HTRF®
A versatile approach for 7TM drug discovery

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Outline

• Medical Research Council Technology, Centre for Therapeutics Discovery

1. Utilising HTRF® assays in an HTS environment

2. HTRF® Tag-lite® technology and secondary assay development

3. Investigational studies for receptor-protein interactions

• Conclusions and future perspectives
Applications of HTRF® to Assay Development/Screening/Profiling

- Establishing relevant HTRF assays at different points in an assay cascade

- Historically have successfully applied HTRF to kinase programmes

- Increased number of 7TM and other receptor targets in our portfolio

- Receptor-relevant HTRF assays specifically for 7TM/GPCR (tyrosine kinase)

- Highlights of HTRF applications
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- **Utilising HTRF® assays in an HTS environment**

- HTRF® Tag-lite® technology and secondary assay development

- Investigational studies for receptor-protein interactions

- Conclusions and future perspectives
**Homogenous Time Resolved FRET (HTRF®):**
A versatile HTS tool

- Flexibility
- Sensitivity
- Throughput
- Low interference
- TR-FRET (lanthanide chemistry)
- Signal stability
- Ratiometric data transformation (correction)

- Stable XC50 (hours-days)
- Low volume assay
- Miniaturisation/Automation-friendly
- Fresh or frozen cells
HTRF® cAMP Assay - dynamic 2 Kit (Eu³⁺ Cryptate):
Melanocortin Receptor 3 (MC3) HTS: Positive Allosteric Modulators (PAM)

MC3
Gs-coupled
γ-MSH
ACTH
α-MSH

• Macrophages
• CNS
• Gut
• Placenta
Cardiovascular Inflammation

• Signal inversely proportional to cAMP
• Wide cAMP concentration range
• Gs or Gi coupled receptors
• Agonist/antagonist screening
• Phosphodiesterase
• Adenylate cyclase
HTRF® cAMP dynamic 2: HTS Assay Performance

- GeneBLAzer® MC3R CRE-bla CHO-K1 Cells (Life Technologies)
- **Formatted as an MC3 potentiator/PAM HTS**
- γ–MSH native ligand (Lys-γ3-MSH)
- EC20 ± 10%
- Frozen cells
- Low volume 384
- Fully automated
- 32 plates/day
- >100K compounds
MC3 HTS Statistics

109760 compounds @ 10μM (1% DMSO)
Mean $Z' = 0.84 \pm 0.06$
Low Control %CV = 5.3 ± 2.7
High Control %CV = 3.6 ± 1.5

- Screened vs EC20 of native ligand ($\gamma$MSH)
- Agonists
- Positive Allosteric Modulators (PAMs)
- Antagonists
MC3 HTS Hit Deconvolution: Agonist vs PAM

- **Agonists**: Active in the absence of native ligand
- **PAMS**: Active only in the presence of native ligand

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**PAM Activity (% Lys-γ3-MSH Max)**

**Agonist Activity (% Lys-γ3-MSH Max)**

**Agonists**

- EC100
- EC0
- EC20
- Test

**Native agonist present**

**Native agonist absent**

**Absence**

**Presence**

- EC100
- EC0
- EC20
- Test
MC3 HTS Hit Profiling: Agonist vs PAM

Increase in [cAMP] in CHO cells stably expressing MC3 receptors

Agonists

PAMs

Agonist-induced increase in [cAMP] in CHO cells stably expressing MC3 receptors

PAM-induced increase in agonist-mediated [cAMP] in CHO cells stably expressing MC3 receptors
MC3 HTS *dynamic 2* Summary

- Target feasibility, assay development, automation/adaptation, HTS, hit follow-up and profiling – **single assay (cAMP dynamic 2)**

- Robustness, signal stability, low interference leading to a dual (tri) HTS format for simultaneous detection of both agonists and PAMs (antagonists)

- The capacity to control an EC20 value throughout a HTS campaign (**potent native peptide ligands**)

- Deconvolution and hit profiling clearly distinguishes specific PAMs and agonists by quantitative pharmacology

