Optimised and scalable reprogramming 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 are able to respond to diverse noxious and pruritic stimuli, and hence are critical for the study of pain mechanisms and neuropathies. It is estimated that 20% of adults suffer from chronic pain, but the current analgesics are limited by short duration, inadequate efficacy, and/or poorly tolerated adverse events. The discovery of novel drugs have been hampered as the efficacy in animal models of pain cannot be reproduced in the clinic. Consequently, drug classes used to treat chronic pain have essentially not evolved over the past 40 years. Thus, there is an unmet need for reliable and scalable human in vitro models to study the molecular mechanisms underlying nociception and develop new, efficacious, and safe pain therapeutics. However, conventional differentiation methods to generate nociceptors from pluripotent cells are complex, inconsistent, and characterised by protracted maturation times.

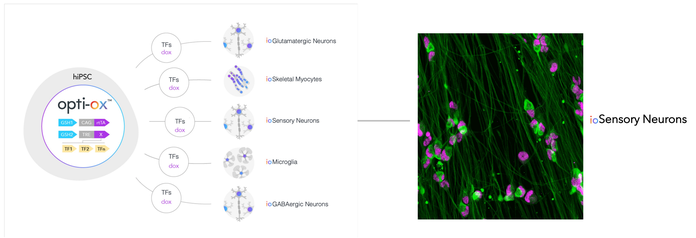
Through our proprietary deterministic cell programming technology (opti-ox™), which enables a robust and controlled expression of transcription factors (TFs), we aimed to generate a rapid and scalable cell culture system for the consistent production of physiologically relevant and functional nociceptor sensory neurons from human iPSCs.

opti-ox engineered iPSC lines expressing a combination of key TFs, consistently and efficiently programme within a week into a homogeneous population of sensory neurons that display critical features of mature nociceptors.

Morphological, transcriptomic and phenotypic characterisation demonstrated that programmed iPSCs acquired a sensory nociceptor identity. Within 7 days, the sensory neurons expressed the key pan-sensory sensory neuron markers ISL1, POU4F1 and PRPH, as well as key nociceptor markers such as NTRK1, TRPV1, TRPM8, and SCN9A. Neurotrophic factors play a critical role in the subtype specification of sensory neurons and by optimising the culture conditions we were able to further enrich for cells expressing key sensory genes including peptidergic nociceptor markers TAC1, and ADCYAP1. Multi-Electrode Array and calcium assays demonstrated that programmed sensory neurons are functional as displaying asynchronous spontaneous activity and responsiveness to diverse noxious stimuli.

In conclusion, with opti-ox deterministic programming, iPSCs are rapidly converted into functional sensory neurons (termed ioSensory 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. Deterministic opti-ox cellular programming of hiPSCs into ioSensory Neurons

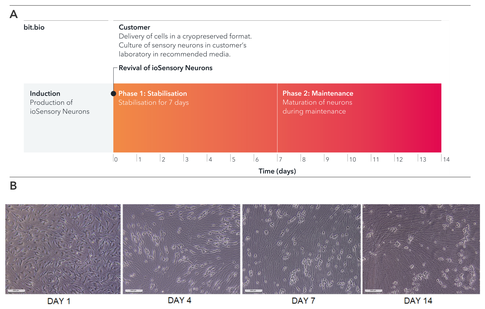


opti-ox technology for the optimal deterministic programming of human iPSCs into defined human cell types, including ioSensory Neurons. opti-ox dual cassette Tet-ON system ensures tightly controlled and homogeneous expression of reprogramming transcription factors (TFs) by preventing silencing of the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

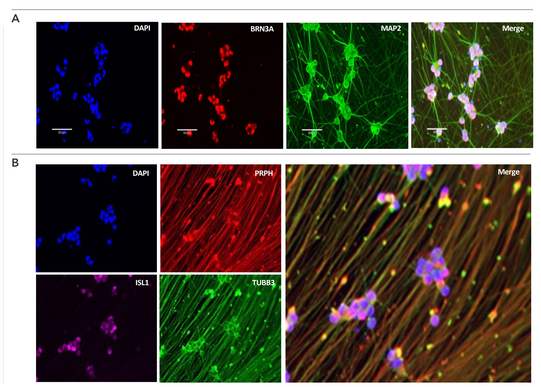
B) Upon reprogramming, ioSensory Neurons show rapid morphological changes with neurons being identified by day 4 and forming visible neuronal networks by day 7. Day 1 to 14 post revival; 10X magnification; scale bar: 200 µM.



