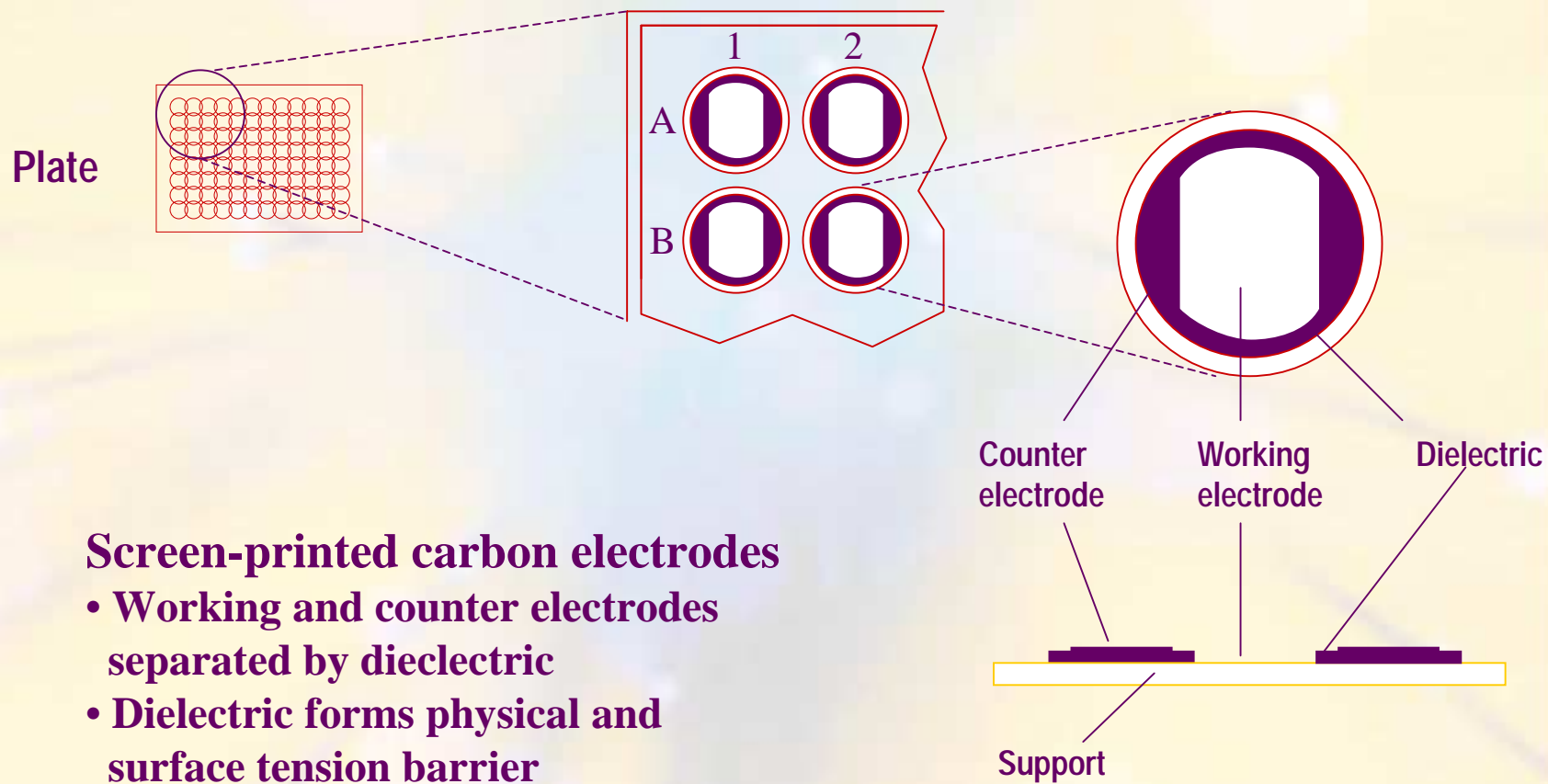
- Ruthenium labeled ligand binding

ECL

Inhibitors decrease ECL intensity by competing binding sites of the receptors.

