Optimised and scalable programming of human iPSCs to generate nociceptor sensory neurons for the study of pain mechanisms and neuropathies

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Abstract

Nociceptive sensory neurons are a specialised subtype of somatosensory cells residing in the dorsal root ganglia. Nociceptors respond to diverse noxious and pruritic stimuli, and hence are critical for the study of pain mechanisms and neuropathies[1]. Around 30% of adults suffer from chronic pain, but the current analgesics are limited by short duration and adverse events[2]. Unfortunately, the efficacy of analgesics in animal pain are poorly translated to humans as clinical trials for pain therapeutics have only a 2% probability of success. Consequently, drug classes used to treat chronic pain have essentially not evolved over the past 40 years[3].

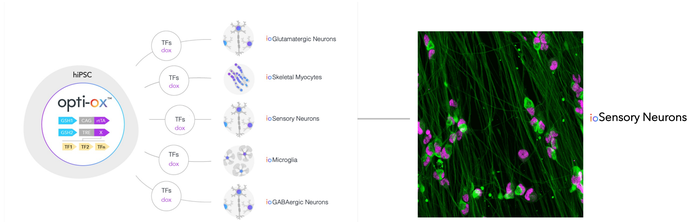
Thus, there is an unmet need for reliable and scalable human in vitro models to develop new, efficacious, and safe pain therapeutics. However, conventional differentiation methods to generate nociceptors from pluripotent stem cells are complex, inconsistent, and characterised by protracted maturation times.

By using our deterministic cell programming technology (opti-ox™)[4], we robustly expressed a combination of transcription factors in iPSCs to generate a homogeneous population of sensory neurons that display critical features of nociceptors. Bulk and single cell RNA-sequencing analysis together with immunocytochemistry showed that within 7 days after the induction of transcription factor expression, the neurons expressed the key sensory markers ISL1, POU4F1 and PRPH. At this early time point, the neurons also expressed the key nociceptor markers such as NTRK1, TRPV1, TRPM8, and SCN9A.

Multi-electrode array and calcium assays demonstrated that these sensory neurons are functional displaying asynchronous spontaneous activity and responsiveness to diverse noxious stimuli. Neurotrophic factors play a critical role in sensory neuron subtype specification and, by adapting culture conditions, we were able to enrich for cells expressing key peptidergic nociceptor markers TAC1 as well as ADCYAP1, and substantially increase the responsiveness to specific noxious stimuli. In addition, using an optimised cocktail of neurotrophic factors, and increased culture length, enhances the percentage of cells responding to TRPM3 and TRPM8 channel agonists in calcium mobilisation assay.

In conclusion, with opti-ox deterministic cell programming, iPSCs are rapidly converted into functional sensory neurons offering a robust and scalable source of human nociceptors that can be used as an in vitro model to study the biology of pain and to develop novel therapies for neuropathies.

1. Precise opti-ox cellular programming of hiPSCs into ioSensory Neurons™

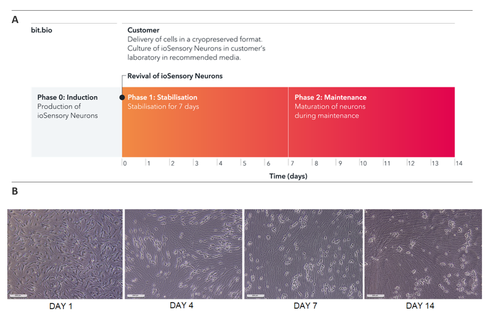


opti-ox technology for the optimal cellular programming of human iPSCs into defined human cell types, including ioSensory Neurons. opti-ox dual cassette Tet-ON system ensures tightly controlled and homogeneous expression of programming transcription factors (TFs) by preventing silencing of the inducible expression cassette after genetic engineering of hiPSCs .

2. Human ioSensory Neurons are ready to use by day 7

A) ioSensory Neurons are delivered in a cryopreserved format and are programmed to rapidly mature upon revival. Cells are revived and cultured in a single medium, with fully disclosed composition allowing modifications to fit customers' bespoke experiments. The protocol for the generation of these cells is a two-phase process: Induction, which is carried out at bit.bio (Phase 0), Stabilisation for 7 days (Phase 1), and Maintenance (Phase 2) during which the ioSensory Neurons mature. Phases 1 and 2 after revival of cells are carried out at the customer's site.

