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

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Outline

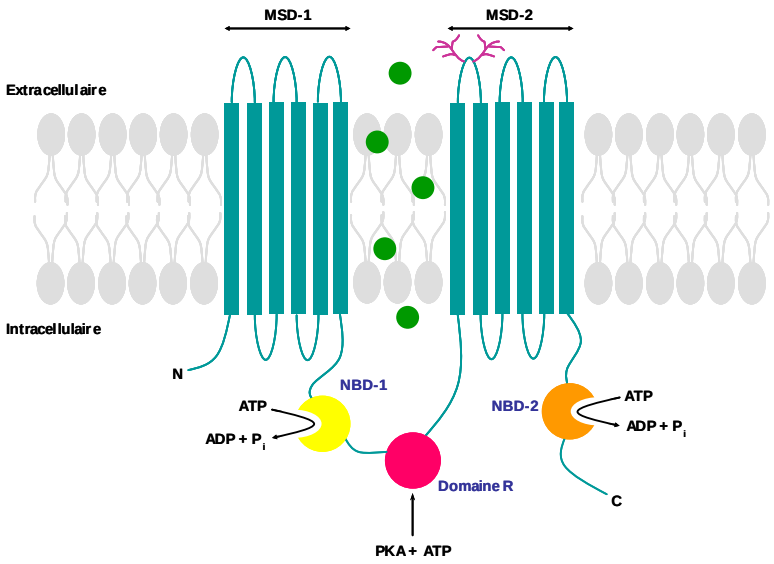
- Introduction to Cystic Fibrosis
  - Disease and cause
  - Approved therapies and remaining challenges
  - Targeting underlying mutations
- Cell-based assays used for CFTR drug discovery
  - YFP-Halide Assay
    - Principle and advantages
    - Assay development on FDSS/µCell
    - HTS to find CFTR 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

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Approved CF Therapies

Targeting the cause

CFTR gene mutation

CFTR protein dysfunction

Defective mucociliary clearance

Stem Cell and Gene Therapy Treatments

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

Targeting the symptoms

Airways obstruction

Inflammation

Infection

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

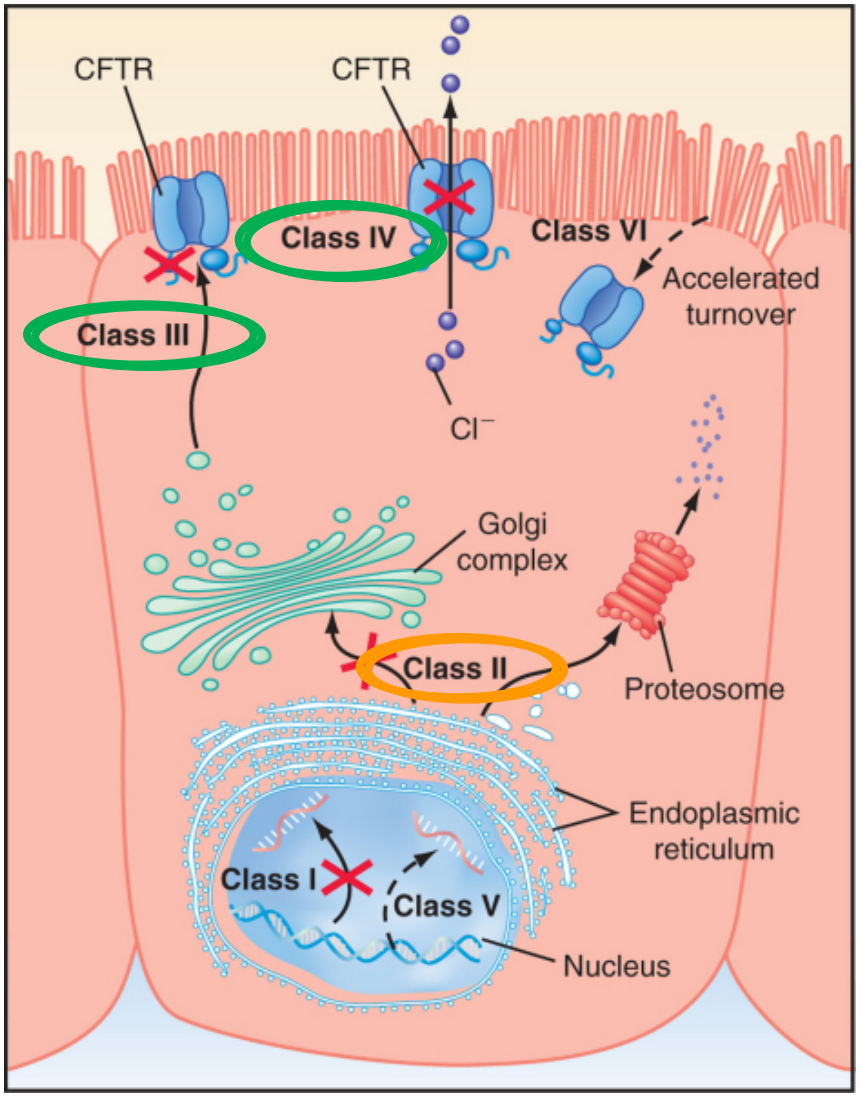
Anti-inflammatory Treatments
Ibuprofen

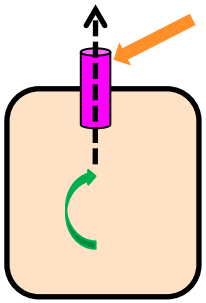
Infection Treatments
Tobramycin, Azithromycin, Cayston®

Challenges in CF drug discovery

- Gene therapy : Lack of efficient gene transfer to cellular targets required to correct *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





$I_{CFTR} = n \times P_o \times g$

n = number of channels

P_o = open probability

g = channel conductance

- 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

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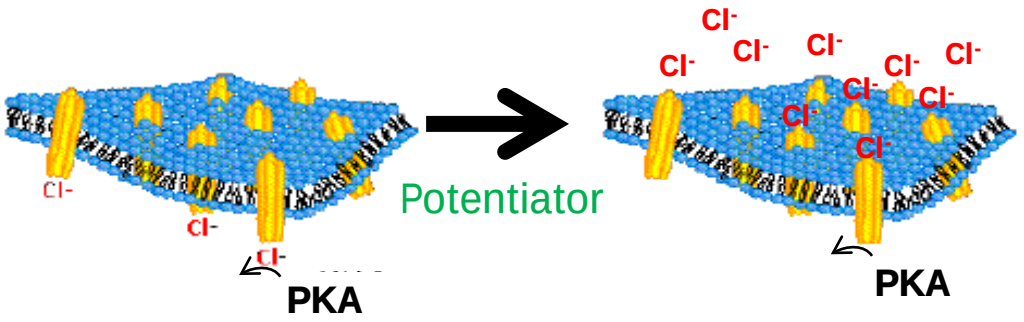
Combination approaches to fix the most severe CFTR mutations

