TSRI-Drug Discovery Graduate Course - Spicer Lecture
Scripps Research Institute Molecular Screening Center

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http://hts.florida.scripps.edu/
Goals for this Lecture

Provide high-level understanding of:
• The concept of HTS and its role in drug discovery
• New technologies in HTS
• Role of automation and technology in HTS
• HTS assay considerations
  • What makes a good HTS assay?
  • Z’ as a measure for HTS assay robustness
• Examples of HTS Campaigns
  • Gonadotropin Releasing Hormone Receptor NRH (GNRH)
  • Malaria-Gametocyte
• New Areas of Exploration
  • 3D HTS
• Success at Scripps!
Understanding the genetic basis of disease profoundly impacts target discovery and clinical trial design.

Yet drug discovery is primarily linear from target validation to candidate selection.

A Paradigm Shift is Required
Where do Lead Molecules Come From?

Special Delivery!
Approaches to Finding New Leads

• Fast-follow on
  – Identify patent gaps around already published structures
  – Make molecules to exploit these gaps

• Structure-based design
  – Requires knowledge of the crystal structure of the protein
  – Design molecules to fit into 3-dimensional pockets on the binding sites or active sites of the target of interest

• Random search (HTS)
  – Test as many molecules as possible to see which ones work
  – Serendipity
The Concept of HTS

- Test as many compounds as possible
  - Typically between 1 and 2 million in large Pharma and 500K to 1 million in biotech for traditional HTS
  - Pooled sets of billions of compounds using DNA-encoded libraries
- Leverage automation and cutting-edge technology
  - Enables millions of tests
  - Enables highly miniaturized assays, beyond what the human could do.
  - Significantly increased throughput
  - Massively reduced cost
- Highly skilled assay designers to develop assays
  - Assays to discover drugs may be very different to traditional biochemical or cellular assays
Traditional HTS Process Flow

100K to >2M
10,000’s
1,000’s
100’s
10’s
3-8
Compounds

Primary
Retest
*CRC
Confirmation

One Replicate
10µM Compound
One Assay

Triplicate
10µM Cpd
1-4 Assays

Triplicate
10pt CRC
1-4 Assays

Purification
Re-synthesis
Orthoganol
Assays

Expansion
Similarity
Purchases

Lead Chemotypes

*CRC=concentration response curve
Well Number

High Control

Compounds

Low Control

Most of the compounds

Single point % Inhibition

Well Number
Hit Selection Criteria

Traditional histogram
- Not very useful
- Too many data points
- Hides the interesting stuff

Scatter Plot
- Much more useful
- Can see all data points
- Highlights the interesting stuff
Hits Highlighted using Ave + 3Stdev Rule

- Negative bias
  - Fluorescent Cpds
  - Increases SD
Introduction to uHTS-Highly Miniaturized Formats

- 1980: 96 wells (50-200ul)
- Mid 1990s: 384 wells (20-50ul)
- Present: 1536 wells (2-10ul)
SRIMSC began in 2005

- Biologists, Biochemists, Programmers, Chemists & Engineers (currently >600 employees)
- Industrial scale HTS lab with Kalypsys/GNF automated platform
  - >646K Proprietary (largest in academia, ~30k unique compounds, focused sub-libraries, professionally curated)
  - >360K Public Domain (NIH)
- Funding is driven by NIH grants and Collaborations with Pharma and Biotech

Where robotics, chemistry and biology join forces to help discover new drugs
Lead ID Screening Workflow

1. **ASSAY DEVELOPMENT**
   - Receive Assay
   - Transfer Protocol

2. **ASSAY DEVELOPMENT**
   - Migrate Data
   - Release to Cheminformatics

3. **Validation Data QC**
   - Cherrypick Hitlist
   - Reformat for uHTS

4. **Primary Data QC**
   - Migrate Data
   - Create Hitlist

5. **Confirmation Data QC**
   - Robotic Setup Calibration

6. **DATABASE**
   - Reagents
   - Supplies
   - Plates

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HTS can be executed against a public domain compound library (MLPCN), the in-house Scripps Drug Discovery Library (SDDL) or against a partner-provided library.

**Assay Development**
- “From test tube to plate”
- Assay Design
- Cell-based assay development
- Biochemical assay development
- Reagent Generation

**Drug Screening**
- Comprehensive HTS/HIT Platforms
- Variety of formats supported
- 1536-well format HTS/HCS
- Million samples/24 hrs
- Pharma-grade IT infrastructure

**Compound Management**
>1MM molecule screening libraries:
- ~640K Scripps’ drug discovery library
- ~370K NIH’s MLPCN library
- LC-MS QC & Sample Auditing
- Cheminformatics Expertise

**Analysis of results; Selectivity Studies; Compound QC (LC-MS), Cheminformatics, Profiling**

**Configure / miniaturize assay(s) in 1536-well microtiter plates**

**Perform HTS/HCS**

**Screening at Lead ID: Generalized Workflow**
We provide cheminformatics, clustering, promiscuity & chemical liability analysis to support decision making all along the project.