- Assay employed as a counterscreen using other non-MC3 cell lines

*MP63: Jerman et al. Identification and pharmacological characterisation of novel positive allosteric modulators (PAM) of Melanocortin 3 Receptors*
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SNAP-Tag Technology and Tag-lite®

SNAP-tag
- SNAP-tag (New England BioLabs)
- O\(^6\)-alkylguanine-DNA alkyltransferase
- Benzylguanine derivatives
- Irreversible covalent labelling of SNAP-tag

Tag-lite®
- Terbium cryptate-labelled SNAP-tag substrate
- Acts as donor in HTRF
- Receptor ligand labelled with d2 - acceptor
Formyl Peptide Receptor (FPR) Receptor Family

- Class A receptors
- $G_i/G_o$ and $G_q/G_{11}$ coupled
- Chemotaxis, superoxide production, pro/anti-inflammatory functions

**FPR1**
- Formyl Peptides
  - WKYMVm
- Annexin 1 Peptides
- Cyclosporins
- Neutrophils
- Immature DC
- Epithelial
- CNS
- Chemotaxis
- Inflammation

**FPR2/ALX**
- Lipoxin A4
- Formyl Peptides
  - WKYMVm
- SAA
- Annexin 1
- Neutrophils
- Monocytes
- Macrophages
- Epithelial
- Immature DC
- Chemotaxis
- Inflammation

**FPR3**
- F2L
- Humanin
- Annexin 1 Peptides
  - WKYMVm
- Monocytes
- Mature DC
- Chemotaxis
- Function (s)?
Why Tag-lite?
Why Not!

• Non-radioactive
• Homogenous
• High-throughput
• HTRF sensitivity
• Low compound and matrix interference
• Different assay endpoints in the same cell line
Generating Tag-lite® FPR Cell Lines: 
*Transient Expression*

**W-peptide (WKYMVm)**
*Synthetic peptide agonist*

- FPR1-3 SNAP-tag constructs (Cisbio)
- HEK293
- Transient transfection
- WKYMVm red HTRF acceptor
- Batch transfection and labelling (Lumi4-Tb)
- 90 minute incubation
Tag-lite® Saturation and Competition Binding: W-peptide (WKYMVm) Transient Expression

### Total Binding

**FPR1**
- Bmax: 87547
- Kd: 30 nM

**FPR2**
- Bmax: 82016
- Kd: 24 nM

**FPR3**
- Bmax: 17366
- Kd: 331 nM

### Specific Binding

**FPR1**
- EC50: 46 nM
- pEC50: 7.3

**FPR2**
- EC50: 20 nM
- pEC50: 7.7

**FPR3**
- EC50: 9600 nM
- pEC50: 5.0

### Competition

**IUPHAR MRCT**
- pKd: 7.5
- 8.7-10.13

### Total Specific Competition pKd

- -14
- -13
- -12
- -11
- -10
- -9
- -8
- -7
- -6
- -5
- -4

### Total Binding NSB Specific

- **FPR1**
- **FPR2**
- **FPR3**

**Freshly cultured or frozen cells**
**Batch transfection/labelling**
Cellul’erk (phospho-Erk) Assay:
*Transiently transfected FPR2 Cells*

- Measures intracellular phosphorylated Erk1/2
- 96-well to 384-well format
- Signal proportional to phosphorylation
- 384-well format being developed
- Success with AlphaScreen/LISA

**Diagram Description**

- GPCR activation by ligand induces ERK1/2 phosphorylation
- Lysis of the cell induces the release of phosphorylated ERK1/2
- Detection of phosphorylated ERK1/2 with HTRF conjugates

**Graph**

- **HTRF Ratio**
  - Green: Compound-43
  - Red: W-peptide

**Ligand [M]**

- **-12 -11 -10 -9 -8 -7 -6 -5 -4**

- **0**
  - 2000
  - 4000
  - 6000
  - 8000
  - 10000
### Generating Tag-lite® FPR Flp-In™ Cell Lines: Stable Expression