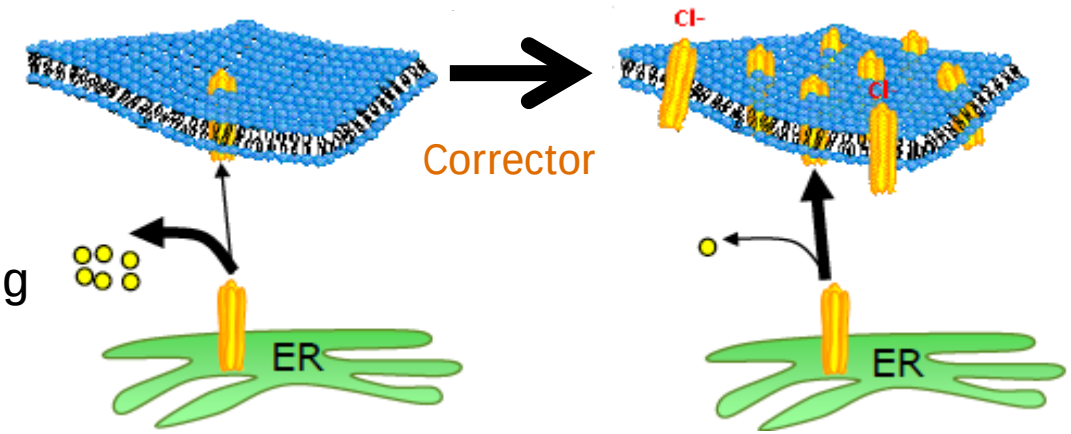
Functional Defects (Class III / IV, eg. G551D, R117H)

Potentiators restore the flow of ions through activated CFTR



Maturation Defect (Class II , eg. F508del)

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



Adapted from Dr. Scott Donaldson's Plenary Session (NACFC, 2013)

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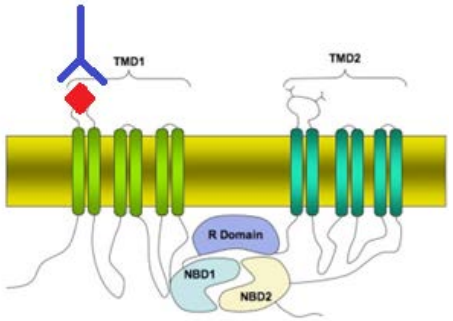
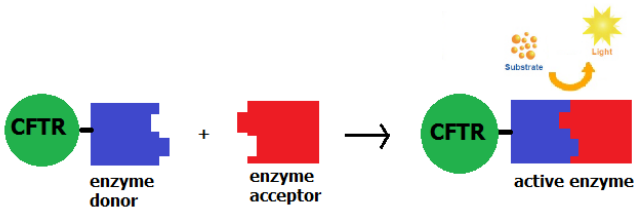
CFTR phenotypic assays

- 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

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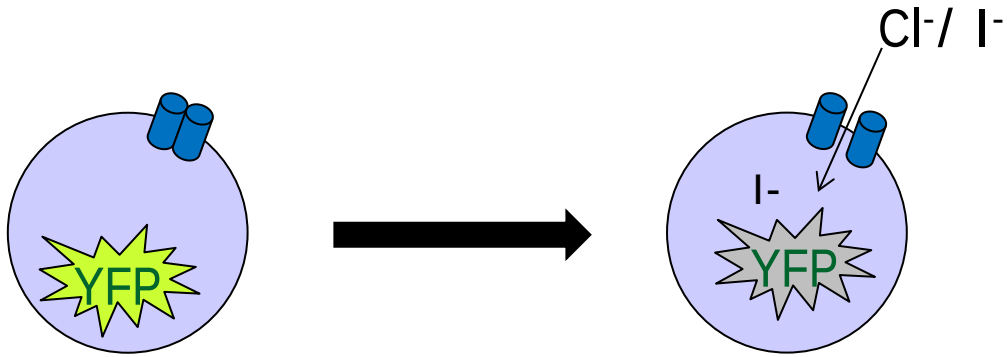
YFP-Halide Assay

- 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
American Journal of Physiology - Cell Physiology Nov 2001, 281 (5)