Hit optimization (SAR & MedChem)
OR
selectivity/profiling
cytotoxicity
optional

Library selection and size can be modified to meet the needs of the program:
• Pilot screening (LOPAC 1280, 10K initiatives, FDA Approved 3200)
• Most go for all-economy of scale
• HCA is an exception (HTS-HCS is a balance)
1.7m Arm Incubators Transfer Dispense/Wash PE Suite FLIPR

- Built specifically for 1536-well plate screening (384-well plate screening also possible)
- Capable of over one million wells screened in a 24-hr period (1536-well format)
- Patented lid prevents evaporation of plate contents
- Long (>96 hr) plate incubation protocols possible
- Plate capacity >1,500 (>2.3 million wells in 1536-well plate format)
- Plate incubation from 4°C to 50°C, 0-100% rH, any gas (CO₂, N₂, Ar, etc.)
- 1536-well plate washers and transfer pipettor enable heterogeneous assays/fixing steps
- Luminescence, BRET, Absorbance, Fluorescence Intensity, FP, TRF, FRET, TR-FRET, AlphaScreen, AlphaLISA, FLIPR, High-Content...
# Lead ID Assay Enablement: HTS Plate Readers

<table>
<thead>
<tr>
<th>Instrument</th>
<th>Common Detection Formats</th>
<th>Examples of HTS Assays Developed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luminescence, BRET, Absorbance, Fluorescence Intensity, FP, TRF, FRET, TR-FRET, AlphaScreen, AlphaLISA</td>
<td>Enzymatic, Reporter Gene, Complementation, DNA binding, Protein conformation, Protein-Protein, Protein Renaturation, Protein-Activity Probe, Protein-Ab, Protein-RNA, Protein-RNA, Receptor Binding, RNA-RNA, Viability</td>
<td></td>
</tr>
<tr>
<td>Kinetic assays Fluorescence Intensity, FRET</td>
<td>Second messenger (Ca$^{2+}$, cAMP, IP3) Membrane Potential (ion channels); multiple pharmacologies/well</td>
<td></td>
</tr>
<tr>
<td>High Content Analysis</td>
<td>Phenotype in 1536-well format</td>
<td></td>
</tr>
<tr>
<td>Monochromator-based Absorbance, Fluorescence Intensity</td>
<td>Excitation &amp; Emission Scan Assays; assays with non-standard fluorophores or chromophores</td>
<td></td>
</tr>
</tbody>
</table>
Cellomics Cell-Insight

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Optics</strong></td>
<td>5x, 10x, 20x</td>
</tr>
<tr>
<td><strong>Light source</strong></td>
<td>LED</td>
</tr>
<tr>
<td><strong>Wavelengths</strong></td>
<td>386, 485, 549 &amp; 650nm</td>
</tr>
<tr>
<td><strong>Imaging modes</strong></td>
<td>Fluorescence, Kinetic</td>
</tr>
<tr>
<td><strong>Compatibility</strong></td>
<td>96, 384, &amp; 1,536</td>
</tr>
</tbody>
</table>

SRIMSC provides HCA- Assay Development
SRIMSC provides HCS implementation and Mechanism-Of-Action [MOA] studies
Key challenge of miniaturizing

- Assay volumes of 2-5ul in 1536-well plates
- 2-5ul is the TOTAL so
  - 1ul enzyme, 1ul substrate, 1ul stop / detection is typical
  - Compound dispense not possible by traditional methods
- Compounds dissolved in DMSO
  - Typical assay tolerance is 1% (v/v)
  - Therefore a 3ul assay needs 30nl of compound
- Solution was acoustic transfer
  - Can transfer 2.5nl-250nl without ever touching the liquid
Acoustic Transfer of Compounds

Assay Plate

Compound Plate
5nl in a 1536 well plate (3.5% CV)
Statistical Parameters: Signal/Background

\[ \text{Signal/Background} = \frac{\text{Mean high signal}}{\text{Mean low signal}} \]

S/B=17.8

Great Assay?
Statistical Parameters: Signal/Background

- But what if data identical but reader baseline is higher by 10000?
- Sometimes a different plate type will give a higher background

\[ S/B = 3.5 \]

- Numbers say BAD Assay!
- BUT I could still find a hit
- Just as good as before
Z’ is an Excellent Measure of Assay Performance

\[
Z' = 1 - \frac{(3 \times \text{Standard deviation of high}) + (3 \times \text{Standard deviation of Low})}{(\text{Mean high} - \text{Mean Low})}
\]

Acceptable Z’ range = 0.5 to 1.0

S/B=17.8

\[Z' = 0.86\]

S/B=3.5

\[Z' = 0.86\]
It is the measurement of the separation of the distribution of high and low signals that is important.

S/B = 3.5

S/B Does not describe the separation of the high and low signals
Statistical Parameters: $Z'$

$$Z' = 1 - \frac{(3 \times \text{Standard deviation of high}) + (3 \times \text{Standard deviation of Low})}{(\text{Mean high} - \text{Mean Low})}$$

**Acceptable $Z'$ range = 0.5 to 1.0**

**PTP1B**

S/B = 3.5

Z' = 0.86

**Validation**

S/B = 3.5

Z' = -0.2
Quality Control in HTS

- Blank QC Plate
- Totals/Blanks
- Control Compounds
Notes on False Positives and False Negatives

- **False Positive**: A compound called as hit but it isn’t really
  - Individual false (+)’s not such a big deal (most of the time)
  - Will be discovered & weeded out eventually
  - Very high overall false (+) rates can drown out real hits & overwhelm capacity for follow-up

- **False Negative**: A real hit that was missed
  - Every single false (−) is potentially much more costly
  - Might have missed the best lead chemotype
  - Will never know what you missed
A hit is a hit is a hit….. Or is it?