<table>
<thead>
<tr>
<th>Cloning</th>
<th>Transfection</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNAP vector (GPCR)</td>
<td>+</td>
<td>Transient transfection</td>
</tr>
</tbody>
</table>

- FPR1 and FPR2 SNAP-tag constructs (Cisbio)
- Sub-cloned in pcDNA5/FRT Flp-In vector (Life Technologies)
- Flp-In™ 293 and HEK293 (Cisbio constructs)
- Transient transfection
- Selection in hygromycin/neomycin
- Isogenic clones selected
- **Screened for receptor expression, binding and function**
Tag-lite® FPR2 Flp-In™ Cell Lines: Receptor Expression – SNAP Fluorescence

Flp-In™ 293 FPR2

Flp-In isogenic clones
Homogenous FPR2 expression

HEK 293 FPR2

HEK293 clones
Differential FPR2 expression

SNAP-Surface®488: non-cell-permeable fluorescent SNAP-tag substrate

(New England BioLabs)
Tag-lite® FPR Flp-In™ Cell Lines:
Receptor Expression – Anti-receptor antibodies

- Commercially available anti-FPR1 and FPR2 monoclonal antibodies
- Further confirmation of surface receptor expression
Tag-lite® FPR Flp-In™ Cell Line Profiling:
Flp-In™ vs HEK293 (high, medium, low MC3) Stable Expression

- Isogenic Flp-In clones – good specific binding
- HEK293 – good specific binding in high/med expression, but not low
- Cellul’Erk data correlates well with Tag-lite binding
HTRF-Based Profiling of FPR2 Tag-lite® Flp-In™ Cell Line

**Saturation Binding**

- **HTRF Ratio**
  - [Red WKYMVm] nM
  - Saturation Binding

**Competition Binding**

- **Log [M]**
  - B/Bo (%)
  - Competition Binding
  - Compound-43
  - W-peptide

**Cellul'Erk**

- **p-Erk (Fold Induction)**
  - W-peptide [nM]

**W-peptide (WKYMVm)**

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Specific</th>
<th>Competition</th>
<th>Erk Phosphorylation</th>
<th>IUPHAR</th>
<th>MRCT</th>
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<tr>
<td>Bmax</td>
<td>120363</td>
<td>116819</td>
<td>6.9</td>
<td>2.04</td>
<td>8.69</td>
<td>8.7-10.13</td>
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<tr>
<td>Kd (nM)</td>
<td>13.27</td>
<td>13.25</td>
<td>8.16</td>
<td>8.7-10.13</td>
<td>7.8</td>
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</tbody>
</table>

- Multiple read-outs from a stable Tag-lite cell line
- Ligand affinity under-estimated (?)
- Amplification of binding affinity with phospho-Erk response
FPR Receptor Summary

- Tag-lite binding assays successfully formatted for FPR receptors
- Reproducible pharmacology in transient and stable format
- Comparable pharmacology to published values*
- Receptor $G_i$ coupling measured with Cellul’erk assay and correlates with binding
- Stable cell lines conveniently generated in Flp-In™ cell background
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Melanocortin Receptor 2-MRAP Interaction

MC2: ACTH receptor responsible for steroidogenesis
MRAP: melanocortin receptor accessory protein

2. Prof Adrian Clark, St Georges Hospital, London
Mechanisms of MRAP-MC2 action

As dimer creating functional receptor complex

- MRAP/MC2R interaction
  - in ER
  - at plasma membrane
- ACTH interaction with MC2R
- ACTH interaction with MRAP

ACTH

Escorting folded Receptor

Endoplasmic Reticulum

Protein folding and/or Post-translational modification

SIGNAL

Plasma Membrane

Courtesy of Prof Adrian Clark, St Georges Hospital, London
MC2-MRAP Proximity Assays: HTRF® Anti-tag Reagents

Anti-HA/FLAG-conjugated Tb/d2 Antibodies

- MC2R-Rluc, c-terminal (pRLuc-N1; PerkinElmer)
- MRAP-EYFP, n-terminal (pEYFP-C1; Clontech)
- HEK-293 cells
- Lipofectamine 2000
- Coelenterazine h
- Fluostar Optima (BMG Labtech)
- Published protocol

