iPSC-derived cells going high throughput:
new strategies for ion channels drug discovery

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**Rationale:**

- Early drug discovery, run in High Throughput Screening format, largely employs heterologous recombinant systems over-expressing a target of interest in a disease-unrelated host cell line.

- An ideal pharmacological profiling would rely on testing the drug candidate against human disease-relevant cells but this has intrinsic limitations.

- Human induced pluripotent stem cells (hiPSCs) represent a valid tool since they could be:
  - obtained from patients
  - *in vitro* cultured and expanded for long periods
  - genetically modified
  - differentiated into virtually any cell type

- Furthermore, merging of optogenetics and iPSC-based technologies provides informative and cost-effective drug screening tool to achieve fast control of specific cell events.
Project Goals:

- Pharmacological characterization of human iPSC-derived neurons for high throughput drug screening (HTS), employing instrumentations and technologies commonly and broadly used for running HTS on recombinant cell lines
- Development of iPSC-derived opto-neurons suitable for high-throughput screening (HTS)
- HTS-compatible platform based on the use of optogenetics for drug screening, using human iPSC-derived cardiomyocytes
Human iPSC-derived Neurons for High Throughput Drug Screening (HTS)
Human iPSC-derived Neurons for HTS

Workflow

1. Assay set up

Cell culture optimization:
- culture protocol
- coating
- cell density
- seeding time
- microtiter plate format

Assay optimization:
- readout
- reference compounds
- instruments

2. HTS assay optimization:
- adaptation from a semi-automated to a fully automated procedure

3. HTS and Hit Confirmation:
- screening of SM library
- data analysis
- hit selection
- hit confirmation
- activity determination
Human iPSC-derived Neurons for HTS: CNS.4U™

Morphological Analysis

DIV1

DIV7

DIV14

DIV20

384 MTP
Test the activity of different key players, with a pivotal role in the pathophysiology of neuronal activity (such as Glutamate Receptors and Voltage-gated Ca^{2+} Channels) in iPSC-derived neurons at FLIPR^\text{TETRA}.

- Physiological expression level of the target protein
- Signal amplitude, robustness and reproducibility
CNS.4U™ Neurons: Glutamate Receptors

Ca²⁺- and MP-Assay: Preliminary Results

**FLIPRTETRA test with a Ca²⁺-dye and a Membrane Potential (MP) dye at:**
- one cellular density (15,000 c/w)
- one coating type (PLO/Laminin)
CNS.4U™ Neurons: NMDA Receptors

Ca²⁺-Assay Preliminary Results

EC₅₀: 191 nM
EC₅₀: 6.4 µM
EC₅₀: 1.23 µM

IC₅₀: 482 nM
In presence of 31.6 µM NMDA

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The functional response of the channels was evaluated by applying increasing concentration of K⁺ to activate the channels.
K⁺ Activation and Inactivation Curve: Preliminary Results

- EC₅₀: 27 mM
- IC₅₀: 19 mM

Promising preliminary results indicating the possibility to use these cells in HTS

Could this process be improved?

Nicardipine

- Resting IC₅₀: 1.02 µM
- Half inactivated IC₅₀: < 100 nM

Optogenetics:
The Use of Light to set up a High-Throughput Screening Assay

Genetically encoded → Controlled by Light
Optogenetics basic concepts

“Sensors”
translate cell physiological signals into optical signals

“Actuators”
transduce optical signals into physiological signals

CALCIUM
VOLTAGE

Less toxic than organic dye

The possibility to have optogenetics sensors able to detect intracellular Ca\(^{2+}\) influxes and actuators such as a channelrhodopsin for a fine tuned light-mediated “electrical modulation” of cell events could allow to control and record Ca\(^{2+}\) variation in a spatiotemporal-specific manner.

Fast and reversible stimulation
Genetically Encoded Optogenetics Proteins: the Evolution

- **Chrimson**: a Red-Light Drivable Channelrhodopsin

  - Chrismson (or CnChR1), was identified from the species *Chlamydomonas nochtigama*, with a spectral peak at 590 nm, is 45 nm more red-shifted than any other previously known channelrhodopsin
  - Chrismson enables temporally precise experiments requiring red light, such as deep tissue targeting, or scenarios where blue light is visually distracting

- **GCaMP6f**: Ultrasensitive and fast protein calcium sensor, it consists of circularly permuted GFP, the calcium-binding protein calmodulin and its interacting M13 peptide
  - Calcium triggers conformational changes and increases EGFP brightness

_Dufour et al. Neurophotonics, 2015_
Development of iPSC-derived Opto-Neurons

Workflow for CRISPR/Cas9 Genome Editing

- *In silico* design of sgRNA and HDR donor
- Sequence analysis of genomic target region