$^*Ru(bpy)_3{}^{2+}$

TPA^* $-H^+$

$Ru(bpy)_3{}^{2+}$ $Ru(bpy)_3{}^{3+}$ TPA TPA^{*+}

e− e−

$Ru(bpy)_3{}^{2+}$-peptide

Membrane receptors

Well bottom with electrode

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The labeling molecule

Ruthenium (II) tris-bipyridine NHS ester
$Ru(bpy)_3^{2+}$

The Plates

Plate

1   2

A

B

Counter electrode

Working electrode

Dielectric

Support

Screen-printed carbon electrodes
- **Working and counter electrodes separated by dieclectric**
- **Dielectric forms physical and surface tension barrier**
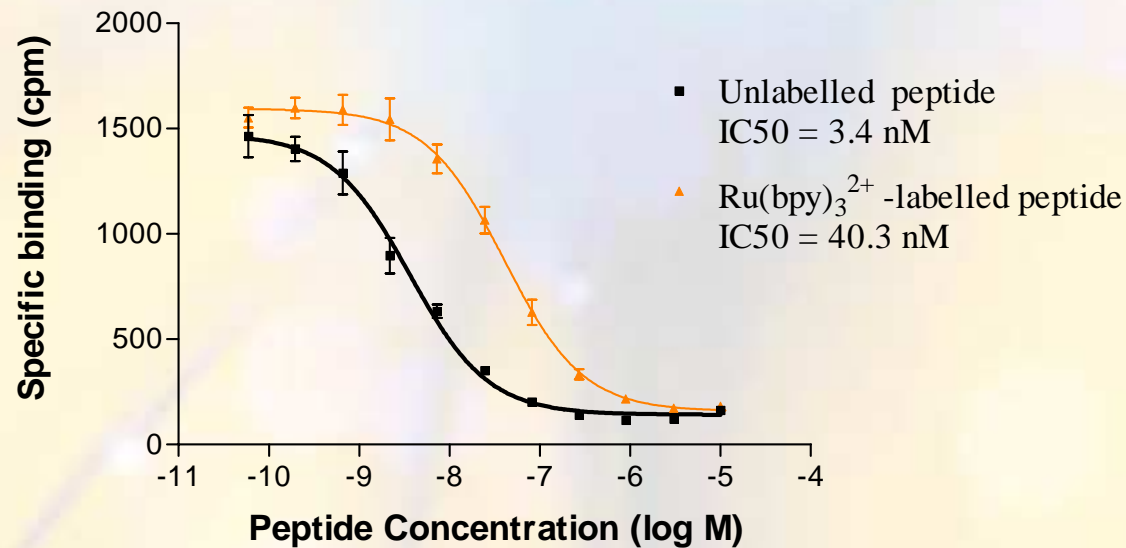
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Binding assay using MSD technology

1. Deposit membranes to assay plates, incubate @ RT for 1 hr **(CyBi-well)**

2. Add Blocker to minimize non-specific binding, incubate @ RT for 30 min **(CyBi-well)**

3. Add test compounds **(CyBi-well)**

4. Add Ru-labeled ligand, incubate @ RT, 1 hr **(multidrop)**

5. Add reading buffer (TPA) **(multidrop)**

6. Read ECL intensity in MSD's instrument

   (Plate crane/Sector)

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Effects of Ru-labeling on the Binding Affinity of the Peptide

^{125}I- binding SPA:

**Labeling caused >10 fold increase of IC$_{50}$ in ^{125}I competition binding.**

- Unlabelled peptide
  IC50 = 3.4 nM

- Ru(bpy)$_3^{2+}$ -labelled peptide
  IC50 = 40.3 nM

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Effects of Ru-labeling on Functional Activity

FLIPR assay:

Labeling caused >10 fold increase of EC_{50} in FLIPR assay

- Unlabeled
  EC50 = 5.5 nM

- $Ru(bpy)_3^{2+}$ -labeled
  EC50 = 61.1 nM
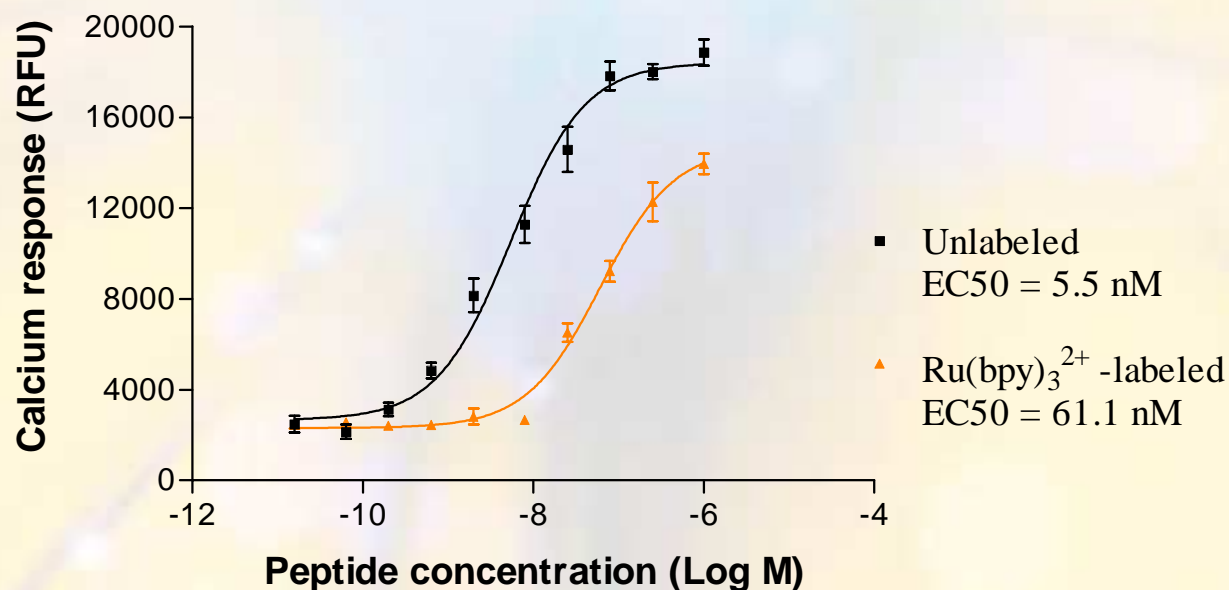
Titration of Membrane Proteins
