# “Application of an optogenetic tool to study Cav1.3 blockers”

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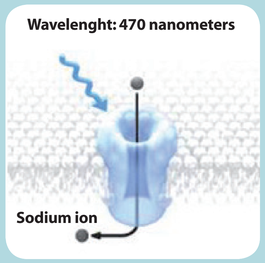
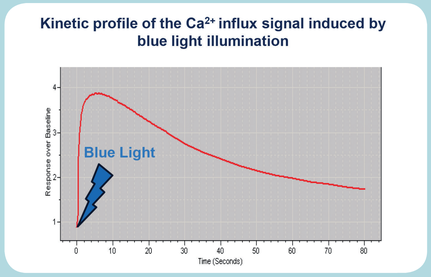
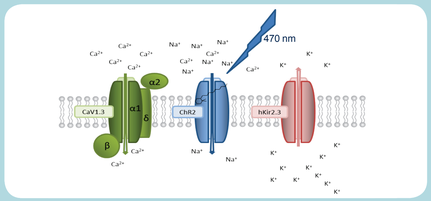
## Overview




• Channelrhodopsin-2 (ChR2) is a microbial non-specific cation channel that opens upon light stimulation and is successfully used to depolarize neurons by the use of blue light (470nm) (Fenno et al., *Annu. Rev. Neurosci.* 2011). In this regard the possibility to optically control the plasma membrane potential opens new exciting perspectives in the quest for new sate-dependent voltage-gated ion channels modulators.
• To verify the applicability of this tool for the study of voltage-gated channels, we generated a stable cell line which expresses both ChR2 and the human Cav1.3 (hCav1.3).
• A ChR2 variant, carrying a single amino acid mutation and requiring less light energy for activation compared to the wild type version, has been used. Thus, the ChR2 mutant results more suitable to be used on the FLIPR based technology.
• The generated cell line was validated by activating the Cav1.3 channel with a depolarizing stimulus either induced by light or high $K^+$ concentration. The influx of calcium ions through Cav1.3 was measured with a calcium sensitive fluorescent dye.
• The pharmacological profile of Cav1.3, in both closed and partial inactivated states, was confirmed using known blockers.
• To further validate the applicability of this technique on the FLIPR, the activity of hits identified in a screening campaign conducted using the potassium activation protocol in search of “state-dependent” Cav1.3 blockers, was confirmed on the optogenetics-based assay upon induction of membrane depolarization by light.
• FLIPR results were in good agreement with electrophysiology data obtained on the QPatch 16X data.

## Introduction




**Target:** human Cav1.3 ($\alpha1/\alpha2\delta1/\beta3$)
**Recipient cell line:** HEK-293 / Kir2.3 / ChR2
**Detection systems:** $Ca^{2+}$ sensitive FLUO-8 No Wash dye
**Stimulus:** depolarization induced by ChR2 activation with blue light pulses
**Blockers:** isradipine, nimodipine
**HTS instrumentation:** FLIPR$^{TETRA}$

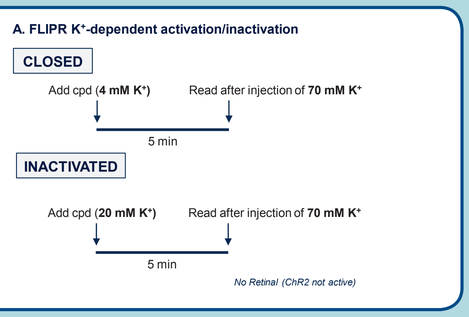
A stable HEK-293 cell line co-expressing:
- the $\alpha1/\alpha2\delta1/\beta3$ subunits of the hCav1.3
- the human inward rectifier Kir2.3 channel
- the channelrhodopsin-2 (ChR2)

was developed.

The Kir2.3 channel sets the plasma membrane potential close to the reversal potential of $K^+$ (hyperpolarized).
The channelrhodopsin-2 allows the modulation of the Cav1.3 activity due to the depolarization events induced by the use of blue light pulses.
Two protocols to study the effect of compounds on Cav1.3 in closed or inactivated states, were optimized using the blue light stimulus provided by the FLIPR.