- MC2R-HA, n-terminal (cDNA.org)
- MRAP-FLAG, c-terminal (AC/LC)
- HEK-293 cells
- Lipofectamine 2000
- Anti-FLAG/Anti-HA labelled antibodies
- Pherastar Plus (BMG Labtech)
- Pilot evaluation
MC2-MRAP Proximity Assays: HTRF® Anti-tag Reagents
Anti-HA/FLAG-conjugated Tb/d2 Antibodies

- HEK293 transient transfection
- MC2-HA or MRAP-FLAG
- Suspension vs adherent
- Anti-tag reagents:

**Combination 1**
- anti-HA-Lumi4-Tb (MC2 donor)
- anti-FLAG-d2 (MRAP acceptor)

**Combination 2**
- anti-FLAG-Lumi4-Tb (MRAP donor)
- anti-HA-d2 (MC2 acceptor)

- Expression (WB), function (cAMP)
- HTRF-based proximity assay

**Delta F Calculation: Specific HTRF Signal**

\[
\text{HTRF Ratio} = (665\text{nm}/620\text{nm}) \times 10000
\]

\[
\Delta F = \frac{\text{Ratio Positive (ACTH)} - \text{Ratio Negative (unstimulated)}}{\text{Ratio Negative (unstimulated)}} \times 100 \%
\]
MC2-MRAP Proximity Assays: HTRF® Anti-tag Reagents: Anti-HA/FLAG-Tb/d2 Antibodies – Suspension Format 4°C

**MC2:MRAP cAMP**

**ACTH (1-24)**

**ACTH (1-39)**

**Delta F (%)**

**Acceptor:Donor Ratio (nM)**

**MC2-MRAP cAMP**

**Delta F %**

**Time (h)**

**Delta F (%)**

**ACTH 1-24 [M]**

**Combination 1**

**Combination 2**

**nM acceptor (donor = 1nM)**

- 3.25
- 7.5
- 14
- 27

**DNA (ng)**

50 100 500 1000

50 100 500 1000

**anti-HA (MC2)**

**anti-FLAG (MRAP)**

**MRAP**

**MC2**

**Delta F % (unstimulated vs ACTH)**

**nM acceptor (donor = 1nM)**

**3:1**

**6:1**

**12:1**

**HTRF® Anti-tag Reagents**

**Anti-HA/FLAG-Tb/d2 Antibodies**

**Suspension Format**

4°C
MC2-MRAP Protein Interaction Summary

• BRET assay unsuccessful
  • small signal, large signal contamination – donor/acceptor (RLuc/EYFP)

• HTRF anti-tag reagents – small, reproducible signal (ΔF ~ 10-15%)
• Ligand (ACTH) concentration-dependent effects
• Further optimisations:
  ✓ Incubation temperature (4°C/22°C)
  ✓ Suspension vs adherent
  ✓ Buffers/matrix
  ✓ Receptor internalisation (sodium azide)
  ✓ Antibody concentrations and ratios
  ✓ Cell number

• Challenging target!
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Conclusions and Future Perspectives

- HTRF is a powerful and versatile tool for interrogating functional 7TM biology
- Robust and reproducible data
- HTRF is applicable to all stages of a project cascade
- Advantages of commonality of assay formats for automation and screening
- Tag-lite a convenient and sensitive format for receptor binding
- Tag-lite cell lines: flexibility, assay multiplexing
- Open assay platform for bespoke assay development
HTRF® 7TM/GPCRs and . . . ?

- HTRF technology is not limited to 7TM biology!
- Kinease™ HTRF assays
- HTRF Transcreener® ADP
- Cortisol (steroid hormone)
- Cytokines (TNFα, IL-1β)
- IP1 (IP-One)

Extracellular targets
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Debbie Taylor

MP63: Jerman et al. Identification and pharmacological characterisation of novel positive allosteric modulators (PAM) of Melanocortin 3 Receptors