1. **Misdispensed**: e.g. Lower substrate or enzyme added
   - Will easily be caught at confirmations (triplicate repeats of compounds that look active)

2. **Assay signal interference**: e.g. compound is fluorescent at the wavelengths used by the fluorescent assay probe
   - Must design a counter screen to filter out these compounds.
   - Run at retest

3. **Mechanistic Interference**: e.g. Enzyme is sensitive to copper ions and copper was used as a catalyst when the compound was made
   - Understand your assay's sensitivity to metal ions
   - Re-purify, remake and retest hits
   - Use chelating agents?
Strategies to Address False Negatives

• Bias protocols to generating false positives
  – Where possible a misdispense should create a hit, rather than miss one

• Pay particular attention to compound addition
  – If the compound is not in the well, it will never be a hit
  – Solubility & dilution ratios are critical

• Mine data for missed compounds due to statistical variation
“Hit Assessment” and Triage

- **Re-tests** — Does the compound hit a 2nd time?
- **Concentration-response curves** — What is the potency of the hit?
- **Null assays** — Does the hit produce the same effect in cells not expressing the receptor/target of interest? (aka “specificity” assay)
- **Selectivity assays** — Is the compound active at similar or related receptors/targets?
- **Confirmation of chemical material** — Did you test what you think you did?
- **Orthogonal confirmation assays** — Confirm compound activity at target of interest
- **Similarity Searching** — Test as many close chemical analogs of hits as possible – including compounds already screened!
Transitioning to Challenging Targets

HTS: The smaller faster cheaper era-till ~2010

- Majority of assays miniaturized to 1536-well
- Cost reduced to ~3-10c/well for consumables
- Fully integrated process, through compound management, HTS and lower throughput secondary assays
- Flexible infrastructure, supporting all major assay technologies
- Significant impact (Med Chem) on 70% of HTS campaigns

Now the challenge has shifted to harder targets:

- Increased complexity
- Increased flexibility
- Target ID becomes a significant challenge now that the low hanging fruit has been picked
Examples of HTS Campaigns

1. Gonadotropin Releasing Hormone Receptor
   a) Genital hypogonadism

2. Gametocyte
   a) Anti Malaria

3. VIM-2 Beta-lactamase
   a) Antibiotic resistance
   b) Drug-Drug synergy
A Phenotypic High Throughput Screening Assay for the Identification of Pharmacoperones for the Gonadotropin Releasing Hormone Receptor

- Mutations in GNRHR are the cause of HH; hypogonadotrophic hypogonadism
This assay uses the Tet-off doxycycline (DOX”-“) responsive promoter to drive expression of the GNRHR.
**A. Add Cells**

- **Add Fluo-8 Dye (no wash)**

**B. Incubate Overnight**
- at 37°C, 5% CO2

**B. Incubate 1 hour**
- at 37°C, 5% CO2
- 30 minutes at room temperature

Start Ag mode

**C. Basal read**
- Pin compounds and controls
- Kinetic read
  - @490ex/530em

Start PAM mode

**C. Basal read**
- Pin EC20 Stimulus and controls
- Kinetic read
  - @490ex/530em

Start ANT mode

**C. Basal read**
- Pin EC80 Stimulus and controls
- Kinetic read
  - @490ex/530em

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HeLa WT vs. E90K GNRHR: Fluo2 Assay Validation N=2

- Add 20ul cell suspension @ 8,500 cells/well in growth medium, spin.
- Add 5ul DMSO or GNRH (500nM Final) in assay buffer (HBSS +20mM HEPES +3% DMSO).
- Kinetic Read for 140 seconds @490ex/530em.
- Baseline Read 5 seconds.
- Add 5ul DMSO or GNRH (500nM Final) in assay buffer (HBSS +20mM HEPES +3% DMSO).
- Kinetic Read for 140 seconds @490ex/530em.
- Add 20ul Fluo-2 dye, spin.
- Incubate 1 hour at 37°C 5% CO2.
- Incubate 30 minutes at Room Temperature.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Scripps EC50/IC50 (ng/ml)</th>
<th>Scripps S:B</th>
<th>Scripps Z'</th>
<th>Assay Provider EC50 (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.47±0.31</td>
<td>2.58±0.18</td>
<td>0.84±0.03</td>
<td>4 to 4.9</td>
</tr>
<tr>
<td>E90K</td>
<td>2.22±1.57</td>
<td>2.17±0.01</td>
<td>0.71±0.08</td>
<td></td>
</tr>
</tbody>
</table>

- IN3 did restore E90K activity with 500nM GNRH.
- IN3 inhibited the GNRH response in the WT cells.
- When IN3 is present in WT cells, it acts as an antagonist.
384 well Compared to 1536

Summary:
- Assay is validated at 1536 well format
- Pharmacology matches expected

Next:
- Pilot screen

IN3 Dose Responses

<table>
<thead>
<tr>
<th>Log IN3 (M)</th>
<th>384 well format</th>
<th>1536 well format</th>
</tr>
</thead>
<tbody>
<tr>
<td>HillSlope</td>
<td>0.4722</td>
<td>1.130</td>
</tr>
<tr>
<td>EC50</td>
<td>3.997e-009</td>
<td>3.526e-009</td>
</tr>
</tbody>
</table>

Z' = 0.77 at 600nM EC100
S:B = 2.47 at 600nM EC100
GNRHR 1536 Well Pharmacoperone Assay
PRIMARY HTS

Summary:
- CRC matches expected over the whole primary HTS
- Hit cut-off is set at 11.40% yielding 4,820 active compounds

Next:
- Show a sample Data File

Control CRCs N=20 plates

Run Stats (N= 519 Plates)
- 646,275 compounds tested nominally at 5.2uM
- Z'=0.79±0.06
- S:B=2.46±0.26
- 11.40% Hit Cutoff=Interval Cutoff
- 4,820 Hits (0.75% hit rate)
Summary:
- CRC matches expected
- Hit cut-off is set at 11.40% yielding 1,668 active compounds