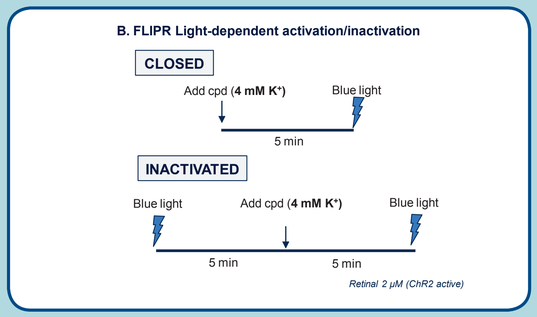
## Methods

**A. FLIPR - K+ protocol**
15000 c/w were seeded in medium and incubated O.N. at 37°C, 5% $CO_2$.
24 hrs after seeding, the culture medium was removed and cells were loaded with 20 µL/w of 0.5X Fluo-8 No Wash dye. The dye solution was prepared according to manufacturer’s instructions in assay buffer at the proper $K^+$ concentration. Plates were incubated for 1h at room temperature.
5 µL/w of test compounds 5X-concentrated were injected by the FLIPR$^{TETRA}$ and the kinetic response was monitored over a period of five minutes.
25 µL/w of high $K^+$ buffer (70 mM final in well and isotonic with $Na^+$) were injected by the FLIPR$^{TETRA}$ and the signal of the emitted fluorescence was recorded for two additional minutes.

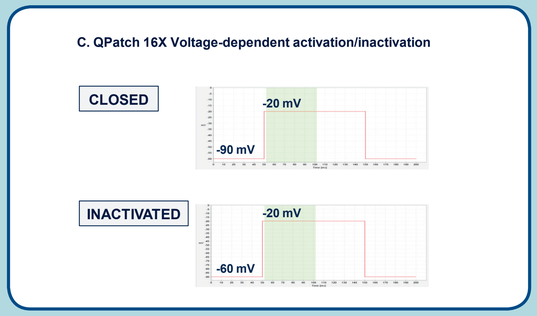



**B. FLIPR - Light protocol**
15000 c/w were seeded in medium containing all-trans retinal (2 µM) and incubated O.N. at 37°C, 5% $CO_2$.
24 hrs after seeding, the culture medium was removed and cell were loaded with 20 µL/w of 0.5X Fluo-8 No Wash dye.
The dye solution was prepared according to manufacturer’s instructions in assay buffer containing all-trans retinal (2 µM).
Plates were incubated for 1h at room temperature.

Both the target activation and inactivation were achieved by stimulation of ChR2 with the blue light emitted by the LEDs of the FLIPR$^{TETRA}$. To study the target in its partial inactivated status, cells were pre-exposed to a flash of blue light.



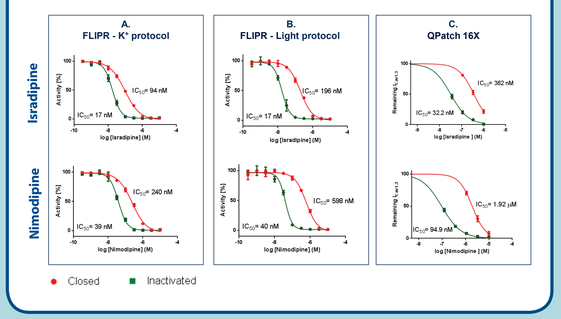

**C. QPatch 16X protocol**
Cells were held at -90 or -60mV to keep the Cav1.3 channels closed or partially inactivated, respectively. 100-ms depolarizing pulses up to -20mV were repeated every 20 seconds, in absence and in presence of the compound under investigation.