3. ioSensory Neurons homogeneously express key pan-sensory markers

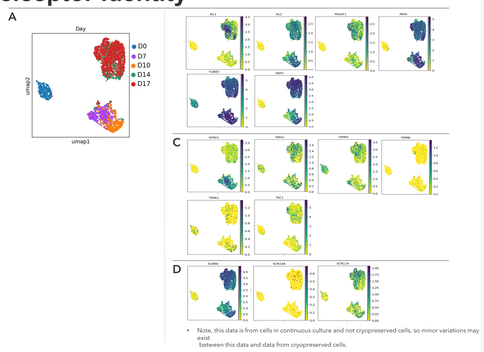
A) Immunofluorescent staining on day 14 post-thaw, demonstrates that reprogrammed ioSensory Neurons are all positive for BRN3A (red), MAP2 (green), and DAPI counterstain (blue). MAP2 positive neurons colocalize with the sensory marker BRN3A suggesting a high purity of neurons with a sensory identity.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

B) Immunofluorescent staining on day 14 post-thaw, demonstrates that reprogrammed 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 7 days, expression of key sodium ion channels (SCN9A/Nav1.7, SCN10A/Nav1.8 and SCN11A/Nav1.9) is also detected further corroborating that ioSensory Neurons display a nociceptor identity.



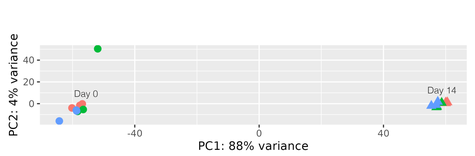
- Note, this data is from cells in continuous culture and not cryopreserved cells, so minor variations may exist between this data and data from cryopreserved cells.

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 reprogramming 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

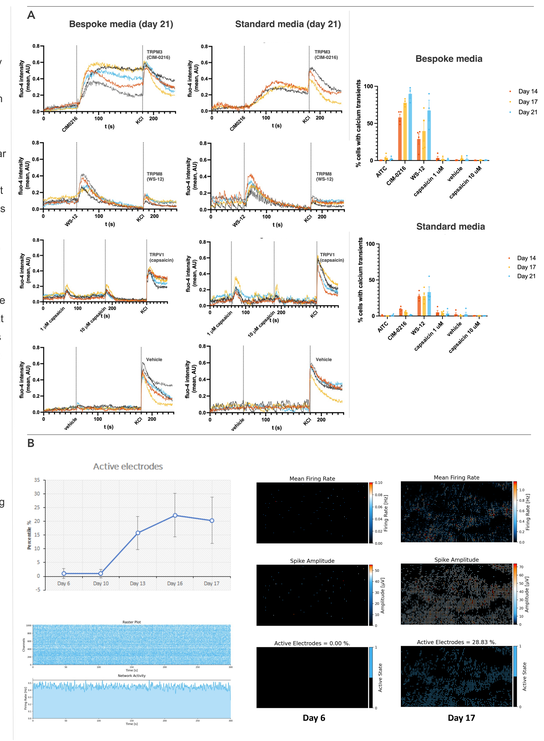
Negligible number of differentially expressed genes between batches

IlogFCI >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 (bespoke and standard), up to day 14, 17 or 21 post-thaw, shows that ioSensory Neurons respond to pharmacological agonists targeting key thermosensitive TRP channels such as TRPV1 (capsaicin), TRPM3 (CIM-0216) and TRPM8 (WS-12). The left panel shows active traces which represent the increase in 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 – the bespoke media and increased culture length appears to greatly enhance the percentage of cells responding to the TRPM3 and TRPM8 agonists, to 90% and 67% at day 21 post-thaw, respectively. Please contact us at technical@bit.bio for further information on the bespoke media.

B) Multi-Electrode Array (MEA) analysis of ioSensory Neurons over a period of 17 days. ioSensory neurons display increased spontaneous activity over time, with neurons firing as early as day 13 of reprogramming. Note, this data is from cells in continuous culture, so minor variations may exist between this data and data from cryopreserved cells.



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 as shown by MEA and display a functional nociceptor phenotype, as demonstrated by responsiveness to selective agonists for TRPV1, TRPM3, and TRPM8.

Cells 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.

Rapidly maturing sensory neurons that are ready to use by 7 days, post-revival.

References:
1. Haberberger, *et al.* Front Cell Neurosci. 2019;13:271.
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4. Pawlowski, *et al.* Stem Cell Reports. 2017 Apr 11;8(4):803–812.