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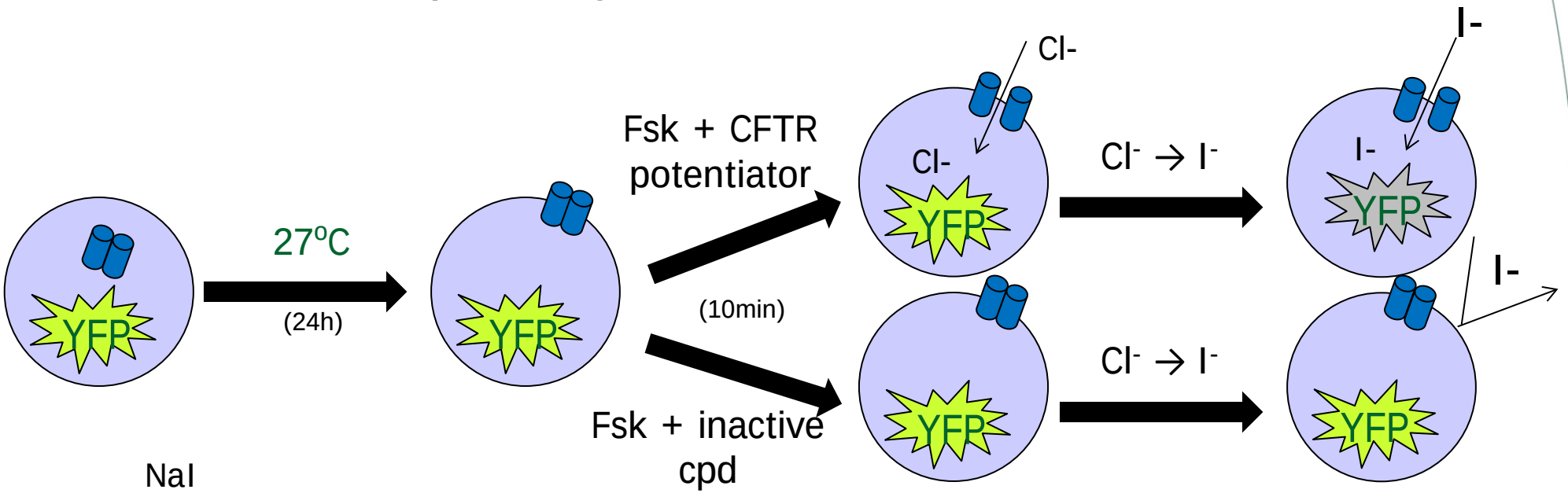
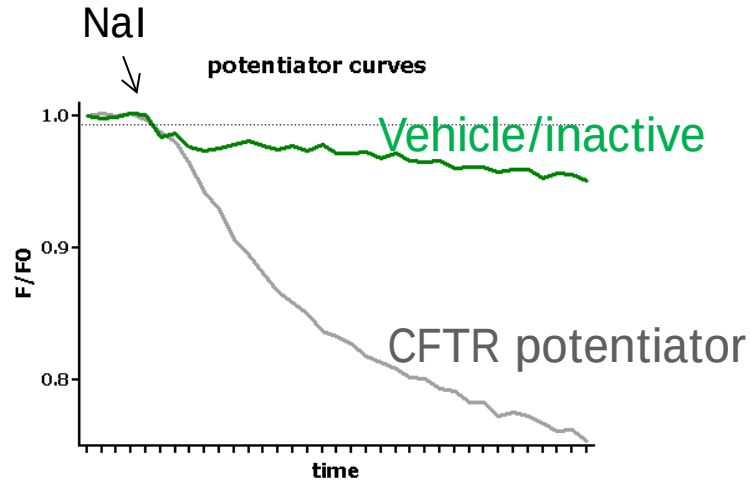
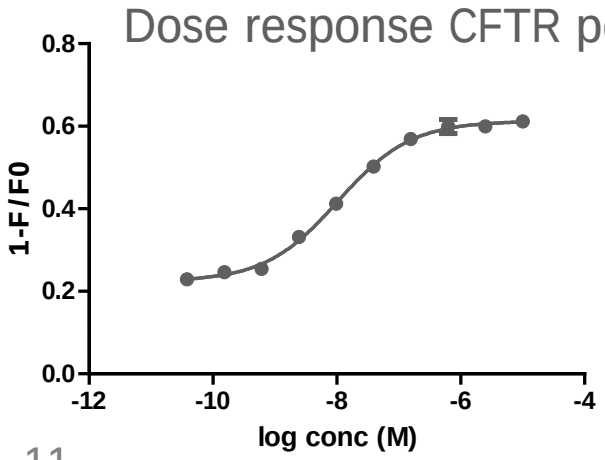
Detection of CFTR potentiators

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







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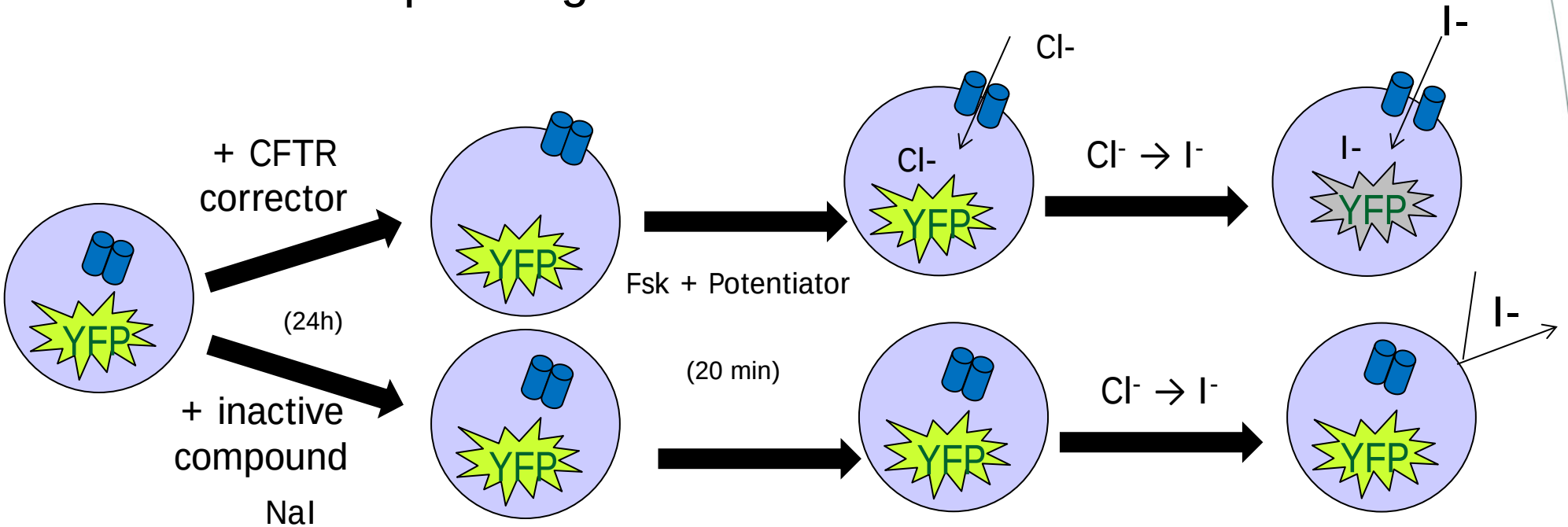
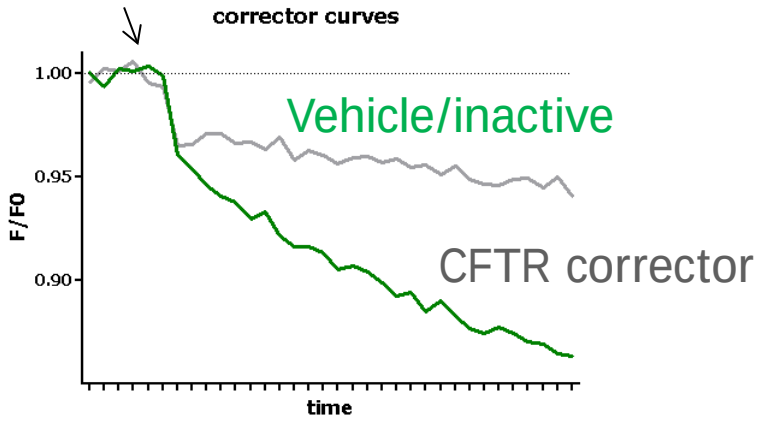
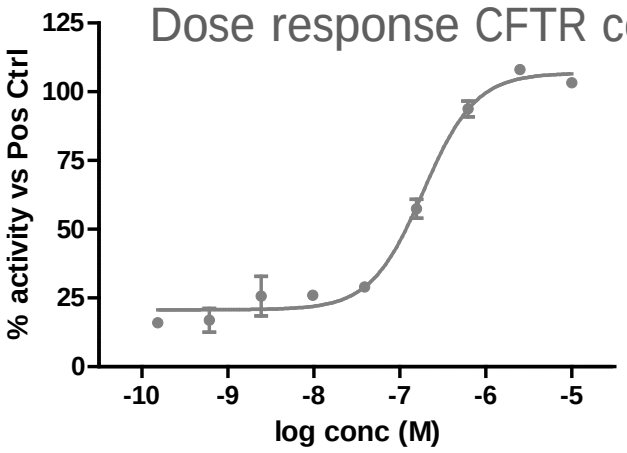
Detection of CFTR correctors

