Optimizing Calcium Flux Assays for Investigating Pain Response with Human iPSC-derived Sensory Neurons

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Poster # C136
Abstract # 9177

NEUROSCIENCE 2024

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Abstract

Chronic pain and peripheral neuropathy are pervasive clinical conditions that affect 10-20% of adults globally, resulting in a tremendous impact on individual well-being and financial burdens for health care systems. Discovery of next-generation compounds and therapeutic targets for effective non-opioid pain management can benefit from the use of human-relevant cellular models during preclinical phases of drug discovery pipelines. Advances in human induced pluripotent stem cell (iPSC) technology and protocols for differentiating them into peripheral sensory neurons has enabled the scalable production and increased accessibility of in vitro human models of pain and neuropathy. As iPSC models become required for drug discovery programs, it is critical to optimize and standardize the use of iPSC sensory neurons in pain-relevant assays, which will ultimately allow for more efficient drug discovery and cross-platform data interpretation. In this study, we employ commercially available iPSC-derived sensory neurons, from male and female donors, within numerous pain and chemotherapy-induced peripheral neuropathy (CIPN) assays, including classic in vitro characterization assays (calcium assays) and advanced assays, such as organ-on-a-chip platforms. We have previously shown that these iPSC-derived sensory neurons have high purity (>80% BRN3A+/UCHL1+) and express hallmark nociceptive channels (i.e., NaV1.7 and NaV1.8) and receptors (i.e., TRPV1 and P2RX).

To evaluate the response of these neurons to sensory receptor specific agonists, we established a high-throughput calcium assay using the Hamamatsu FDSS/µCell. We demonstrated that iPSC-derived sensory neurons cultured for ≥21 days show consistent responses to capsaicin (TRPV1 agonists) across technical replicates and across manufactured lots of sensory neurons, establishing these cells as robust and reproducible. To improve assay efficiency, we also investigated critical parameters that could affect the calcium assay consistency, including cell density, extracellular matrix, assay media, day of assay, and calcium indicators. Last, we investigated the use of iPSC-derived sensory neurons in organ-on-a-chip platforms, which offer the ability to compartmentalize conditions and establish co-cultures, including sensory neurons and macrophages to model neuroinflammatory pain. These data further establish the utility of iPSC-derived sensory neurons for high-throughput drug screening and provide methods for pain and CIPN assay standardization.

Keywords: Pain models, iPSC, and Ca^{2+} signaling

Materials Methods

FUJIFILM CDI recently released a new **iCell® Lab Note** featuring specific details on the Calcium Flux Assay with iCell Sensory Neurons. Highlights to the protocol are listed below:

- iCell Sensory Neurons Base Medium and Supplement are provided with the complete kit
- Day 0: Coat culture plate with PDL solution (50 µg/mL for 1 h at 37ºC), wash 3X with sterile water, and then coat with Laminin-511 (2.5 µg/mL for 1 h at 37ºC).
- Use lot-specific CoA information to determine the viable # of cells/vial, then thaw and plate cells
  - Recommended cell density is 14-19K per well in 384w plates; 45-60K per well in 96w plates.
- Day 1: Perform a complete 100% media change.
- Day 4: Change 50% media 2X per week until day of assay.
- Day 21 or later: Prepare assay buffer with 1X HBSS (-/-), 2 mM $CaCl_2$ and 20 mM HEPES.
- Remove culture media and replace with Calcium 6 dye (in assay buffer); incubate ≥2 h at 37ºC.
- Prepare dose-response of capsaicin (or another agonist)
- Pre-treat cells with antagonists for 30-60 min, if desired.
- Proceed with calcium flux assay on FDSS/µCell or FLIPR Penta.
- Analyze data for Max Ratio signal for each [compound].

FDSS/µCell

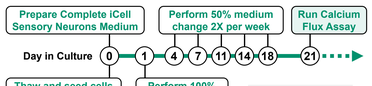
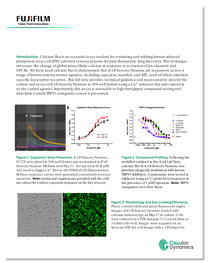


Table 1. Materials Needed.