## Pharmacology of reference compounds

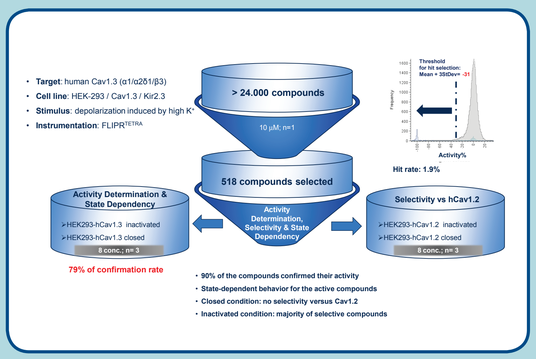
The pharmacology of two known Cav1.3 state-dependent bockers, isradipine and nimodipine was evaluated using two different protocols on the FLIPR and in parallel on the QPatch 16X.

**A: FLIPR – $K^+$ protocol.** The closed and the partial inactivated states of Cav1.3 were induced by incubating the cells in the presence of 4 or 20 mM $K^+$, respectively. The Cav1.3 channel activation was then achieved by injecting a high $K^+$ buffer (70 mM KCl).
**B: FLIPR – Light protocol.** In this set of experiments the target activation and inactivation were achieved through stimulation of ChR2 with the blue light emitted by the LEDs of the FLIPR$^{TETRA}$. For studying the target in its partial inactivated condition, cells were pre-exposed to blue light to induce cell membrane depolarization.
**C: QPatch 16X.** The closed and inactivated states were measured by clamping the cells at -90mV and -60mV, respectively.
Each point is the average± SEM.



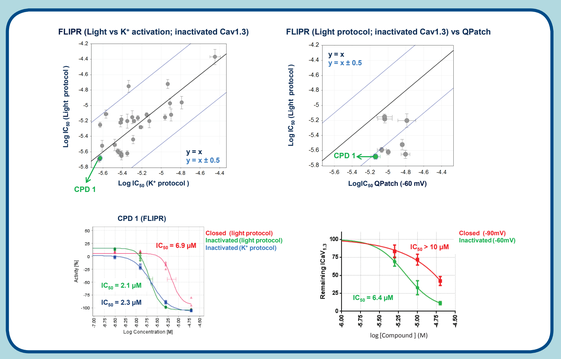

## Identification of Cav1.3 state-dependent blockers

A screening campaign was performed in search of state dependent blockers of hCav1.3 using a potassium-dependent activation protocol on the FLIPR.
The majority of the identified hits confirmed their activity in potency determination experiments.
The tested compounds showed a good state dependency as well as selectivity versus the Cav1.2 channel.




## Potency comparison: FLIPR (light/$K^+$) – QPatch 16x

The activity of the 30 most potent compounds has been compared using the two alternative FLIPR protocols. For all the compounds a good correlation was obtained in terms of potency, with deviations below 3 fold (LogIC50 within ± 0.5). Among these compounds, 13 were selected and also assayed by electrophysiology at the QPatch 16X. 8 compounds inhibited CaV1.3 by more than 50% when the membrane potential was clamped at -60mV (partial inactivated state).




## Conclusions

An optogenetics-based cellular assay has been developed to study the L-type voltage gated $Ca^{2+}$ channel Cav1.3.

The use of the light gated ion channel *Channelrhodopsin-2* allowed to achieve the optical control of the cell membrane potential. This tool has been used to induce cellular depolarization as stimulus for Cav1.3 activation. The calcium ion influx through Cav1.3 in either its closed or inactivated state, was monitored on the FLIPR upon cell loading with a $Ca^{2+}$-sensitive fluorescent dye.

Known state dependent blockers were tested against the closed and inactivated states of Cav1.3 with the two alternative activation protocols and in parallel on the QPatch 16X. The FLIPR results were in agreement with electrophysiology data.

The optogenetics tool was used to validate hits identified using a $K^+$ dependent activation of Cav1.3 on the FLIPR$^{TETRA}$.

The results obtained indicates a good reproducibility of the pharmacological profile of best hits among the different methodologies, suggesting that the optogenetics tool is suitable to study calcium channel pharmacology on the FLIPR.

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