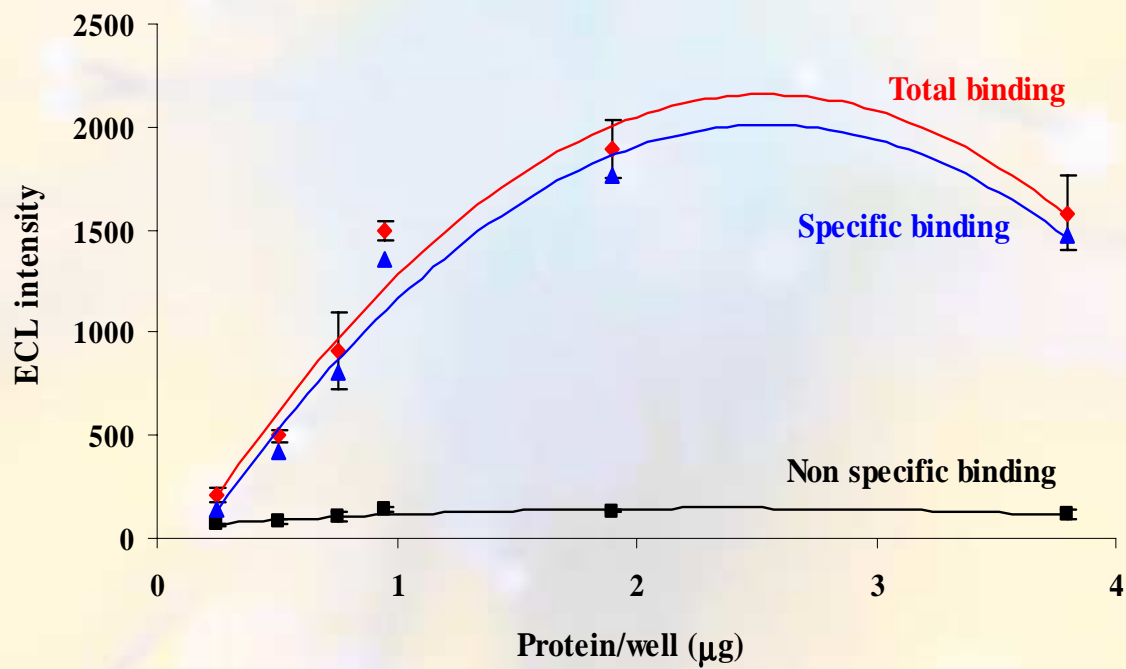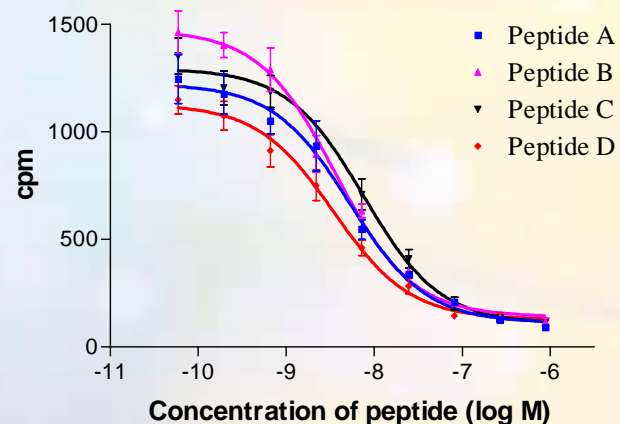
Saturation Binding
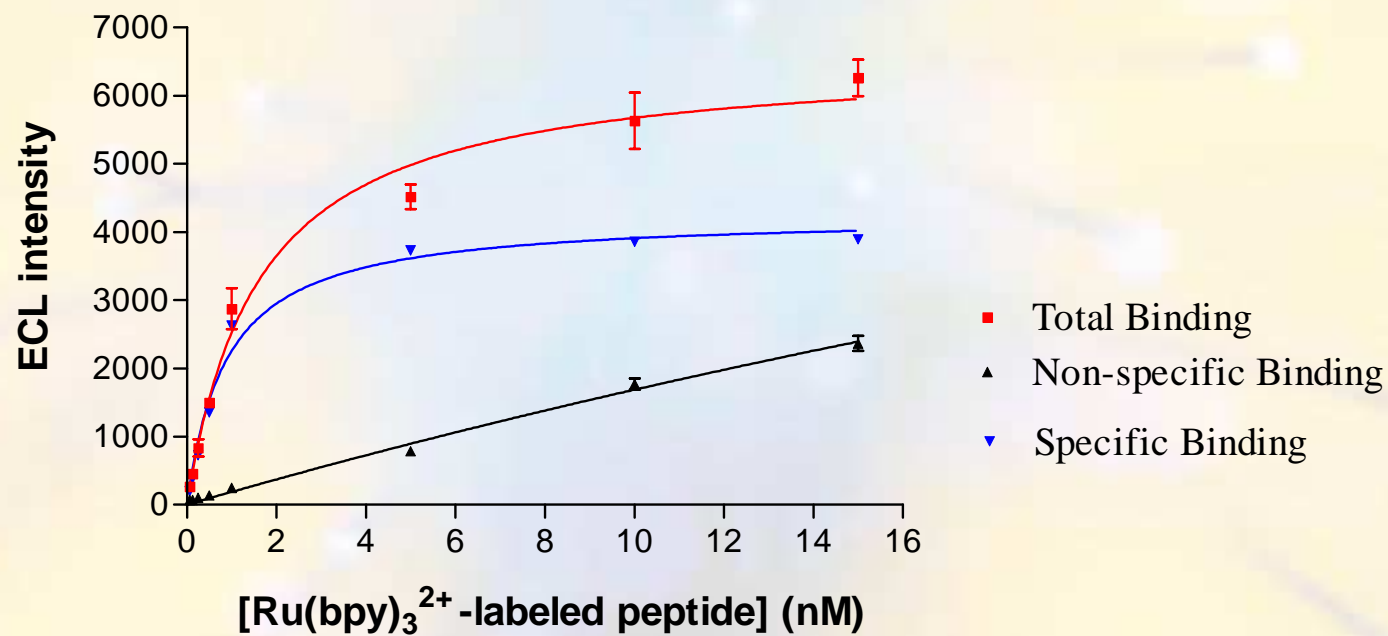
Kd $= 0.89 \pm 0.14$ nM
Bmax $= 4.0$ pmol/mg protein