Next:
- IPONE Confirmation Screen

Run Stats (N= 15 Plates)
- 4,809 compounds tested 3X nominally at 5.2μM
- Z’=0.76±0.03
- S:B=2.42±0.04
- 11.40% Hit Cutoff=Primary Cutoff
- 1,668 Hits (34.7% hit rate)
GNRHR 1536 Well Pharmacoperone Assay
IP-ONE Secondary Screen

1536 wpf
Corning SWST

Add 3ul cell suspension @ 1,500cells/well in growth medium spin

Pin 30nL compound, control, or DMSO
Incubate overnight at 37C 5% CO2

Add 3ul of 1000nM GNRH in 2x Stim buffer or stim buffer only
Incubate 2 hour 37C 5% CO2

Add 1.5ul IP-One D2 (1:20 Dilution)
Spin
Add 1.5ul IP-One Cryptate (1:20 Dilution)
Spin

Incubate RT for 1 hour in dark

Viewlux

Read on Viewlux @ 618nm/ 671nm

V_assay=6ul
GNRHR 1536 Well Pharmacoperone Assay
IPONE-Confirmation Screen

Summary:
- CRC matches expected
- Hit cut-off is set at 21.87% yielding 812 active compounds

Next:
- +DOX counterscreen

Control CRCs

Run Stats (N= 15 Plates)
- 4,809 compounds tested 3X nominally at 4.3uM
- Z'=0.78±0.02
- S:B=1.72±0.01
- 21.87% Hit Cutoff=DMSO sample field + 3SD (5 plates)
- 812 Hits (16.9% hit rate)
**GNRHR 1536 Well Pharmacoperone Assay**

**1536 well Counterscreen Protocol**

**FLIPR Tetra**

- Add 3μl cell suspension @ 1,500 cells/well in growth medium (+/- dox), spin
- Baseline Read 5 seconds
- Add 50nL DMSO or GNRH (500nM Final) (75/25 DMSO)
- Kinetic Read for 140 seconds @490ex/530em

**1536 w pf Greiner SBCTL**

- Pin 30nL compound, control, or DMSO
- Incubate overnight at 37°C 5% CO2
- Add 2μl of Fluo-2 dye, spin
- Incubate 1 hour 37°C 5% CO2
- Incubate 10 minutes at Room Temperature

**+ Dox cells (Counterscreen):**

2μg/ml Dox for extended passaging to block receptor production

**V\text{assay} = 5μl**

[DMSO] = 0.75%

6μM Screening Concentration
**Summary:**

- Control CRC shows SR-435409 is slightly more potent than during the pilot phase, causing a bigger response in the + DOX cells.
- IN3 is less potent (stock is old) but, still no response at ECMAX which validates the cells/assay.
- Hit cut-off is set utilizing the average plus 3 stdev of the SR-435409 EC100 "+" DOX control ("pink dots") at 41.6% yielding 32 active compounds.
  - more compounds move forward than if we use the low control.

**Next:**

- Venn of overlapping hits of all 3 assays

---

**Run Stats (N=15 Plates):**

- 4,809 compounds tested 3X nominally at 5.2uM
- Z'=0.81±0.02
- S:B=2.83±0.05
- 41.6% Hit Cutoff= average of EC100 + DOX + 3SDs
- 32 hits (0.67% hit rate)

---

**Control CRCs:**

<table>
<thead>
<tr>
<th>Compound</th>
<th>EC50</th>
<th>HillSlope</th>
</tr>
</thead>
<tbody>
<tr>
<td>SR-01000435409-1 no DOX</td>
<td>2.118</td>
<td>2.170</td>
</tr>
<tr>
<td>SR-01000544741-1 no DOX</td>
<td>2.170</td>
<td>2.170</td>
</tr>
<tr>
<td>SR-01000435409-1 + DOX</td>
<td>2.118</td>
<td>2.170</td>
</tr>
<tr>
<td>SR-01000544741-1 + DOX</td>
<td>2.170</td>
<td>2.170</td>
</tr>
<tr>
<td>SR-01000435409-1 no DOX</td>
<td>2.118</td>
<td>2.170</td>
</tr>
<tr>
<td>SR-01000544741-1 no DOX</td>
<td>2.170</td>
<td>2.170</td>
</tr>
<tr>
<td>SR-01000435409-1 + DOX</td>
<td>2.118</td>
<td>2.170</td>
</tr>
<tr>
<td>SR-01000544741-1 + DOX</td>
<td>2.170</td>
<td>2.170</td>
</tr>
</tbody>
</table>

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**HillSlope**

<table>
<thead>
<tr>
<th>Ratio (Max/Min)</th>
<th>log [Compound] (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>-8</td>
</tr>
<tr>
<td>1.5</td>
<td>-7</td>
</tr>
<tr>
<td>2.0</td>
<td>-6</td>
</tr>
<tr>
<td>2.5</td>
<td>-5</td>
</tr>
<tr>
<td>3.0</td>
<td>-4</td>
</tr>
<tr>
<td>3.5</td>
<td>-3</td>
</tr>
<tr>
<td>4.0</td>
<td>-2</td>
</tr>
</tbody>
</table>

**IN3 NO DOX**

- Inhibitory potency

**IN3 + DOX**

- Inhibitory potency

721 compounds are active in both assay formats and inactive in + DOX counterscreen. 662 of these compounds are PAINS free.
Summary:
- Cells for the + DOX counterscreen were derived from a Conn stock vial sent recently—Scripps now has multiple freeze-backs incremented about 1 passage
- CRC matches expected, same compounds in the hit selection as controls respond similarly
- DOX blocks response almost completely
- 399 compounds are active in both Fluo2 and IPONE and inactive in + DOX at EC50<5uM

