YFP-halide assays for CFTR drug discovery using the FDSS/μcell

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Outline

• Introduction to Cystic Fibrosis
  ➢ Disease and cause
  ➢ Approved therapies and remaining challenges
  ➢ Targeting underlying mutations

• Cell-based assays used for CFTR drug discovery
  ➢ YFP-Halide Assay
    ▪ Principle and advantages
    ▪ Assay development on FDSS/µCell
    ▪ HTS to find CFTR modulators

• Conclusion
Cystic Fibrosis: Facts & Biology

• Cystic Fibrosis (CF) is the most frequent life-threatening autosomal recessive disease in the Caucasian population
  ➢ 1 in 2500 newborns diagnosed with CF
  ➢ 1 in 25 Caucasians carry at least 1 CF allele

• Characterized by thick mucus in lumen of several organs
  ➢ Airways, pancreas, gastro-intestinal tract, reproductive tract
  ➢ Frequent lung infections, sinus infections, poor growth, and infertility
  ➢ Life expectancy: mid-30s

• Caused by mutations in the CFTR gene (~1900)
Cystic Fibrosis Transmembrane Conductance Regulator (CFTR)

- Chloride channel from ABC transporter family, expressed on the apical membrane of epithelial cells
- In normal cells, water follows chloride ions onto surface of lung, hydrates lung surface and cilia beat normally
- Defective CFTR channels don’t transport chloride ions out of cells
- Reduced hydration of lung surfaces impairs normal functioning of cilia

CFTR: ATP-gated Cl⁻ channel
CFTR gene mutation

CFTR protein dysfunction

Defective mucociliary clearance

Targeting the cause

Airways obstruction

Targeting the symptoms

Inflammation

Infection

Stem Cell and Gene Therapy Treatments

Small-Molecule CFTR Modulators Kalydeco®, Kalydeco® /Lumacaftor combination (ORKAMBI™ FDA decision July 5, 2015)

Mucus Alteration and Airway Surface Liquid Modulation Therapy Hypertonic saline, Pulmozyme®

Infection Treatments Tobramycin, Azithromycin, Cayston®

Approved CF Therapies

Targeting the cause

Approved Therapies

Infection Treatments

Anti-inflammatory Treatments Ibuprofen

Anti-inflammatory Treatments Ibuprofen

Targeting the symptoms

Approved Therapies

Infection Treatments

Anti-inflammatory Treatments Ibuprofen

Infection Treatments

Anti-inflammatory Treatments Ibuprofen
Challenges in CF drug discovery

- Gene therapy: Lack of efficient gene transfer to cellular targets required to correct \textit{in vivo} CFTR function -> 25 failed clinical trials to date
- Lack of good animal models
- Paucity of structural information on full-length wild-type and mutant CFTR
- Complexity of defects caused by various CFTR mutations
Classes of CFTR mutations

- **Class 1 - Synthesis**
  - Premature stop codon, ~10% freq

- **Class 2 - Maturation**
  - Includes F508del, ~70% freq

- **Class 3 - Regulation**
  - Includes G551D, ~3-5% freq

- **Class 4 - Conductance**
  - Includes R117H, ~2% freq

- **Class 5 - Quantity**

- **Class 6 - Turnover**

\[
I_{CFTR} = n \times P_o \times g
\]

- \( n \) = number of channels
- \( P_o \) = open probability
- \( g \) = channel conductance
Combination approaches to fix the most severe CFTR mutations

**Functional Defects (Class III / IV, eg. G551D, R117H)**

Potentiators restore the flow of ions through activated CFTR

Correctors restore the processing of CFTR from the ER to the surface

Adapted from Dr. Scott Donaldson’s Plenary Session (NACFC, 2013)
CFTR phenotypic assays

• CFTR cell surface expression:
  - Epitope-tagged CFTR detection on cell surface via ELISA
  - Beta galactosidase enzyme fragment complementation for detection of membrane localized enzyme fragment fusion CFTR construct (DiscoveRx™)

• CFTR channel function:
  - Membrane potential or halide-sensitive fluorescent dyes to measure CFTR-dependent chloride influx
  - YFP-Halide assays to measure CFTR-dependent halide influx
YFP-Halide Assay