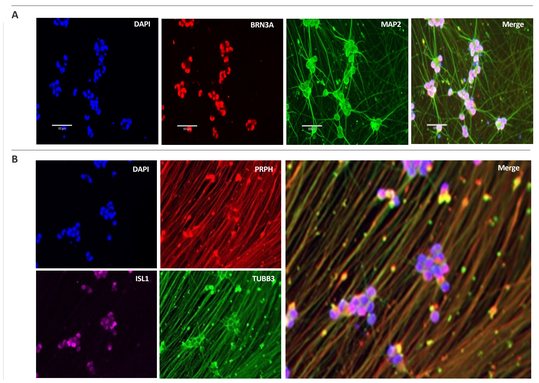
B) Upon programming, ioSensory Neurons show rapid morphological changes with neurons being identified by day 4 and forming visible neuronal networks by day 7. Day 1 to 14 post revival; 10X magnification; scale bar: 200 µM.



3. ioSensory Neurons homogeneously express key pan-sensory markers

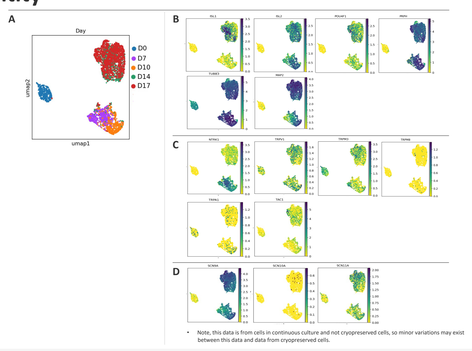
A) Immunofluorescent staining on day 14 post-thaw, demonstrates that programmed ioSensory Neurons are all positive for BRN3A (red), MAP2 (green), and DAPI counterstain (blue). MAP2 positive neurons colocalize with the sensory marker BRN3A suggesting a high purity of neurons with a sensory identity. 10X magnification, scale bar: 60µm.

B) Immunofluorescent staining on day 14 post-thaw, demonstrates that programmed ioSensory Neurons are all positive for ISL1 (magenta), PRPH (red), TUBB3 (green), and DAPI counterstain (blue). TUBB3 positive neurons co-localize with the sensory markers ISL1 and PRPH indicating that neurons have a sensory identity. 10X magnification.



4. ioSensory Neurons form a pure population (>99%) of sensory neurons with a defined nociceptor identity

A) Single cell RNA-sequencing analysis was performed with ioSensory Neurons at five specific timepoints (day 0, 7, 10, 14 and 17). By day 7, the population has a distinct expression profile indicating a pure population (>99%) of post-mitotic sensory neurons. Gene expression was assessed by 10x Genomics single cell RNA-sequencing. **B)** By day 7, the expression of key sensory marker genes (ISL1, ISL2, POU4F1/BRN3A, and PRPH), and the pan-neuronal markers TUBB3 and MAP2, could be detected. **C)** Within 7 days, the expression of key nociceptor marker genes (NTRK1, TRPM3, TRPM8, TRPV1, and TRPA1) is detected in a high proportion of ioSensory Neurons. By day 10 expression of neuropeptide genes such as TAC1 are also detected, indicating a subset of cells with a peptidergic nociceptor identity. **D)** Within 14 days, expression of key sodium ion channels (SCN9A/Na_V1.7, SCN10A/Na_V1.8 and SCN11A/NaV1.9) is also detected further corroborating that ioSensory Neurons display a nociceptor identity.



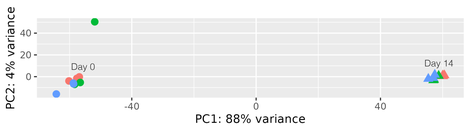
Note, this data is from cells in continuous culture and not cryopreserved cells, so minor variations may exist between this data and data from cryopreserved cells.

5. ioSensory Neurons show high lot-to-lot consistency

Bulk RNA sequencing analysis was performed on three independent lots of ioSensory Neurons at different time points throughout the programming protocol. Principal component analysis shows high consistency between each lot of ioSensory Neurons at each given timepoint.

Differential gene expression analysis shows only 37 or less differentially expressed genes between lots, less than 1% of the total 25,000 genes within a human cell, at day 14 post-thaw.

Pure populations of ioSensory Neurons with equivalent expression profiles can be generated consistently from every vial, allowing confidence in experimental reproducibility.