Next:
- Venn Overlap of active compounds
399 compounds are active in both assay formats and inactive in + DOX counterscreen

Next:
Sample Data File
## Example of the file format

Note all compounds were PAINS free and had no metadata associated.
GNRHR HTS Campaign

**PRIMARY HTS**
- 646,275 compounds tested | 4,820 hits
- Hit-cutoff = 11.40% Activity
- ~5.2uM screening concentration

<table>
<thead>
<tr>
<th>Compounds tested</th>
<th>Hits</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>646,275</td>
<td>4,820</td>
<td>11.40%</td>
</tr>
</tbody>
</table>

Selected 4,820 hits for testing in secondary assays

**SECONDARY HTS**
- 11 compounds unavailable

<table>
<thead>
<tr>
<th>Screen Type</th>
<th>Compounds tested</th>
<th>Hits</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>GNRHR no DOX Confirmation screen</td>
<td>4,809</td>
<td>1,668</td>
<td>34.7%</td>
</tr>
<tr>
<td>GNRHR IPONE Screen</td>
<td>4,809</td>
<td>812</td>
<td>16.9%</td>
</tr>
<tr>
<td>GNRHR + DOX Counterscreen</td>
<td>4,809</td>
<td>32</td>
<td>0.67%</td>
</tr>
</tbody>
</table>

**TITRATION ASSAYS**
- 662 compounds available

<table>
<thead>
<tr>
<th>Screen Type</th>
<th>Compounds tested</th>
<th>Hits</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>GNRHR no DOX Confirmation screen</td>
<td>662</td>
<td>420</td>
<td>63%</td>
</tr>
<tr>
<td>GNRHR IPONE Screen</td>
<td>662</td>
<td>466</td>
<td>70%</td>
</tr>
<tr>
<td>GNRHR + DOX Counterscreen</td>
<td>662</td>
<td>0</td>
<td>0%</td>
</tr>
</tbody>
</table>

**399 Compounds Selective For GNRHR**
Live/dead Assays for the Purpose of Discovering Molecular Probes that Inhibit Plasmodium falciparum Gametocytes

- A high-throughput assay for the identification of drugs against late-stage Plasmodium falciparum gametocytes.
Plasmodium falciparum—the most virulent of the four malaria parasites infect humans destroying red blood cells in the liver. It digests a cell's hemoglobin, multiplies inside to the point of rupturing the cell, and rapidly spreads a new generation of infection.

- Infects about 300 - 500 million cases per year with about 2 million of deaths.
- 36% of global population live in areas of risk from malaria.
- Symptoms include recurrent cycles (every one to three days) of fever, chills, muscle aches, headaches; nausea, vomiting, and jaundice also may occur.
- The incubation period for malaria symptoms is about one to three weeks but may be extended to eight to 10 months after the initial infected mosquito bites occur.

- This was a collaborative MLPCN driven effort between Scripps Florida, Queensland Institute of Medical Research (Australia) and University of Kansas Specialized Chemistry Center.
- CPDP objective per Grant # 1R21NS075609-01
  “Develop A High-throughput screen to identify new agents which are active against Plasmodium falciparum late stage gametocytes.”
Most commonly affects elderly and children:

**Current Treatments:**
- Currently there are only a handful of drug choices for the treatment of malaria (asexual blood stage):
  - chloroquine, atovquone, artemether, quinine, mefloquine, quinidine, tetracycline, doxycycline and clindamycin as designated by the World Health Organization.
  - Optimally one would design a drug that defeats the malaria parasite resistance and renders it incapable of further transmission.

**GAMETOCYTES are required for TRANSMISSION:**
- Primaquine is directed at gametocytes and is used clinically
  - Contraindication for G6PD patients-elevated RBC lysis
  - Drug resistance is huge problem
  - Anti-gametocidal agents wont reduce the parasite burden but will help prevent transmission
    - Other approaches are mozzie-nets

**Sexual stage cycle in mosquito midgut**

![Female anopheles mosquito image]
Probe Development Strategy

Primary HTS
- Primary Assay (Single Concentration in singlicate).
- Cheminformatic Analysis (>% Hit cutoff)
- *In silico* comparison to remove non-specific hits.
- Proceed with the active hits from the primary assay. (<2,500)

Secondary Assays
- Confirmation and Counterscreen Assays (Single Concentration in triplicate.)
- Cheminformatic Analysis ( > % Primary Hitcutoff).
- *In silico* comparison to remove non-specific hits.
- Proceed with the active hits from the confirmation assay. (<128)

Tertiary Assays
- Dose Response and Counterscreen Assays (10-point dilution series in triplicate)
- Cheminformatic Analysis ( IC50 < 10uM vs.GAMs; >10uM in Counterscreen).
- *In silico* comparison to remove non-specific hits.
- Proceed with the potent compounds against the primary assay.