Competition Binding

ECL binding assay
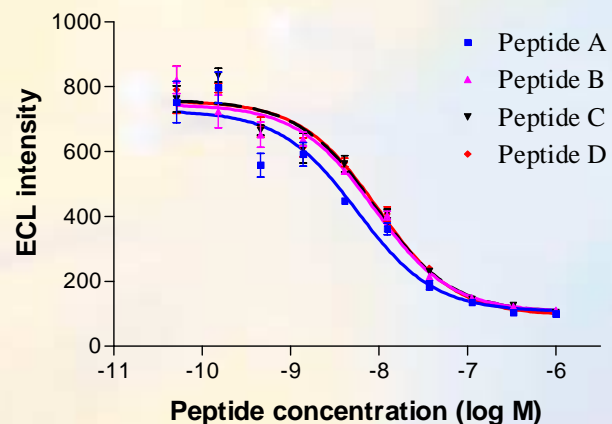
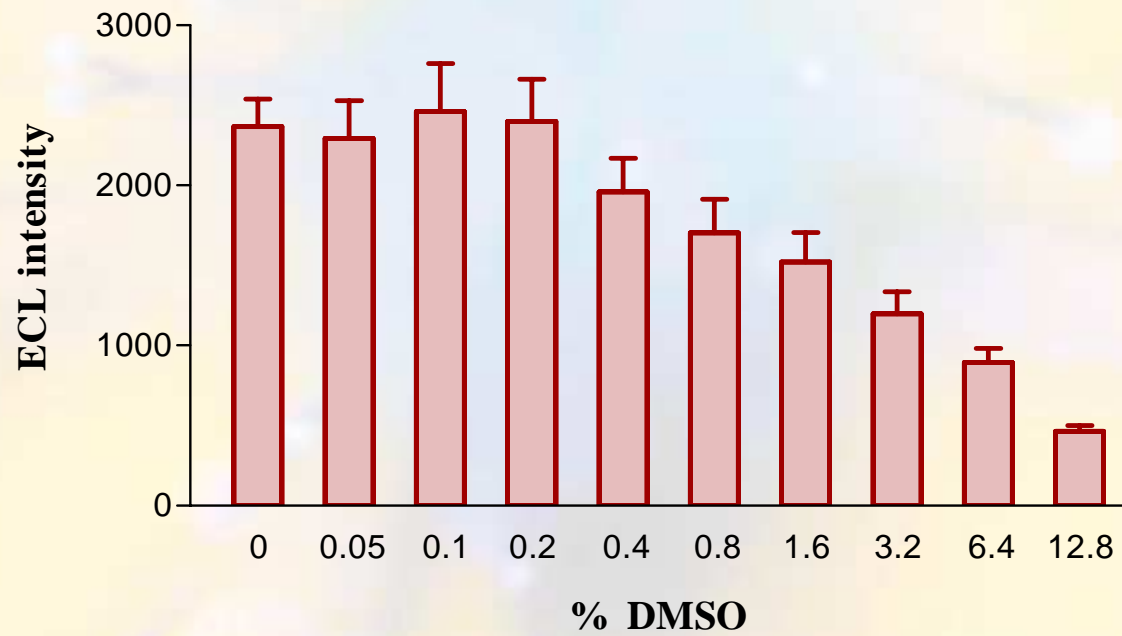### ^{125}I-SPA assay