- Fluorescence of Yellow Fluorescent Protein mutant (YFP-H148Q/I521L) quenched by halides (selectivity: iodide > chloride)
- Cell lines expressing mutant CFTR and YFP are generated, CFTR activated via cAMP ↑ (Forskolin) in the presence of iodide
- Compound mediated restoration of anion channel function estimated by fluorescence quenching of iodide

Cell-based assay for high-throughput quantitative screening of CFTR chloride transport agonists
Luis V. J. Galietta, Sujatha Jayaraman, A. S. Verkman
Detection of CFTR potentiators

Cells over-expressing F508d-CFTR and YFP-H148/I152L

![Diagram showing the detection of CFTR potentiators](image-url)

**Figure:**
- Cells are grown at 27°C for 24 hours.
- Fsk + CFTR potentiator activates YFP.
- Fsk + inactive cpd does not activate YFP.
- Nal inhibits CFTR potentiator.

**Graph:**
- Dose response CFTR potentiator with Vehicle/inactive and CFTR potentiator curves.

**Legend:**
- YFP
- Cl- → I-
- Fsk + CFTR potentiator
- Fsk + inactive cpd
- Nal
- Vehicle/inactive
Detection of CFTR correctors

Cells over-expressing F508del-CFTR and YFP-H148/I152L

- + CFTR corrector
- + inactive compound

(24h)

Fsk + Potentiator

(20 min)

Cl⁻ → I⁻

CFTR corrector

Vehicle/inactive

Dose response CFTR corrector

% activity vs Pos Ctrl

log conc (M)

Dose response CFTR corrector

CFTR corrector

Vehicle/inactive
Advantages

• Sensitive quantitative assay to measure restoration of deficient cellular chloride transport - HTS compatible

• Excellent optical properties and retention in cells

• Fluorescence indicator loading and washing not necessary

• Applicable to physiologically relevant bronchial epithelial cells - show minimal basal halide permeability before stimulation

• Anion co-transporters like NKCC and exchangers like AE1 transport I- poorly, whereas CFTR transports I- efficiently -> better selectivity

• Can be run in different flavours: to detect potentiators, correctors and compound synergies
HTS to identify CFTR potentiators
Assay optimization

• Evaluation of HEK-293 cells vs CF bronchial epithelial (CFBE41o-) cells
• Transfection/transduction conditions
• Using the μcell/FDSS for increased throughput - comparison with EnVision®
• Analysis method
HEK-293
Cell number and YFP transfection conditions

Baseline fluorescence and Z’ in HEK-293 cells transfected with YFP-H148/I152L

Z’ is generally poor for HEK-293 transfected cells
CFBE41o- cells
Cell number and YFP transduction conditions

Baseline fluorescence and Z’ in CFBE cells transduced with YFP-H148/I152L

- Adenoviral transduction in CFBE cells gives more uniform YFP signals
- More physiologically relevant cell line for CF
## Comparison of instruments

<table>
<thead>
<tr>
<th><strong>EnVision® (Perkin Elmer)</strong></th>
<th><strong>FDSS/ μCELL (Hamamatsu)</strong></th>
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<tbody>
<tr>
<td>Dual-detector multilabel Plate reader</td>
<td>Kinetic plate reader for fluorescence and luminescence</td>
</tr>
<tr>
<td>Label specific optical mirror modules and filter optics</td>
<td>Filter optics</td>
</tr>
<tr>
<td>Temperature control</td>
<td>No temperature control</td>
</tr>
<tr>
<td>Well per well reading</td>
<td>High speed, high - sensitivity CCD camera for detection of entire plate</td>
</tr>
<tr>
<td><strong>High precision dispenser unit:</strong></td>
<td><strong>384 well dispenser head:</strong></td>
</tr>
<tr>
<td>Well per well dispensing -96w plates</td>
<td>Simultaneous 384w dispensing</td>
</tr>
<tr>
<td>Dispense volume: 2 - 475 µL</td>
<td>Dispense volume: 1 - 30 µL</td>
</tr>
<tr>
<td>Dispense speed: 100 - 500 µL/sec</td>
<td>Dispense speed: 2 - 50 µL/sec</td>
</tr>
<tr>
<td></td>
<td>Multiple compound/ligand dispensing</td>
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<tr>
<td></td>
<td>Active wash station allowing reuse of tips, with wipe function</td>
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</tbody>
</table>
Comparison of methods