MOA
- Lead Optimization: SAR, medicinal chemistry and low-throughput assays.
- FACS-based Gametocyte Growth Inhibition Counterscreen.
- Asexual Blood Stage Activity Counterscreen.
- Membrane Feeding Mosquito Counterscreen
- Exflagellation Inhibition Counterscreen
- *In vivo* RagC6 Mouse Antigametocidal Counterscreen
- Probes should inhibit the above assays with an IC50 <10uM

*Compounds need to meet the probe criteria to consider them as “possible leads”.*
Optimum number of gametocytes for the BacTiter-Glo Assay

1. Dispense 4uL of gametocytes 250 cells/well
2. Add 30nL test compound
3. Incubate plate with compounds for 24hrs, 5% CO2 at 37°C
4. Add 4uL Bac-Titer Glo substrate
5. Incubate for 10 minutes (RT)
6. Read Lumi

We monitor the Relative Luminescence Units =RLU
The y-axis shows the ATP production in relative luminescent units (RLU).
The x-axis shows the number of gametocytes used in thousands.

Simple, homogenous and sensitive assay

Example of DMSO plate

Average Z’=0.66±0.02; S:B=5.23±0.51; N=3

See: Mol Biochem Parasitol. 2011 Dec;180(2):127-31
The number of viable gametocytes available limited the number of plates for the primary HTS.

The primary HTS went well for a difficult target.

The controls used were no gametocytes (100% Inh.) vs. gametocytes only (0% Inh.).

CRC analysis of the non specific control (Methylene Blue) demonstrates the expected results.

A low number of hits was identified (100).

**Top 275 hits requested and to proceed directly to dose response testing**

- PubChem AID (743093)
Jurkat T-cell Cytotoxicity Counterscreen

1. Cells
   - Dispense 4uL of cells
     - 500 cells/well

2. Pinning
   - Add 30nL test compound

3. Substrate
   - Incubate plate with compounds for 24hrs, 5% CO2 at 37°C
   - Add 4uL CellTiter Glo substrate

4. Read Lumi
   - Incubate for 10 minutes (RT)

**Simple, homogenous and sensitive assay**

- The number of viable cells per well is quantified using CellTiter-Glo ATP detection system
- This counterscreen should eliminate generally cytotoxic compounds
- The controls used were no cells + DOX (100% Inh.) vs. cells only (0% Inh.)
- CRC analysis of the control (DOX) demonstrates the expected results
- This assay was used to counterscreen those available from the top 275 requested primary hits

<table>
<thead>
<tr>
<th>Jurkat Cytotoxicity Counterscreen</th>
<th>Control Compound</th>
<th>IC50/CC50</th>
<th>Z'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin</td>
<td>38.7 nM</td>
<td></td>
<td>0.73</td>
</tr>
</tbody>
</table>

CRC analysis of the control (DOX) demonstrates the expected results.
### 244/275 available compounds were tested in both the Gametocyte assay and the Jurkat Cytotox Assay

- Compounds are ranked with most potent first.
- 2 most “selective” compounds are identified in yellow.
- Only 2 compound were found active and based on IC50<10uM criteria
- PubChem AID 1117278
- SR-751192 was of interest for KU to order as powder along with 2 analogs
Secondary Assay Strategy

Antimalarial Drug Discovery: Assays for Compound Screening

**Secondary Assays**

<table>
<thead>
<tr>
<th>In vitro</th>
<th>In vivo</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FACS based <em>P. falciparum</em> Gametocyte Inhibition Assay</strong></td>
<td><strong>P. falciparum</strong> Asexual Stage Assay</td>
</tr>
<tr>
<td>Numeric activity cutoff - IC50 &lt; 5 µM. Screening 50 - 100 compounds</td>
<td>Numeric activity cutoff - IC50 &lt; 5 µM. Screening 50 - 100 compounds</td>
</tr>
<tr>
<td><strong>Mosquito Infectivity Assay</strong></td>
<td><strong>Exflagellation Assay</strong></td>
</tr>
<tr>
<td>Numeric activity cutoff - IC50 &lt; 5 µM. Screening 5 - 10 compounds</td>
<td>Numeric activity cutoff - IC50 &lt; 5 µM. Screening 5 - 10 compounds</td>
</tr>
<tr>
<td><strong>P. falciparum Gametocyte Mouse Model</strong></td>
<td></td>
</tr>
<tr>
<td>Numeric activity cutoff - IC50 &lt; 5 µM. Screening 5 - 10 compounds</td>
<td></td>
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</tbody>
</table>

The efficacy of probe candidates on asexual stage parasites will be determined using a [3H] hypoxanthine assay. Compounds causing red cell lysis or disruption of red cell membranes as determined by light microscopy will not be tested further.

Compounds active in the mosquito infectivity assay will also be tested for their ability to prevent gametocyte exflagellation to elucidate MOA.

We have developed a novel *in vivo* mouse model using immunocompromised splenectomised RagC57 mice, to study the *in vivo* effects of compounds on *P. falciparum* gametocytes.

This gametocyte assay will confirm that powder samples of probe candidates shown to inhibit late stage gametocyte viability by HTS can inhibit gametocyte viability using a FACS-based assay.

The ability of the compounds to prevent gametocyte uptake by mosquitoes and thus prevent oocyst development will be determined by membrane feeding of *Anopheles stephensi* mosquitoes. A colony of these mosquitoes is maintained under quarantine at QIMR.
Summary:
• The MLPCN HTS campaign for the gametocyte assay is complete.
• Screening with gametocytes is challenging (batch consistency, etc.)
• 244 compounds proceeded directly to dose response from the primary assay.
  • One compound, SR-01000751192-1, appears to be ~>9 fold selective vs. the counterscreen, has limited promiscuity, and is not a PAINs inhibitor.
  • SR-01000599986-1 also shows ~>3 fold selectivity but has some promiscuity but, also is not a PAINs inhibitor.
• SR-01000751192 appears to reconfirm but analogs didn’t improve potency
• The MLPCN phase of this project is closed

Next Steps:
• A 10K follow-up pilot of the SDDL was also tested as part of the R21 effort
  • These are unique from the MLPCN library compounds
• 25 hits were identified and titrated for CRC determination in both gametocyte assay and Jurkat assay
• Analyze these results and determine if 2nd assays are warranted in AUS
• Write HTS manuscript
Key Assays of the Future (or back to the future?)