Ki values (nM) of peptide ligands

	Peptide A	Peptide B	Peptide C	Peptide D
Meso Scale	3.01	4.95	5.26	3.67
SPA	4.38	3.01	6.31	2.87

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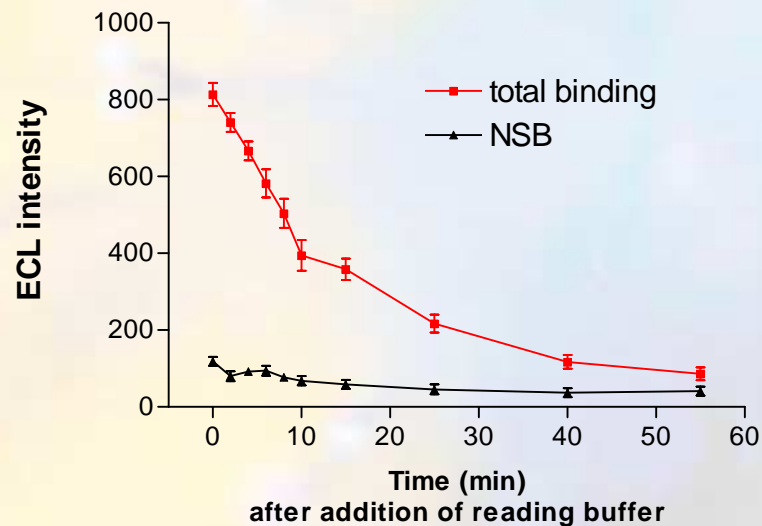Thinking Ahead in CNS

Effects of DMSO

Technical Challenge 1

- Signal drops after the addition of reading buffer

**ECL Signal Declination
after Addition of Reading Buffer**

Time (min)	% signal left
0	100.0
2	95.1
4	82.6
6	70.2
8	61.4
10	47.0
15	43.1
25	24.6
40	11.5
55	6.5

- **Reason:**
  Reading buffer caused irreversible dissociation of receptor-ligand complex.

- **Solution:**
  Read 1 min after addition of reading buffer.

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HTS Assay Conditions

- **Total reaction volume = 25 μl**

- **Total membrane protein = 0.9 μg/well**

- **Final labeled ligand concentration = 0.5 nM**

- **Final Compound concentration =10 μM**

- **Final BSA concentration = 0.2%**

- **Final DMSO concentration = 0.2%**

- **Positive control peptide = 1 μM**

- **Reading buffer (TPA) = 1 X**

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Signal Declination during Read Time

	Bottom left	Top left	Whole plate
Avg. ECL	2773.6	2302.7	2483.1
SD	176.4	230.0	254.2
% CV	6.4	10.0	10.2

Overall Assay window: 19.2
Z' factor: 0.7

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Technical Challenge 2: Membrane deposition

- Shape matters

• Volume matters

Bad Membrane Deposition

Plate AZW04008: 7 hits in row A. None confirmed.

11 plates of 977 plates screened had this phenomenon
88 of 267 hits were from these wells