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







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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

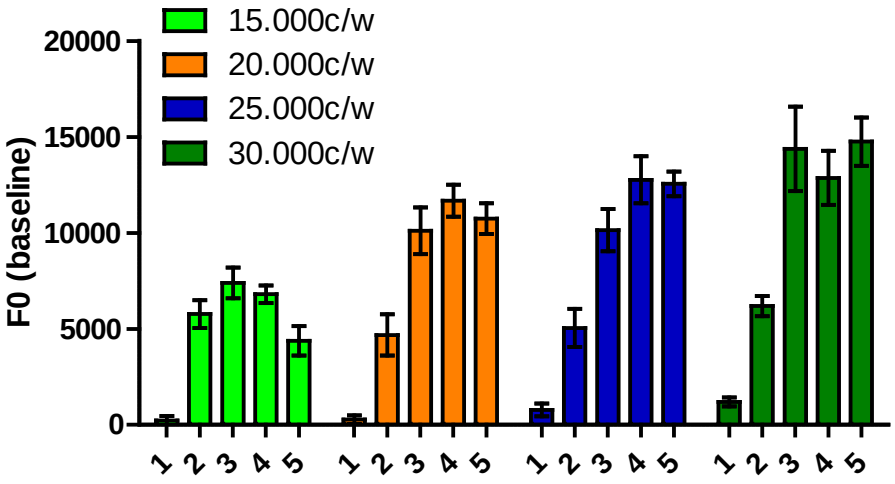
Assay optimization

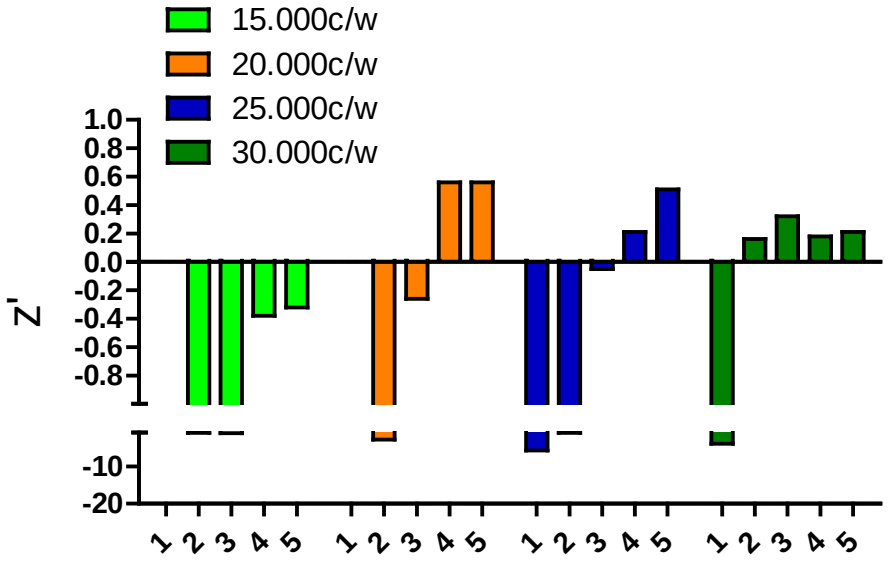
- Evaluation of HEK-293 cells vs CF bronchial epithelial (CFBE41o-) cells
- Transfection/transduction conditions
- Using the µcell/FDSS for increased throughput - comparison with EnVision®
- Analysis method