<table>
<thead>
<tr>
<th>EnVision®</th>
<th>Assay conditions</th>
<th>FDSS/µCELL</th>
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</thead>
<tbody>
<tr>
<td><strong>Day 1: Cell seeding</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>96 well</td>
<td>Plate format</td>
<td>384 well</td>
</tr>
<tr>
<td>6,000 cells/well in 100 µL</td>
<td>Cell density</td>
<td>2,000 cells/well in 50µL</td>
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<tr>
<td><strong>Day 2: Transduction of cells</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MOI 20</td>
<td>YFP virus</td>
<td>MOI 30</td>
</tr>
<tr>
<td>MOI 30</td>
<td>CFTRdelF508 virus</td>
<td>MOI 30</td>
</tr>
<tr>
<td></td>
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<td></td>
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<tr>
<td><strong>Day 3: Low temperature correction</strong></td>
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<td></td>
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<tr>
<td>20 hours incubation at 27 °C, 5% CO₂</td>
<td></td>
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<tr>
<td><strong>Day 4: Washing of cells, compound addition &amp; activation of CFTR</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Manual washing (2 x 40 µL)</td>
<td>Washing of cells with DPBS</td>
<td>Plate washer (5x 90 µL, 20 µL remaining after last wash)</td>
</tr>
<tr>
<td>Addition of compounds and forskolin using multichannel (40 µL added on dry cells)</td>
<td>Compound addition + activation of CFTR with 10 µM forskolin</td>
<td>Compound and forskolin addition using FDSS/µCELL (10 µL added on cell plates containing 20 µL PBS)</td>
</tr>
<tr>
<td>150 µL/sec</td>
<td>Injection of I⁻</td>
<td>30 µL/sec</td>
</tr>
<tr>
<td>16 minutes (34 readings per well every 0.2 sec) with excitation at 485 nm and emission at 530 nm</td>
<td>Reading</td>
<td>3 minutes including washing of tips (384 wells read simultaneously) with excitation at 480 nm and emission at 540 nm</td>
</tr>
</tbody>
</table>
Analysis method

- Analysis based on slope
- Analysis based on fluorescence at 36 sec
- Analysis based on fluorescence at 105 sec

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<thead>
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<th>Analysis based on slope</th>
<th>Analysis based on fluorescence at 36 sec</th>
<th>Analysis based on fluorescence at 105 sec</th>
</tr>
</thead>
<tbody>
<tr>
<td>S/B</td>
<td>1.7</td>
<td>2.2</td>
<td>1.4</td>
</tr>
<tr>
<td>Z'</td>
<td>0.67</td>
<td>0.76</td>
<td>0.76</td>
</tr>
</tbody>
</table>

$F_0$ = fluorescence before injection
$F$ = fluorescence at a fixed time point after injection.

Dose response CFTR potentiator
Comparison of compound activities

Good correlation between pEC$_{50}$ values obtained on both platforms
Comparison of QC parameters

• Z’ values comparable between platforms
• S/B considerably reduced on the FDSS/µCELL but more stable over runs
HTS to identify potentiatiors of F508del-CFTR

• Library
  ➢ A diverse selection of 76,000 compounds was screened (40 plates/day, 6 runs)

• HTS QC criteria
  ➢ Plates accepted if $Z’ \geq 0.35$
  ➢ Pharmacology QC by reference compound $IC_{50}$
    ▪ $+/-$ 3 fold the historical average
YHA potentiator HTS
Normalized data

- High hit rate observed
- Consistent data after normalization over the different runs
- Reproducible pharmacology for reference compounds
• Good assay S:B and plate Z' for most plates screened
• Only 1 plate rejected for the whole screen
YHA potentiator HTS
Hit identification

- Asymmetric distribution due to high hit rate, hit calling using IQR method
- Percentage activation $\geq Q3 + 3.5 \times IQR$ (62%)
- 909 hits identified with > 62% activity, being further profiled
Conclusions

- Quantitative functional assay to identify compounds that improve chloride channel function in CFBE cells
- Robust protocol with an average S/B of 1.8 and a Z’>0.5.
- Optimized protocol using the plate washer and the FDSS/µCELL led to a 20-fold increase in the assay throughput - suitable for HTS
- Good correlation between EC$_{50}$ data obtained on the EnVision® and the FDSS/µCELL
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