- High Content Microscopy
- HT-Flow cytometry
- RT-PCR
- HT-Mass Spec
- Microfluidics
- Arrays
- Label Free detection

A 1536-Well 3D Viability Assay to Assess the Cytotoxic Effect of Drugs on Spheroids

Franck Madoux, Allison Tanner, Michelle Vessels, Lynsey Willetts, Shurong Hou, Louis Scampavia, Timothy P. Spicer  
First Published January 13, 2017  research-article
Pancreatic cancer remains a leading cause of cancer-associated death, with a 5-year survival rate less than 10%.

Genetic KRAS (Ki-ras2 Kirsten rat sarcoma viral oncogene homolog) mutations are found in more than 90% of pancreatic cancer patients.

Genetic alterations in tumor suppressors TP53, CDKN2A, SMAD4, ARID1A and MLL3 also accumulate in the pancreatic cancer patient.

R. Siegel, et.al. CA CANCER J CLIN 2013, 63: 11-30
Primary human pancreatic ductal cells hM1-2D, hF2-2D, hT1-2D, and cancer-associated fibroblasts hM1-CAF and hT1-CAF, were generated from tissues of pancreatic cancer patients in the laboratory of Dr. Tuveson, M.D. at CSHL.

2D monolayer cells were conditioned from established pancreatic 3D organoids.

Targeted sequencing analysis of human pancreatic organoids reveals their genetic makeup.
Why Culture Cells in 3D?

• Evaluation of drug cytotoxicity traditionally relies on use of cell monolayers.

• Three-dimensional (3D) cell models have been described as more physiologically relevant for a variety of applications.

• Numerous methods have been developed to produce spheroids.

• We are funded to help develop the N3D and Greiner BioOne technology

M. Zanoni, et.al. Scientific Reports, 2017, 6:19103
H. Tseng, et.al. Scientific Reports 2017, 5:13987
Greiner 384 well plates readily form spheroids using cells combined with Nanoshuttle (A). This can be done on the top side of plates or the bottom side of the plates using a magnetic driver (B). Note that the shape of the bottom of the wells is compatible with automated confocal microscopy.
n3D Spheroid Technology for Drug Test Protocol

In Flask Labelling

Without NS

With NS

n3D Spheroid Technology for Drug Test
384-well plate

1. Add nanoshuttle to cells in flask, incubate cells overnight | 37°C 5%CO₂ 95% RH
2. Seed xx cells in 25 μL culture medium to 384 Greiner cell repellent plate
3. Put the plate atop of the spheroid drive, incubate the plate on drive for xx hrs
4. Inculate cells for 2 days | 37°C 5%CO₂ 95% RH
5. Pintool transfer 100nL compounds
6. Inculate cells for additional 2 days | 37°C 5%CO₂ 95% RH
7. Image spheroids using HCS
8. Dispense 25 μL CellTiter-Glo 3D Reagent, Shake and incubate for 60min at RT, Read on ViewLux

Final volume = 25 μL

→ This 384-well protocol will be adapted into 1536-well format.
### n3D Technology to Generate Spheroids

<table>
<thead>
<tr>
<th></th>
<th>4h on drive</th>
<th>24h off drive</th>
<th>48h off drive</th>
<th>72h off drive</th>
<th>Corning spheroid plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>hT29</td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
</tr>
<tr>
<td>PANC-1</td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
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<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
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<tr>
<td>hM1-2D</td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
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</table>

#### Condition:
- n3D technology: 2500 cells per well, 4hrs printing time.
- Using n3D technology, hT29 and PANC-1 can form typical spheroids, while hM1-2D cannot.
hT29_Nanoshuttle

PANC-1_Nanoshuttle

HT29 Z-stack analysis

→ Nuclei staining with Hoechst then 5 micron image slices for spheroids with INCell 6000 confocal imager.
Primary Pancreatic Cancer Cells to Form Spheroids

Pancreatic cancer cells do not form typical 3D spheroids, but formed smaller 3D structures, which were confirmed to be organoids by CSHL.

Fibroblasts are able to form more typical 3D spheroids.

Images were taken at 72hrs post seeding
Confocal images (1 micron image slices) using Hoechst staining confirmed the formation of 3D structure in hM1-2D, hT1-2D and hT1-CAF culture.
## 3D Cell Culture: Spheroids vs. Organoids

There is a difference

<table>
<thead>
<tr>
<th>Definition</th>
<th>Image</th>
<th>Techniques</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multicellular spheroids (cellular aggregates) mimic the architectural and functional characteristics of native tissue through self-assembly.</td>
<td><img src="image1" alt="Spheroids Image" /></td>
<td>Using gravity-enforced self-assembly, centrifugal force or other external force to promote cell-to-cell aggregation. Techniques include hanging drop, pellet culture, liquid overlay, spinner culture, rotating wall vessel, external force. Spheroids are usually generated from cells initially cultured as monolayers.</td>
<td>T.M. Achilli, et.al. Expert Opin Biol Ther. 2012, 12(10): 1347–1360</td>
</tr>
<tr>
<td>The term ‘organoid’ literally means organ-like, reflecting the ability of organoid culture conditions to prompt cells to self-organize into structures mimicking the architecture of the organ from which they were derived.</td>
<td><img src="image2" alt="Organoids Image" /></td>
<td>Organotypic culture usually relies on artificial extracellular matrices (Matrigel and collagen) to facilitate their self-organization into structures that resemble native tissue architecture. Organoids are generated from primary tissues, ESCs and iPSCs.</td>
<td>L.A. Baker, et.al. Trends in Cancer. 2017, 2(4): 176–190</td>
</tr>
</tbody>
</table>

→ The descriptions above represent the general understanding of spheroids and organoids.
→ They share a lot of similarities and the term organoid is often used interchangeably in literature but are actually quite distinct from each other.
→ Both spheroids and organoids are 3D models, filling the gap between monolayer culture and in vivo study.
The Ability of Cells to Form Spheroids

Molecular mechanism responsible for the difference in cell aggregation?