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

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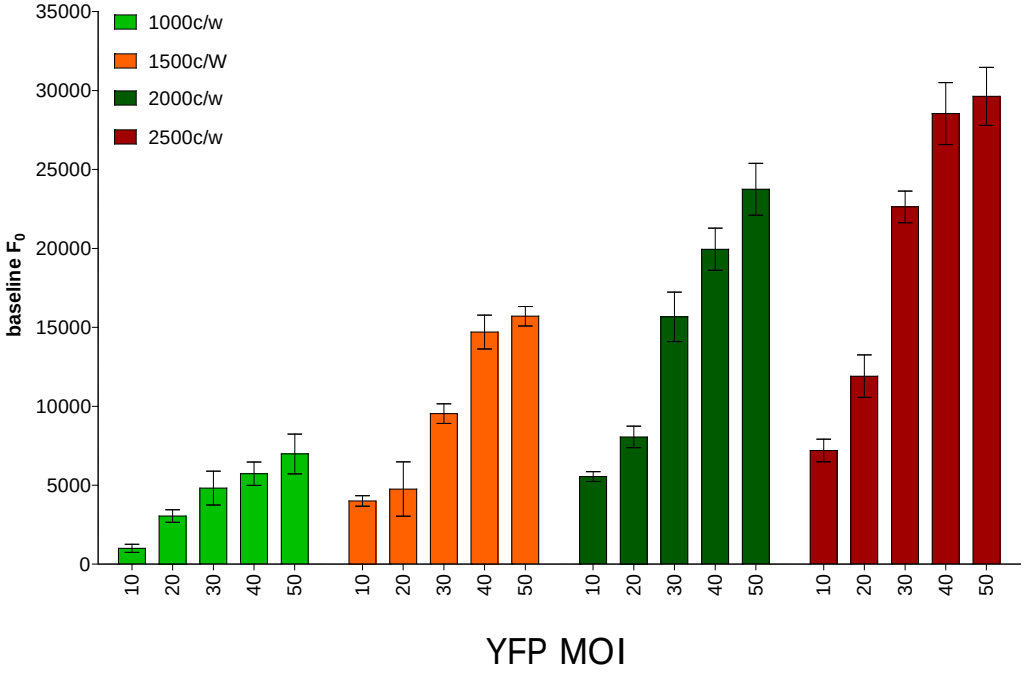
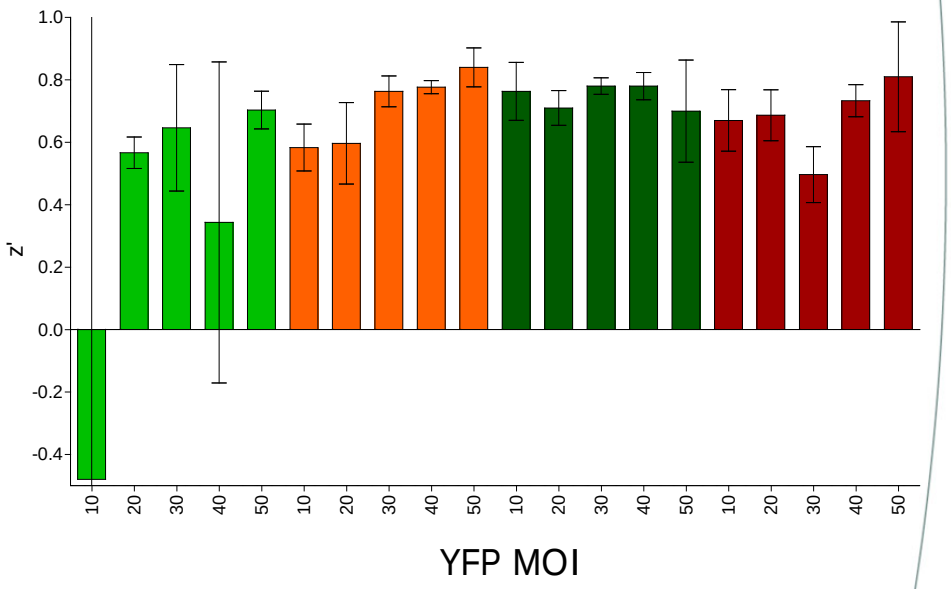
CFBE41o- cells

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

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Comparison of instruments

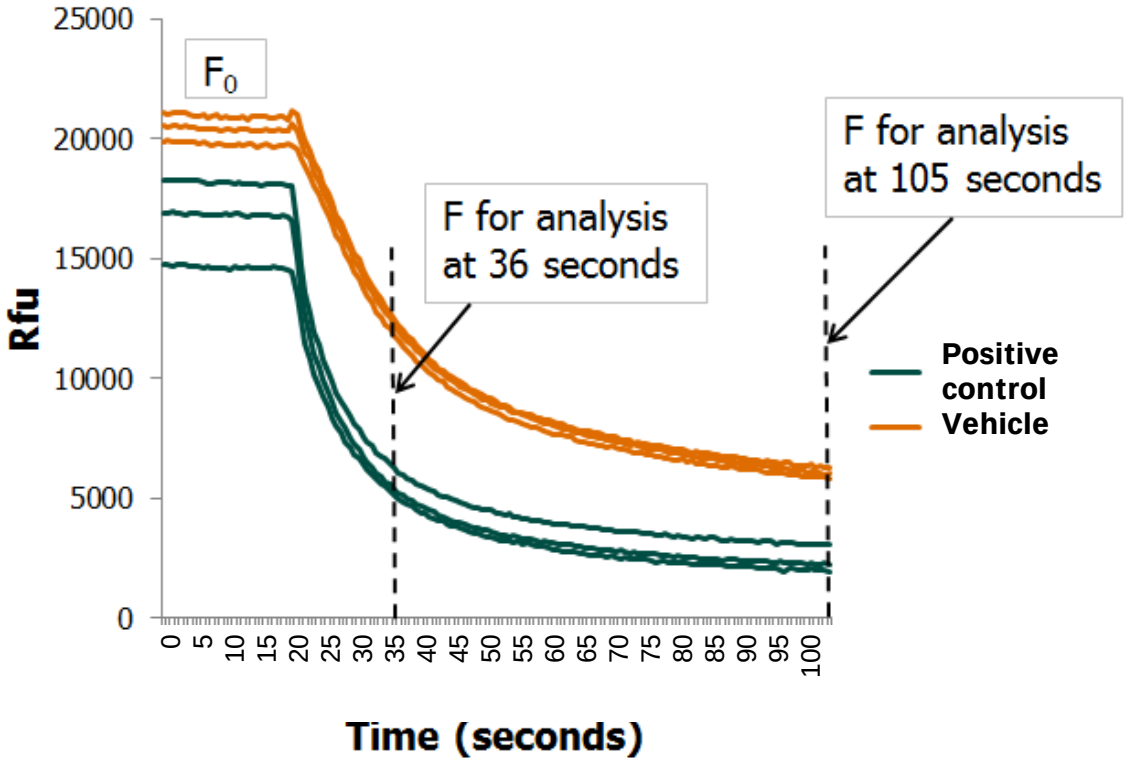
EnVision® (Perkin Elmer)
Dual-detector multilabel Plate reader
Label specific optical mirror modules and filter optics
Temperature control
Well per well reading
High precision dispenser unit:
Well per well dispensing -96w plates
Dispense volume: 2 - 475 µL
Dispense speed: 100 - 500 µL/sec

FDSS/µCELL (Hamamatsu)
Kinetic plate reader for fluorescence and luminescence
Filter optics
No temperature control
High speed, high - sensitivity CCD camera for detection of entire plate
384 well dispenser head:
Simultaneous 384w dispensing
Dispense volume: 1 - 30 µL
Dispense speed: 2 - 50 µL/sec
Multiple compound/ligand dispensing
Active wash station allowing reuse of tips, with wipe function