Product	Vendor	Cat. #
iCell Sensory Neurons Kit, 01279 [1]	FCDI	R1250
• iCell Sensory Neurons Base Medium	(incl. in kit)	M1052
• iCell Sensory Neurons Supplement (100X)	(incl. in kit)	M1053
Poly-D-Lysine (PDL) Solution [2]	GIBCO	A3890401
Recombinant Laminin iMatrix-511 Silk [3]	AMSBIO	AMS.892021
384-well Cell Culture Plate [3]	Greiner	781091
FLIPR Calcium 6 Assay Kit [3]	Molecular Devices	R8190
Capsaicin [3]	Sigma	PHR1450

[1] iCell Sensory Neurons from female Donor 21527 (R1252) are also available.
[2] Manual coating of cell culture plate with PDL solution is recommended over the use of PDL pre-coated plates for this application.
[3] Alternative materials, plates, calcium dyes, and agonists from other sources may be compatible in this assay but have not been fully tested.



Characterization of Human iPSC-derived Sensory Neurons

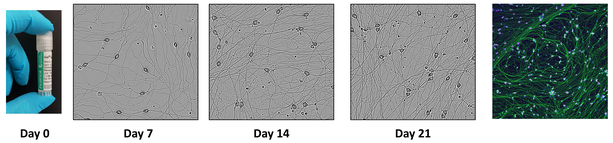


Figure 1. Neuronal Morphology. iCell Sensory Neurons were plated onto Geltrex-coated 6-well plate (1x10^6 cells/well). Sensory neurons mature with a bright cell body, extensive neurite networks, and pseudo-unipolar morphology.

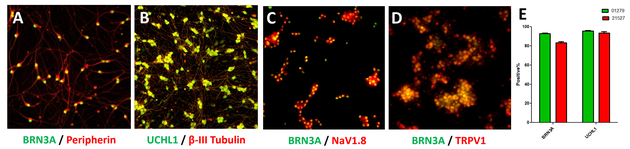


Figure 2. Neuronal Purity by ICC. iCell Sensory Neurons express pan-neuronal and sensory neuron markers. Staining for (A) BRN3A/peripherin and (B) UCHL1 β-III Tubulin at day 7. Sensory neurons also express mature sensory neuron markers (C) NaV1.8 and (D) TRPV1. (E) Quantification by flow cytometry of % positive neurons across five (5) lots for each donor – 01279 and 21527.

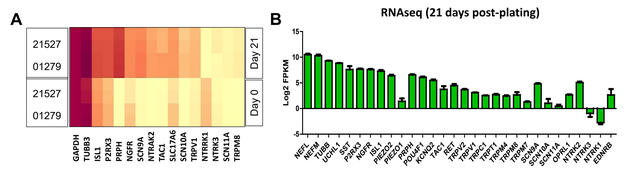


Figure 3. Neuronal Gene Expression. (A) mRNA from both donor of iCell Sensory Neurons (01279 and 21527) was collected at day 0 and day 21. qPCR shows consistent expression of pan-neuronal genes (TUBB3) and sensory neuron-specific genes (P2RX3, TRPV1, NaV1.7) across donors and increased expression at day 21. (B) RNAseq data (Log2FPKM) collected at day 21 corroborate qPCR data and confirm expression of additional neural markers (NEFL, NEFM) and the presence of other sensorineural TRP channels, Na channels (SCN), and mechanoreceptor receptors (PIEZO1/2).

Calcium Flux Assay with iCell Sensory Neurons

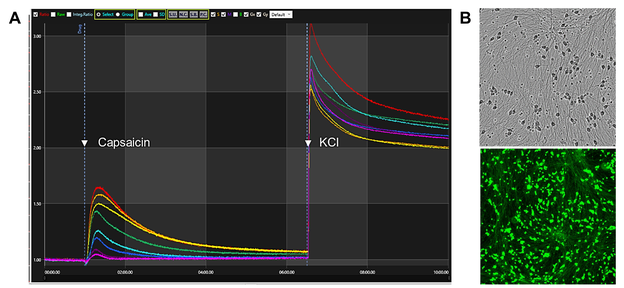


Figure 4. Calcium Flux. Monitoring changes in intracellular [Ca^{2+}] in response to activation of ion channels and GPCRs is a common technique for tracking neural activity and is extremely useful for utilizing iPSC-derived sensory neurons in pain therapeutic drug discovery. Our goal was to use this assay to demonstrate that iCell Sensory Neurons are responsive across a range of known sensory neuron agonists. We selected the response to Capsaicin as the metric to optimize around. (A) Raw data from the FDSS/µCell looks like this. Different colored traces are from a titration of Capsaicin (0→8 µM). The time when capsaicin or KCl were added is indicated by the white arrows. Elevated extracellular [KCl] (50 mM) triggers neuronal depolarization and is used a control step in this assay after 10 minutes of recording the agonist response as it confirms the presence of functional cells. (B) Phase and fluorescence images (from Calcium 6 dye) can also be used to demonstrate that sensory neurons are happy and healthy in each well.