- β1-Integrin expression: All three relatively high and close to equal
- E-cadherin expression: Hep3B > HepG2 > PLC/PRF/5 (8.5 : 4.1 : 1)

Figure 2. Comparative analysis of spheroid-forming capability among three hepatoma cell lines

Figure 3. Formation and activation of fibroblast spheroids depend on fibronectin–integrin interaction

Cells in 2D monolayer and in 3D spheroid/organoid format demonstrated different responses to therapeutic compounds. Drug resistance was observed in 3D model.
Summary and Future Directions

- **3D structure formation:**
  - 2 pancreatic cancer patient derived fibroblast are able to form spheroids.
  - 3 Pancreatic cancer cell lines can not form typical spheroids, but are able to form organoids.

- Compared to monolayer cells, 3D spheroids display a different response to therapeutic compounds.

- The same set of drugs as 2D screen will be tested on 3D format of these 5 cell lines.

- The screening will be adapted into 1536 well format, and larger scale HTS (~150K compounds) will be performed.

- Expanding tumor models by co-culture of cancer associated fibroblast and cancer cells to better mimic cancer biology.
Phenotypic Analysis and HCS

Assay development focused on capture of quantitative, project relevant parameters
Advancing Biological Understanding and Therapeutics Discovery with Small-Molecule Probes


Cell 161, June 4, 2017
Jumping into the game

Envoy to seek new Parkinson's disease therapy with Scripps Research Institute

By David Mutze
JUPITER, Fla.—A new player in drug discovery is teaming up with a familiar name to identify new drugs for Parkinson's disease (PD) that have greater efficacy and safety compared to current therapies.

Envoy Therapeutics Inc., a recently formed drug discovery company, recently announced that it has begun a research collaboration with the Scripps Research Institute to identify new drugs for PD. Using funding provided by Envoy, scientists at the two organizations will apply Scripps high-throughput screening (HTS) capabilities to discover compounds that modulate a target protein discovered by Envoy.

"The parties are jointly developing and evaluating various assays in order to optimize the efficiency and effectiveness of the screening," says Dr. Stephen Hitchcock, senior vice president of drug discovery at Envoy. "Subsequent to initial screening, the parties will identify a subset of hits which will be the focus of additional validation and optimization studies."

According to Scott Forrest, director of business and technology development at the Scripps Research Institute, what makes Envoy Therapeutics an attractive partner for this collaboration is a combination of excellent science, aggressive pursuit of novel targets (as a small biotech), Envoy can quickly get into "fresh" target space more rapidly than Big Pharma and a multidisciplinary approach to drug discovery that combines strong biology with HTS and Envoy contented on page 52.

"Many neurological disorders are defined by deficiencies in specific populations of neurons and neuronal circuitry. However, the intermingling of many different cell types within brain regions makes identifying targets that modulate specific pathways very challenging."

—Dr. Stephen Hitchcock, senior vice president of drug discovery at Envoy

A detailed listing of relevant media can be found here:


Takeda snaps up CNS drug developer Envoy Therapeutics in $140M deal

November 6, 2012 | By John Carroll

Japan's Takeda has moved in to snap up a Florida biotech equipped with some novel CNS drug technology and backed by a Nobel Prize-winning scientist who's played a prominent role in the field.

The Japanese pharma giant will pay up to $140 million for Envoy Therapeutics in an upfront and milestone payments, possibly laying out some healthy returns for the investors behind a drug developer launched three years ago with $8 million in venture cash, including money from Takeda's venture arm.

Envoy was founded by Nobel laureate Paul Greengard—who had focused his research work on dopamine—and Howard Hughes investigator Nathaniel Heintz. The biotech had highlighted its role in mapping genetic engineering and molecular biology in the search for new protein therapeutics that could tackle a long line-up of severe ailments such as Parkinson's and schizophrenia. Just last May the Michael J. Fox Foundation stepped up with $1.2 million to support the move to clinical development of a new oral therapy that could provide the benefit of dopamine replacement therapy without the usual side effects.

[Image of various universities and research institutions]
Impact: Research Publications

Highly diverse areas of focus have led to research contributions among numerous publications (currently 93 total and counting; 65 probe reports)
• HTS involves testing very large numbers (e.g., millions) of compounds in order to identify biologically active molecules
• HTS utilizes automation, cutting-edge technology and highly miniaturized assays to test large #s of compounds in a cost-effective way
• Assay performance must be measured throughout HTS
• HTS assay robustness is typically expressed in terms of a Z’ score
• Follow-up assays including re-tests, concentration-response curves, null/specificity assays, selectivity assays and other orthogonal assays are important for HTS follow-up
• Confirmation of chemical mater, confirmatory assays and similarity searching also important in HTS follow-up
SRIMSC Team