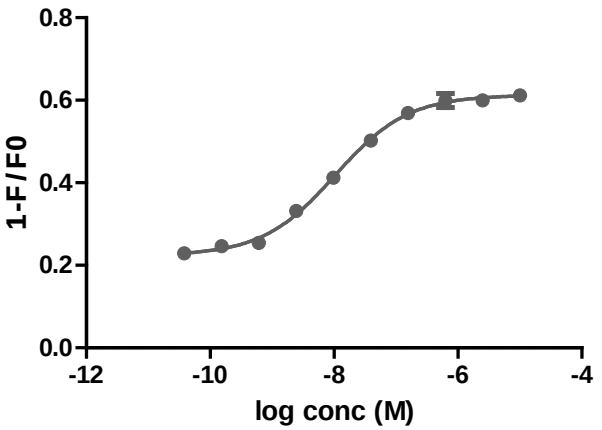
Comparison of methods

EnVision®	Assay conditions	FDSS / µCELL
Day 1: Cell seeding		
96 well	Plate format	384 well
6,000 cells/well in 100 µL	Cell density	2,000 cells/well in 50µL
Day 2: Transduction of cells		
MOI 20	YFP virus	MOI 30
MOI 30	CFTRdelF508 virus	MOI 30
Day 3: Low temperature correction		
20 hours incubation at 27 °C, 5% CO_2		
Day 4: Washing of cells, compound addition & activation of CFTR		
Manual washing (2 x 40 µL)	Washing of cells with DPBS	Plate washer (5x 90 µL, 20 µL remaining after last wash)
Addition of compounds and forskolin using multichannel (40 µL added on dry cells)	Compound addition + activation of CFTR with 10 µM forskolin	Compound and forskolin addition using FDSS/µCELL (10 µL added on cell plates containing 20 µL PBS)
150 µL/sec	Injection of I-	30 µL/sec
16 minutes (34 readings per well every 0.2 sec) with excitation at 485 nm and emission at 530 nm	Reading	3 minutes including washing of tips (384 wells read simultaneously) with excitation at 480 nm and emission at 540 nm

Analysis method



Dose response CFTR potentiator



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

	Analysis based on slope	Analysis based on fluorescence at 36 sec	Analysis based on fluorescence at 105 sec
S/B	1.7	2.2	1.4
Z'	0.67	0.76	0.76

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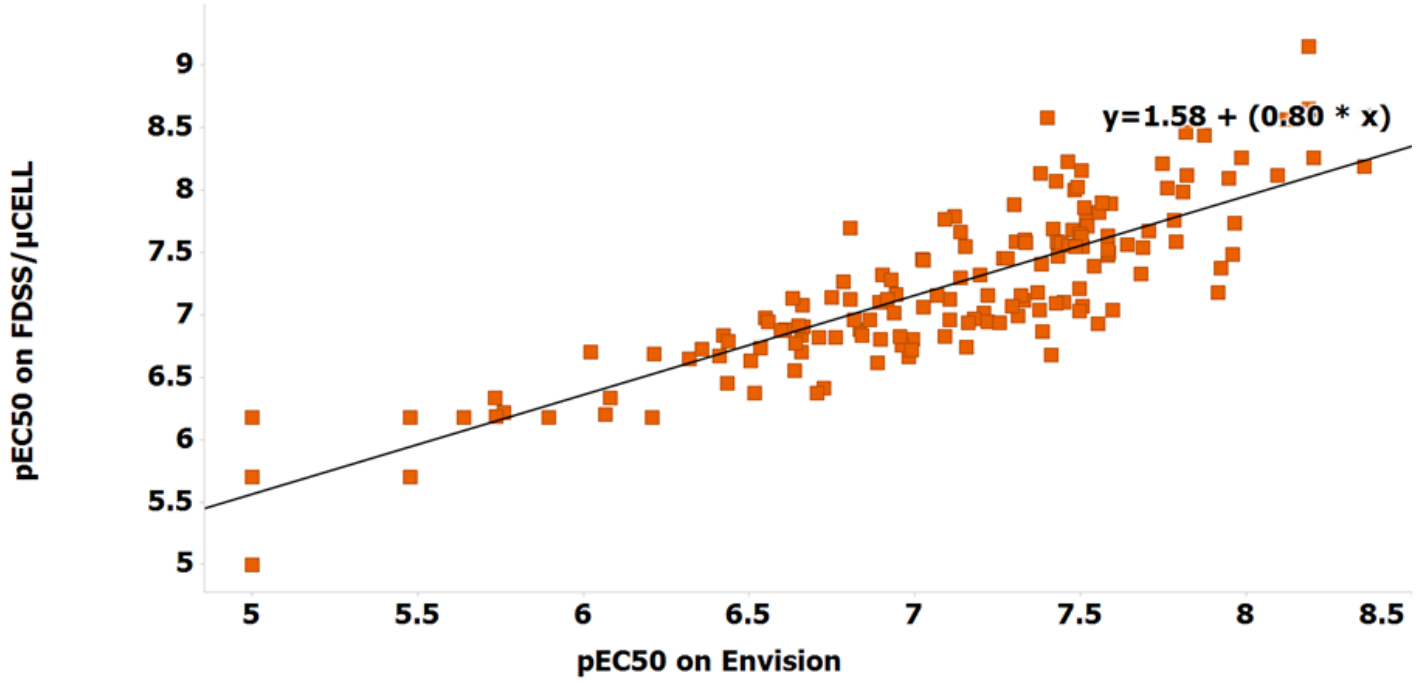
Comparison of compound activities