Optimization of Calcium Flux Assay

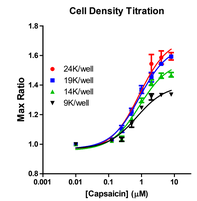


Figure 5. Cell Density Titration. iCell Sensory Neurons were cultured at different densities in 384w format: 9K, 14K, 19K, 24K cells per well. The recommended plating density for this cell type ranges from 0.3-1.9 x 10^5 cells/cm^2 depending on the application. Cells were assayed on DIV 21 using an 8-point dose-response of Capsaicin. These data suggest that 19K (**blue**) gives the most robust and consistent response. More cells (24K in **red**) does not provide additional benefit. Fewer cells (14K in **green**) is acceptable as the Ca^{2+} signal is still strong.

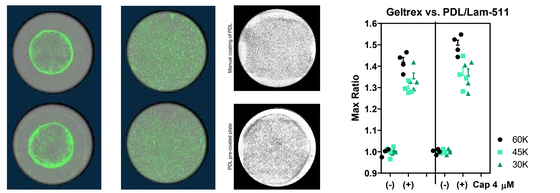


Figure 6. ECM Comparison. An important factor in developing cell-based assays with iPSC-derived neurons is the extra-cellular matrix (ECM). Options range from poly-D-lysine (PDL) to polyethylenimine (PEI), and from Matrigel to laminin. Here, we compared Geltrex (LEFT wells) to PDL plus recombinant Laminin-511 (CENTER wells) in 96-well format. While both ECM supported iCell Sensory Neurons viability, morphology, and function (see graph comparing -/+ 4 µM Capsaicin, the long-term attachment with PDL/Laminin-511 was more consistent across wells and is recommended for extended culture duration. Also, manual coating of plates with PDL is preferred over PDL pre-coated (RIGHT wells).

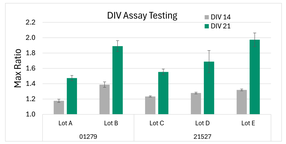


Figure 7. DIV Testing. In general, the response to various sensorineural agonists improves with time in culture for iCell Sensory Neurons. Of course, a faster time to assay post thaw is desirable. This experiment shows that cells do respond to 4 µM Capsaicin at DIV 14, but the signal is higher and more consistent when assayed on DIV 21.

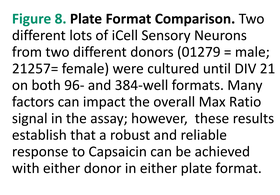


Figure 8. Plate Format Comparison. Two different lots of iCell Sensory Neurons from two different donors (01279 = male; 21257= female) were cultured until DIV 21 on both 96- and 384-well formats. Many factors can impact the overall Max Ratio signal in the assay; however, these results establish that a robust and reliable response to Capsaicin can be achieved with either donor in either plate format.

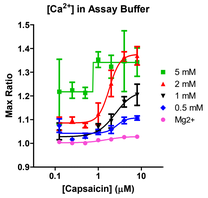


Figure 9. Assay Buffer Optimization. Robust Ca^{2+} signal is not consistently observed in maintenance medium (data not shown), so the day of assay buffer was examined. Since this is a calcium signaling assay, culture media was exchanged with HBSS (-/-) and increasing [Ca^{2+}] in the buffer were tested. It was observed that 2 mM $CaCl_2$ final concentration yielded the highest signal in the assay. Interestingly, use of HBSS (+/+) that contains Mg^{2+} did not support a functional response of iCell Sensory Neurons.

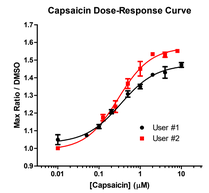


Figure 10. Assay Reproducibility. Under these optimized conditions, the Calcium flux assay was performed by two independent operators on different days but with the same lot of iCell Sensory Neurons. As shown on the graph to the right, similar results were generated from a ≥8-point dose-response of Capsaicin, which highlights the assay reproducibility.