Differential Gene Expression at day 14	
Lot 2 vs Lot 3	9
Lot 1 vs Lot 2	10
Lot 1 vs Lot 3	37

Negligible number of differentially expressed genes between batches

llogFCl >0.5 and FDR < 0.01

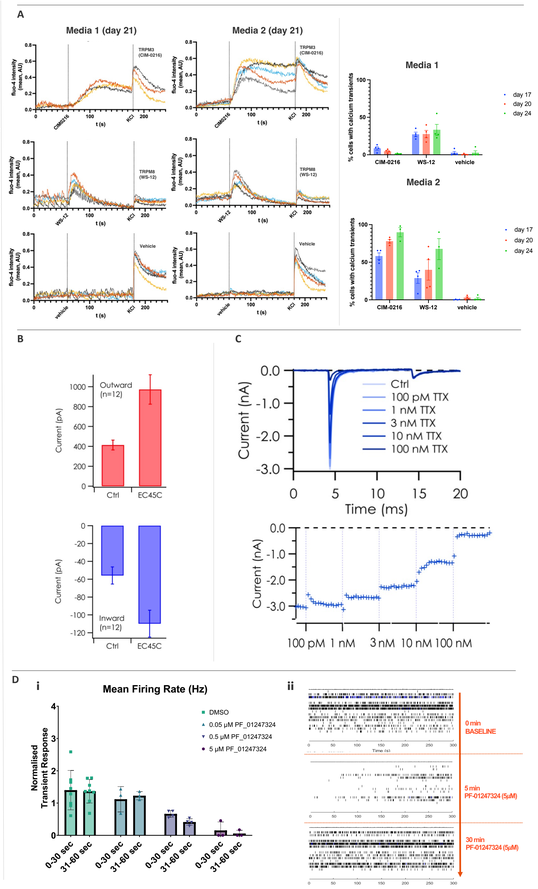
6. ioSensory Neurons display a functional nociceptor phenotype

A) Calcium mobilisation imaging, performed with ioSensory Neurons cultured, under two different media conditions (1 and 2), up to day 14, 17 or 21 post-thaw. ioSensory Neurons respond to pharmacological agonists targeting key thermosensitive TRP channels such as TRPM3 (CIM-0216) and TRPM8 (WS-12). The left panel shows active traces of increased intracellular calcium mobilisation of individual cells, at day 21 post-thaw, upon exposure to noxious agonists but not to vehicle, indicating that cells display features of functional nociceptors. The right panel shows the percentage of responding cells at day 14, 17, or 21 post-thaw - Media condition 2 and increased culture length appears to greatly increase the percentage of cells responding to TRPM3 and TRPM8 agonists.

B) Medium-throughput automated patch clamp analysis (Patchliner, Nanion Technologies) of Day 21 ioSensory Neurons showing a 2-fold increase in outward (red) and inward (blue) currents in response to culture media heated to 45°C, indicating heat responsiveness.

C) Patchliner (Nanion Technologies) of Day 21 ioSensory Neurons showing block of Na_V channels by TTX. The IC50 of TTX was approximately 7.5 nM (n = 5) showing the presence of TTX-sensitive Na_V currents. The highest concentration of 100 nM blocked the majority of the current but some functional TTX-resistant sodium current remained (Na_V1.5, Na_V1.8 or Na_V1.9). B and C data provided in partnership with Nanion.

D) Multi-Electrode Array (MEA) data analysis of Day 20 ioSensory Neurons treated with the selective Na_V1.8 antagonist PF-01247324. (i) Acute (within 1 min) dose-dependent reduction in mean firing rate following PF-01247324 treatment. (ii) Spike activity before treatment (top), 5 minutes after application (middle), and after 30 minutes (bottom). Most electrodes show reduced activity over 5 min recording time post-treatment, indicating selective Na_V1.8 inhibition. Electrical activity returns to comparable level at baseline after 30 minutes post-treatment, confirming that the cells retain viability and functionality.



Summary & conclusions

ioSensory Neurons show **>99% purity** for the expression of key sensory neuron markers including PRPH, POU4F1 (BRN3A), ISL1, and TUBB3 as characterized by single cell RNA sequencing and ICC.

ioSensory Neurons have a defined **nociceptor identity** as shown by the expression of key nociceptor marker genes, NTRK1 and TRP ion channels, including TRPV1.

These cells display spontaneous activity by MEA (data not shown) and display a **functional nociceptor phenotype**, as demonstrated by responsiveness to heat and selective agonists for TRPM3, and TRPM8.

ioSensory neurons demonstrate expression of the **functional TTX-resistant sodium channels**, characteristic of nociceptors, and respond to Na_V1.8 blockers.

ioSensory Neurons **mature rapidly** and are easy to culture using a simple 1-medium, 2-step mitomycin C-free protocol – ideal for researchers without iPSC expertise.

ioSensory neurons show **batch-to-batch reproducibility** and homogeneity, as shown by bulk RNA-sequencing.

References:
1. Haberberger, *et al.* Front Cell Neurosci. 2019;13:271.
2. Goldberg, *et al.* BMC Public Health. 2011 Oct 6;11(1):770.
3. Woodcock, et al. Nat Rev Drug Discov 2007 6: 703–710
4. Pawlowski, *et al.* Stem Cell Reports. 2017 Apr 11;8(4):803-812.