Good correlation between pEC_{50} values obtained on both platforms

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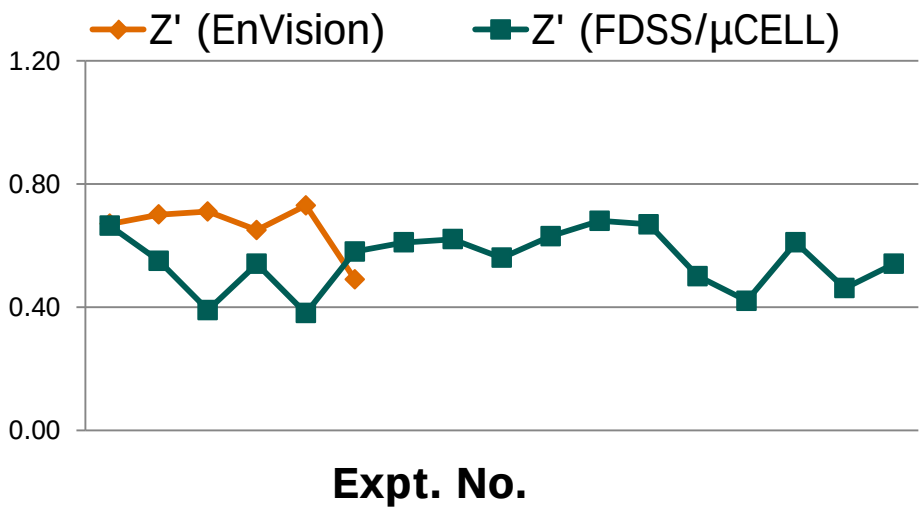
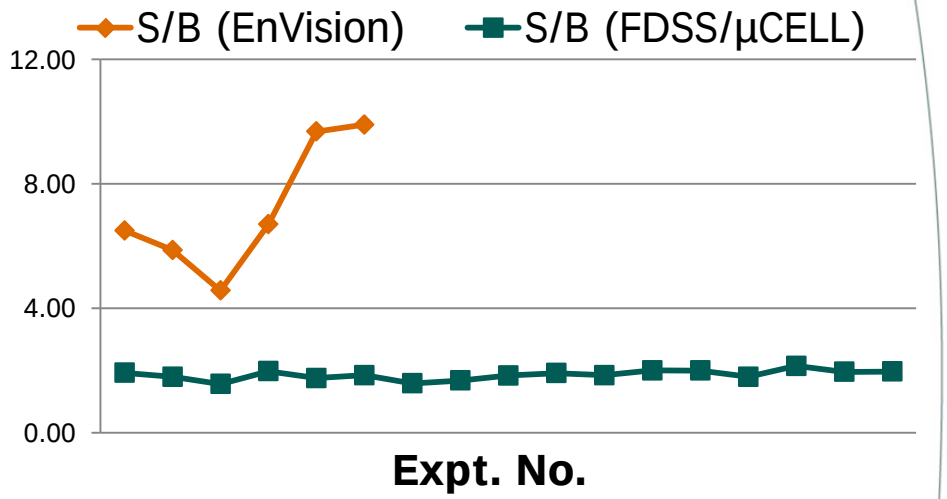
Comparison of QC parameters





- Z’ values comparable between platforms
- S/B considerably reduced on the FDSS/µCELL but more stable over runs

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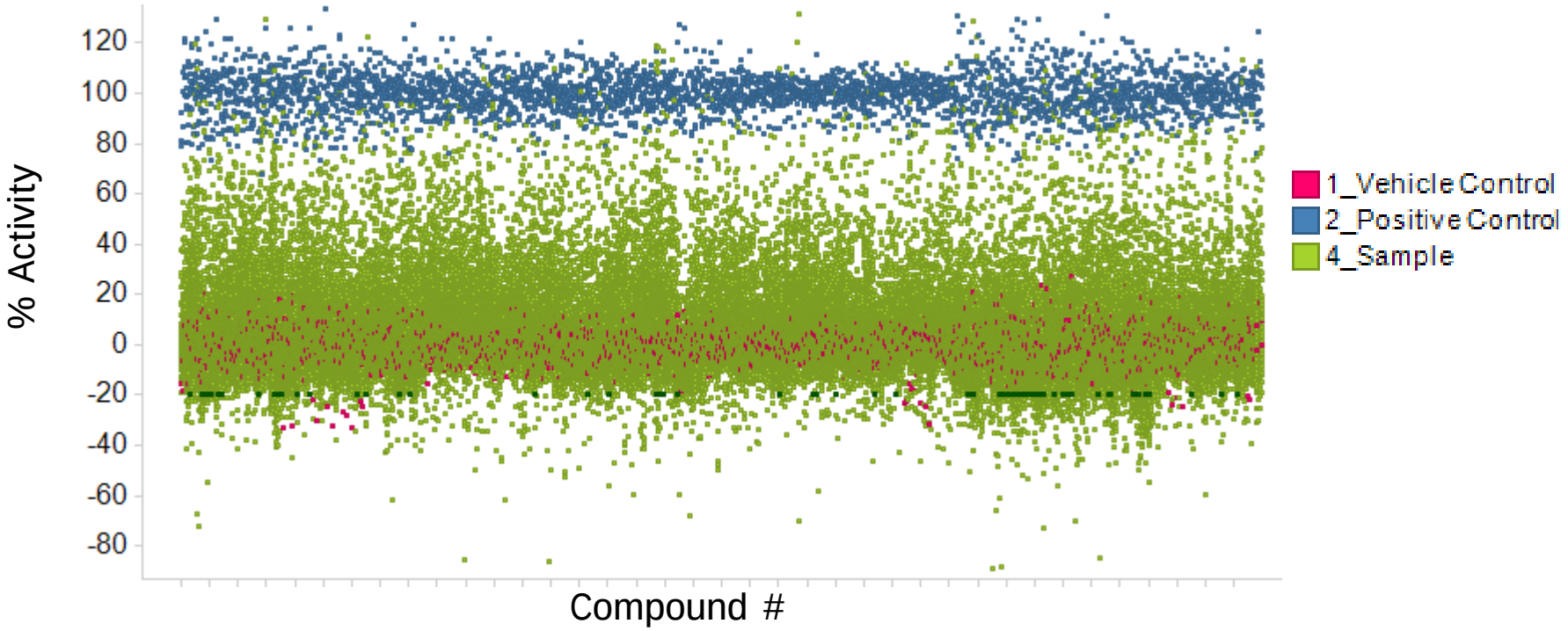
HTS to identify potentiators of F508del-CFTR

- Library
  - A diverse selection of 76,000 compounds was screened (40 plates/day, 6 runs)
- HTS QC criteria
  - Plates accepted if Z'>=0.35
  - Pharmacology QC by reference compound IC_{50}
    - +/- 3 fold the historical average

YHA potentiator HTS

Normalized data

HTS data normalized to positive control



- High hit rate observed
- Consistent data after normalization over the different runs
- Reproducible pharmacology for reference compounds