Construct Generation:
- CRISPR sgRNAs/Cas9
- HDR donor

Transfection of hiPSCs

Recovery, enrichment and plating at low density

Selection of clones

Screening of clones and characterization

KM, CK
### EuroTransBio: 11th Transnational Call

<table>
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<tr>
<th>Grant</th>
<th>Title</th>
<th>Partner</th>
<th>Axxam contribution in iPSC</th>
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</thead>
</table>
| NeurOptics | Development of iPSCs derived Opto-neurons suitable for high-throughput screening (HTS) | • Ncardia | • Generation of Opto-iPSC  
• Validation of Opto-Neurons for HTS |

![Diagram showing the process of iPSC development and screening with GCaMP6f](image.png)
HTS-compatible Platform-based on the Use of Optogenetics for Drug Screening:

iPSC-derived Cardiomyocytes
Voltage-gated Ion Channel Light-pacing by “OPTO-FLIPR”

Standard FLIPR\textsuperscript{TETRA} + Custom-made modification = «OPTO – FLIPR »

- 2 exc/em
- Alternated sampling

- 2 exc/em
- Flexible temporal pattern

Robust and specific \( \text{Na}_V^{1.x} \) pacing by fast kinetics ChR2

Syncytium assays

Functional response of «Target A» (which includes changes in ion or small molecule concentrations) can be transferred to «Cell line B» through Gap Junctions (Connexin proteins) and can modulate the activity of «Target B», or can be monitored by Genetically encoded indicators expressed in «Cell line B»

- ChR2 exc
- Dye imaging
- No blocker
- Amitriptyline 30\( \mu \text{M} \)

% \( \Delta F \) (membr. potential DYE)

- HEK-293
- MP dye

Target A

Stimulus A

Target B

CX-43

Cell line A

Ions / small molecules

Cell line B

unpublished"
Voltage-gated Ion Channel Light-pacing by “OPTO-FLIPR”

Standard FLIPR^{TETRA}

- 2 exc/em
- Alternated sampling

+ Custom-made modification = «OPTO – FLIPR »

- 2 exc/em
- Flexible temporal pattern

Robust and specific $Na_{v}1.x$ pacing by fast kinetics ChR2

![Graph showing % ΔF (membrane potential, DYE) with and without ChR2 excitation and Dye imaging.]

Use-dependent blockers’ validated with $Ca_{v}1.x$ pacing by fast kinetics ChR2 (syncytium assay)

![Graph showing % ΔF (Ca^{2+}, DYE) with and without use-dependent blockers.]

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“OPTO-cardiac Safety” with Light-paced iPSC-derived Cardiomyocytes

**Standard FLIPR**

- **Cor.4U®**
  - Calcium dye
  - GOOD spontaneous activity

**Cor.4U®**

**Isoproterenol 1µM** (44 bpm; +50% ΔF)

**No synchronization**

(1 well replicate)

**Perfect synchronization & Light-pacing up to 1.5 Hz**

- **<«OPTO-FLIPR»>**
  - **Cor.4U®** + HEK/ChR2<sub>fast</sub>
    - (syncytium assay)
  - Calcium dye

**0.5 Hz**

- Frequency / use-dependent blocker

**1 Hz**

**1.5 Hz**

- No blocker

(4 wells replicate)

(unpublished)
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<th>Axxam contribution in iPSC</th>
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</table>
| OPTEL | A novel optogenetic electrophysiology platform for ion channel and transporter screening | • Nanion Technologies GmbH  
• University of Bonn | Optogenetic pacing of Cor.4U cardiomyocytes: gene transfer of ChR2 using adeno-associated viruses or co-culture with ChR2-expressing HEK293 cells |

End: Nov 2017
Concluding Remarks

Opto-Neurons in HTS

- The functional presence of **glutamate receptors** was shown using both a Ca\(^{2+}\) and membrane potential dye.

- Among the different glutamate receptor it was demonstrated the presence of **NMDAR**, using specific agonists and antagonist.

- Applying different concentration of K\(^{+}\) it was possible to measure the presence of **voltage-gated Ca\(^{2+}\) channels** and in particular of the L-type Ca\(^{2+}\) channels by testing specific state dependent blocker.

- It is ongoing the generation of an opto-neurons system, for a fast and less toxic Ca\(^{2+}\) measurement and for setting up fine tuned light-mediated **use/state-dependency FLIPR\(^{TETRA}\) assays**

Light-paced iPSC-derived Cardiomyocytes

- **Syncytium assays** validated for optogenetic control of recombinant / native ion channel function.

- **ChR2 light-pacing** of:
  - Recombinant voltage-gated Na\(^{+}\) or Ca\(^{2+}\) channels
  - iPSC-derived cardiomyocytes beating up to 1.5 Hz

- **Frequency / use-dependency** studies enabled.
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