Screen Performance

A typical daily results summary (70 plates)
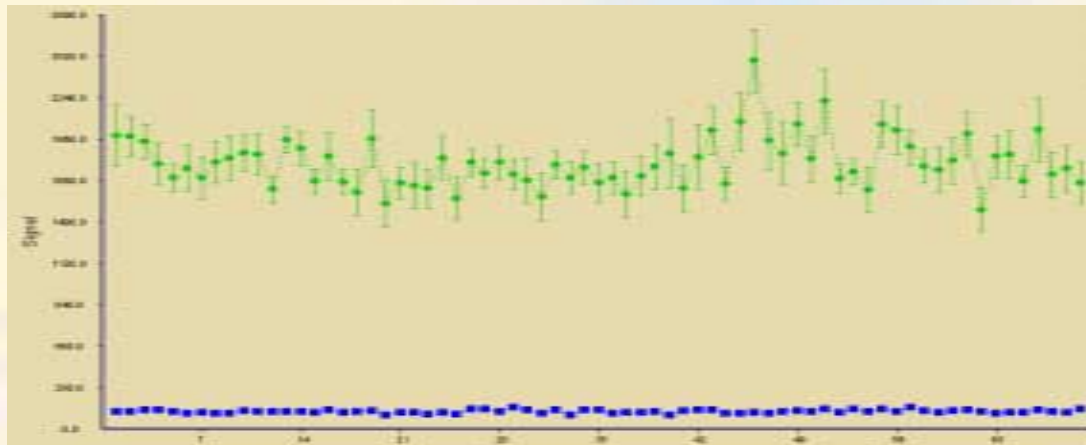
0% activity control
Avg. ECL = 1795.8 $\pm$ 224.1

100% activity control
Avg. ECL = 114.2 $\pm$ 15.8
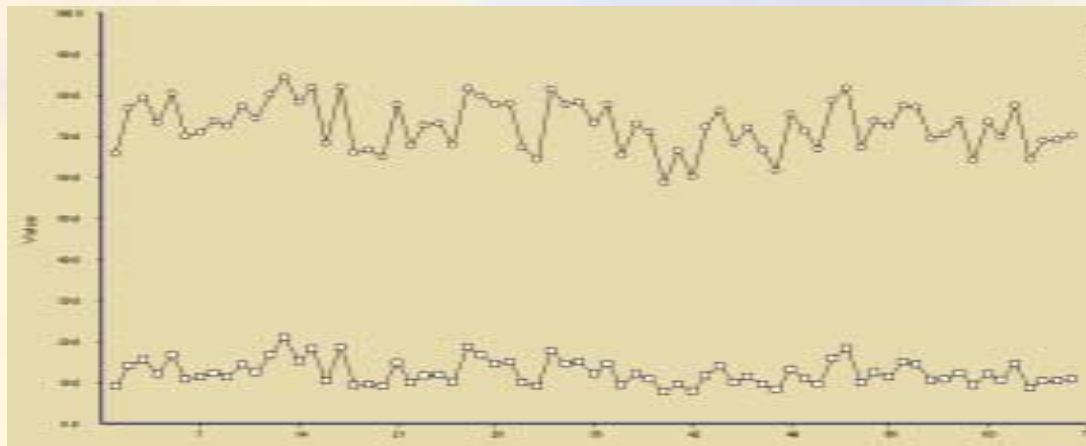
Z' factor (x 100)
Avg. Z' = 0.73