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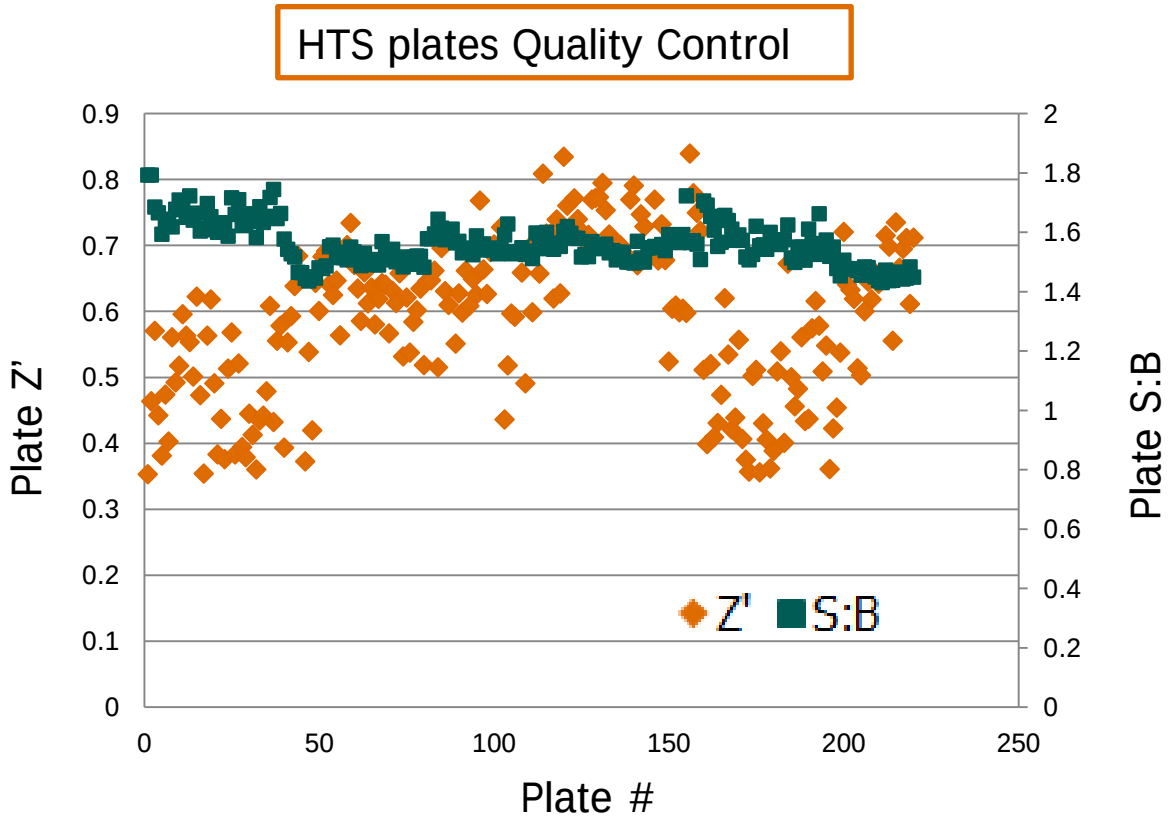
YHA potentiator HTS

QC results



- Good assay S:B and plate Z' for most plates screened
- Only 1 plate rejected for the whole screen

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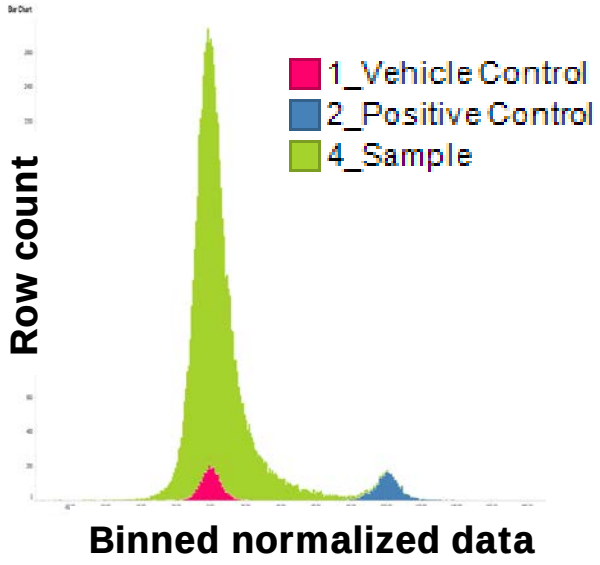
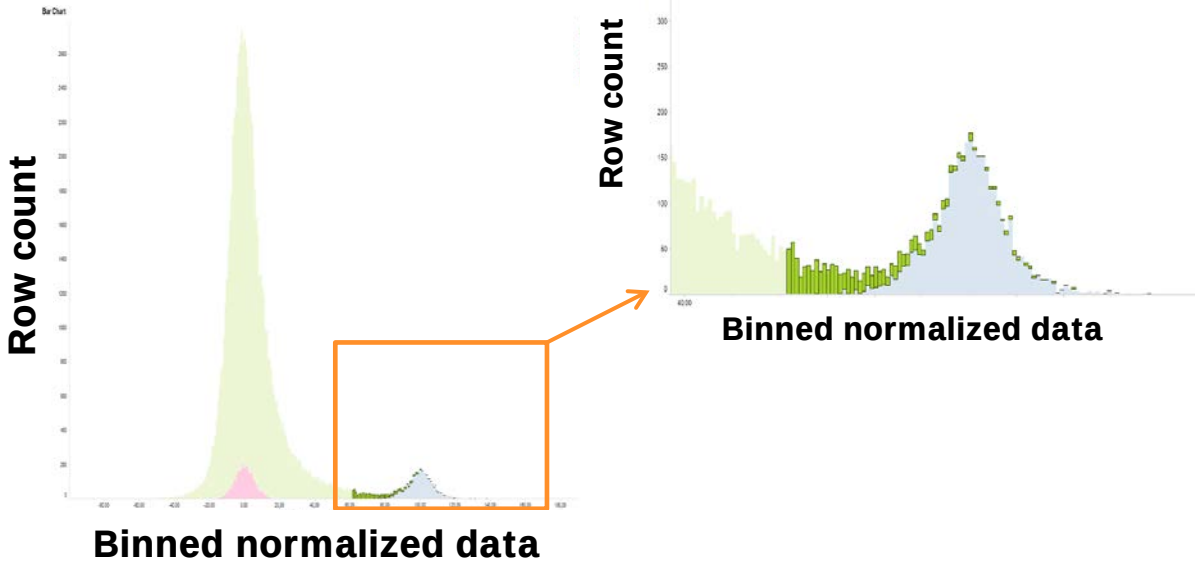
YHA potentiator HTS

Hit identification

% activity distribution for controls and samples



Hits selection at 62%



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

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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

Acknowledgments

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