Compound Profiling with Calcium Flux Assay

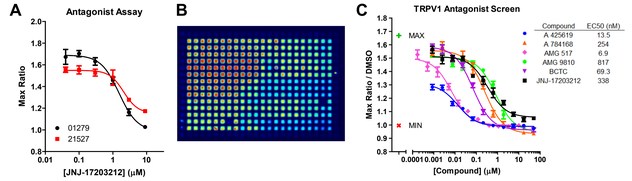


Figure 11. Compound Profiling. (A) Following the optimized assay protocol presented here, calcium flux was pharmacologically modulated with the known antagonist, JNJ-17203212, using both donors of iCell Sensory Neurons (01279 and 21527). No differences were noted. (B) Testing was expanded to 384-well format to profile a panel of six different TRPV1 antagonists (all from Tocris). (C) Compounds (11-pt dose-response, n=3) were incubated for 1 h prior to stimulation with 1 µM Capsaicin. All inhibitor EC_{50} values were sub-micromolar and are listed in the table.

Additional Responses to Sensorineural Stimulation

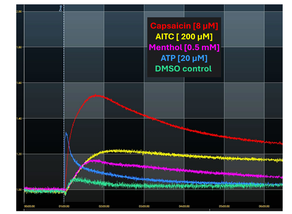


Figure 12. Alternate Agonists. The most robust signal in the Ca^{2+} flux assay with iCell Sensory Neurons comes from Capsaicin (TRPV1 agonist). Additionally, cells respond to AITC (TRPA1 agonist), Menthol (TRPM8 agonist), and ATP (P2RX3 agonist). Data featured here was generated with donor 01279 in 384-well format on Day 35. Note: the media & supplements provided with iCell Sensory Neurons are critical for these functional responses on Day 21 or later.

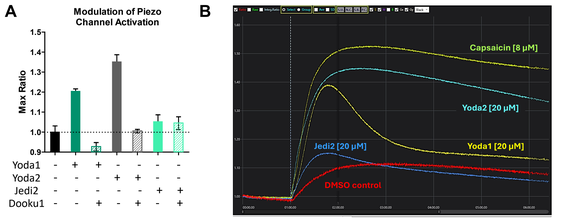
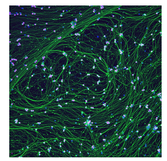


Figure 13. Mechanoreceptor Agonists. Piezo channels are expressed in iCell Sensory Neurons, with Piezo2>Piezo1 (see Fig. 3B). Cells were cultured until Day 28 and then treated with either 20 µM Yoda1 (Tocris #5586), 20 µM Yoda2 (Tocris #8051), or 20 µM Jedi2 (Tocris #6614). (A) The graph illustrates that Yoda1 and Yoda2 activate Piezo channels and result in calcium flux. The response to Jedi2 is different than DMSO control, but it is low. Mechanoreceptor activation has not been as rigorously tested as compared to stimulation with Capsaicin. Pre-treatment with the Piezo antagonist Dooku1 (10 µM; Tocris #6568) reduced the calcium signal from both Yoda1 and Yoda2, but no change with Jedi2.

Summary and Future Directions

FUJIFILM CDI has developed a robust differentiation protocol for large-scale manufacturing of human iPSC-derived sensory neurons. These cells have been rigorously characterized and were shown to be sensitive to drugs for modeling chemotherapy induced peripheral neuropathy (CiPN) and responsive to pain modulators in Substance P / CGRP peptide release assays (data presented elsewhere). Calcium flux is another essential assay readout for evaluating and utilizing these cells for pain therapeutic drug discovery. We have used this assay to demonstrate that iCell Sensory Neurons respond to a range of known sensory agonists, including capsaicin, AITC, menthol, ATP, and Piezo activators (e.g., Yoda2); each of which stimulate specific nociceptive receptors. Capsaicin response was used as the primary metric to establish an optimized assay readout, testing numerous variables such as cell density, ECM, time in culture, assay buffer, etc. Importantly, this assay is amenable to high throughput compound testing in 384-well format. iCell Sensory Neurons are available from both male and female iPSC donors to enable studies on sex-type differences. Next, we look forward to co-culture studies with iPSC-derived macrophages to study neuroinflammation as well as using "organ-on-a-chip" systems to compartmentalize these cells for more spatial and temporal control over their interactions.



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