Assay window
Avg. = 12.4

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Screen Performance

Overall screen performance (summary of ~1000 plates)
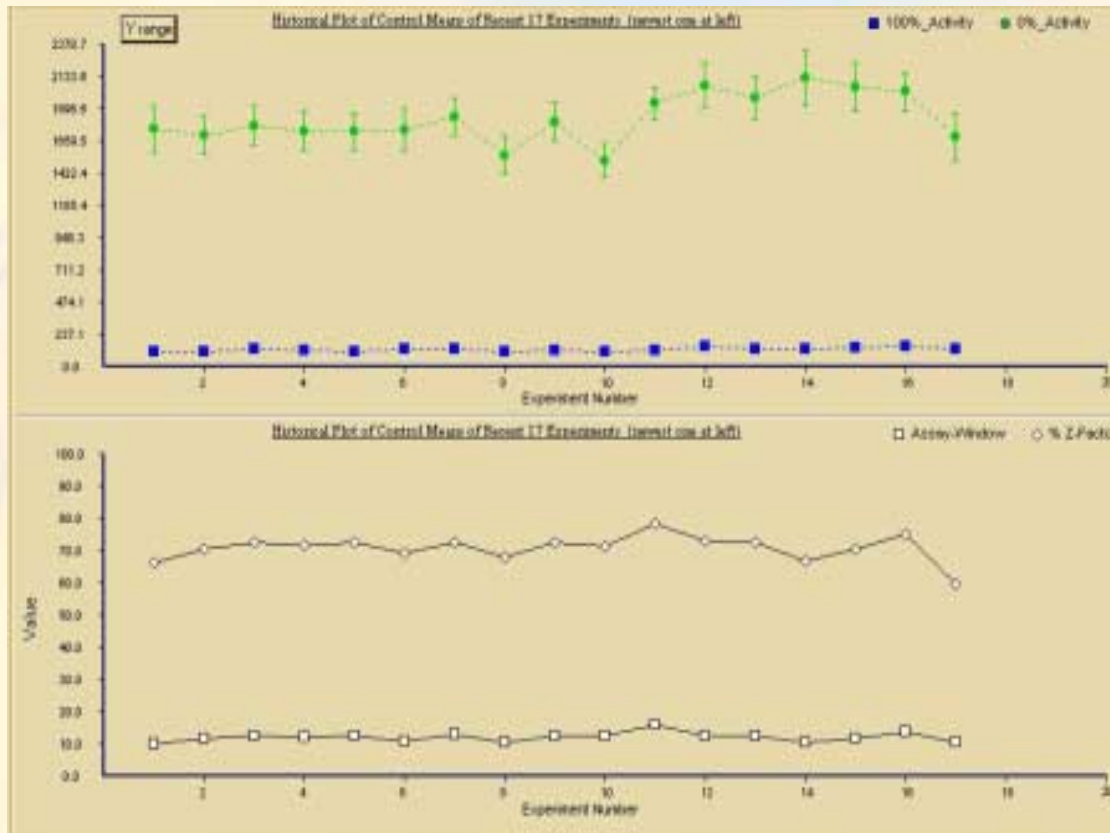
0% activity control
Avg. ECL = 1818.4 $\pm$ 152.6

100% activity control
Avg. ECL = 119.9 $\pm$ 12.6

Z' factor (x 100)
Avg. Z' = 0.70

Assay window
Avg. = 11.9

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HTS Results

- Screened a total of 977 plates, 308,613 compounds.

- Throughput was 100 plates (384-well) per day, one person, one Sector HTS reader.

- Confirmed Hits

Compound	Meso Scale % Activity	% Light Quench	SPA % Activity
A	51.21	< 1	< 40
B	48.35	10	53.68
C	52.12	18.1	< 40
D	73.51	27.4	< 40
E	56.37	8.6	< 40
F	68.65	25.7	66.34
G	44.38	13.7	< 40
H	42.13	20.3	< 40
I	48.22	9.5	< 40
J	55.88	13.5	< 40

- Data shown were averages of triplicates.
- All hits showed minimal interference with ECL intensity.

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Comparison of Binding Assay Methods

	Non Radioactive			Radioactive	
	DELFIA**	**FP**	**MSD**	**SPA, LeadSeeker**	**SPA, TopCount**
Labeling molecule	Europium	Bodipy-TMR	$Ru(bpy)_3^{2+}$	^{125}I	^{125}I
Effects of labeling*	lost activity	10 times less active	10 times less active	no effect	no effect
Assay Window	N/A	1.4	> 10	5	> 10
Z'	N/A	< 0	0.7	0.5	0.7
Protein (μg/well)	N/A	10	1	5	10
Throughput (reading time: min/plate)	N/A	2	2	5	40

* Effects of labeling were measured using FLIPR assay for functions (EC_{50}) and ^{125}I competition binding (IC_{50}).

** DELFIA: Dissociation-Enhanced Lanthamide FluoroImmuno Assay

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Summary

- **Best binding assay amenable to high throughput screening for this membrane bound receptor**

- **Assay sensitivity rivals that of radioligand binding**

    - Needed only 1 ug membrane protein compared to 5 ug for SPA

    - Could detect binding to < 0.5 fmole receptor

- **Good assay performance with assay window > 10 and Z' > 0.7.**

- **Throughput was 100 plates (384-well) per day, one person, one Sector HTS reader. Can be improved with automation.**