A NOVEL HIGH-THROUGHPUT COMBINED VOLTAGE-CLAMP/CURRENT-CLAMP ANALYSIS OF SINGLE PRIMARY NEURONS

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INTRODUCTION

The patch-clamp technique is the gold-standard methodology for biophysical investigations of channels and receptors studied by voltage-clamp, and for analysis of excitability of cells, such as neurons studied via current-clamp. However, the throughput of manual patch-clamp is slow, and high-throughput robotic patch-clamp, while helpful for assessments, such as drug screening, has been primarily used to study channels and receptors expressed in heterologous expression systems. In this study, we introduce a novel approach to automated high throughput patch-clamping that substantially enhances high throughput analysis of excitable cells at the channel and cellular level. As a proof-of-concept, we apply this approach to investigate the detailed biophysical properties of voltage-gated sodium (Nav) channels in dorsal root ganglion (DRG) neurons, which are among the most diverse and complex neuronal cells. Our approach enables high throughput, unbiased, fast, simultaneous, and head-to-head electrophysiological recordings from a wide range of primary neurons. Furthermore, our approach eliminates the need for culturing of cells on coverslips and provides the ability to perform both voltage- and current-clamp recordings on the same neuron. This approach can be used for many applications, including both physiological and pharmacological analyses of primary DRG neurons.

MATERIALS & METHODS

Whole-cell patch-clamp experiments were performed on Sophion Qube-384. 3-4 C57BI/6 mice at the ages of 7-9.5 weeks old were used (males and females were used). DRG neurons were dissociated and isolated using protocols previously described (1-2). Cells were purified and further isolated using a combination of Mesh (70 µM) filtering and 15% BSA gradients. Probability curves were fit with both double and single Boltzmann equations, and the neurons were binned based on capacitance. A custom Python script was developed for fitting, and binning data. Recovery from inactivation required a biexponential fit. Analysis and plotting was performed with Sophion Analyzer, Graphpad Prism 9.

Workflow

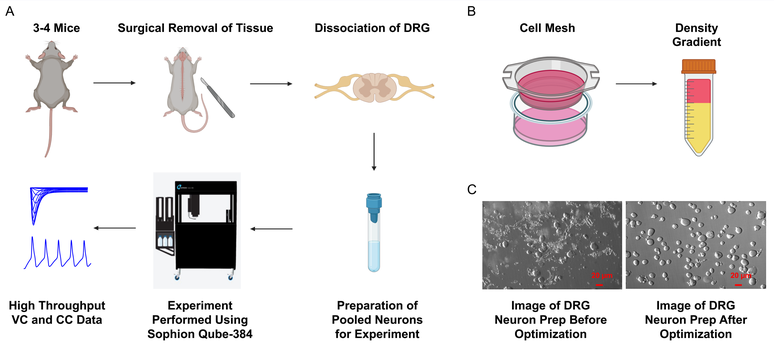

Figure 1 – Workflow of the assay and cell preparation
A) We start off by harvesting DRG tissue from 3-4 adult mice and preparing neuronal cell suspension using standard methods. B) Cell suspension if filtered using a 70 µM cell mesh, followed by a two rounds of cell purification on 15% BSA density gradient in DRG media. C) Images of DRG neuronal cultures using the standard protocol and after implementing the filtration and purification of the neuronal suspension on density gradients. We aimed to make sure we get as many neurons as possible while maintaining purity, healthy-looking membranes, and diversity of cell sizes. Finally, cells were loaded onto the instrument to run VC/CC experiments.

Activating Properties of DRG Neurons

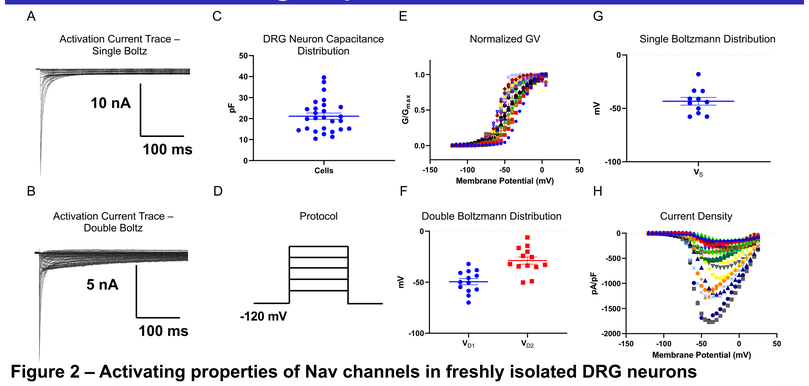

Figure 2 – Activating properties of Nav channels in freshly isolated DRG neurons
A-B) Sample traces of data that were either better fit with single or double Boltzmann equations. C) The distribution of capacitances of the cells that were used in our final analysis. D) The standard VC protocol that was used. Cells were held at -120 mV, followed by depolarizing step-pulses. E) GV data from each neuron. Relationships display normalized conductance as a function of membrane potential. Relationships in this panel were run with both single & double Boltzmann functions, and F) if the fit quality mathematically was better with double, V_{D1} (i.e., $V_{1/2}$'s) and V_{D2} were binned in panel F; G) if single Boltzmann worked better, the V_S was put into the bin in panel G. H) Shows current densities.

Inactivation of DRG Neurons

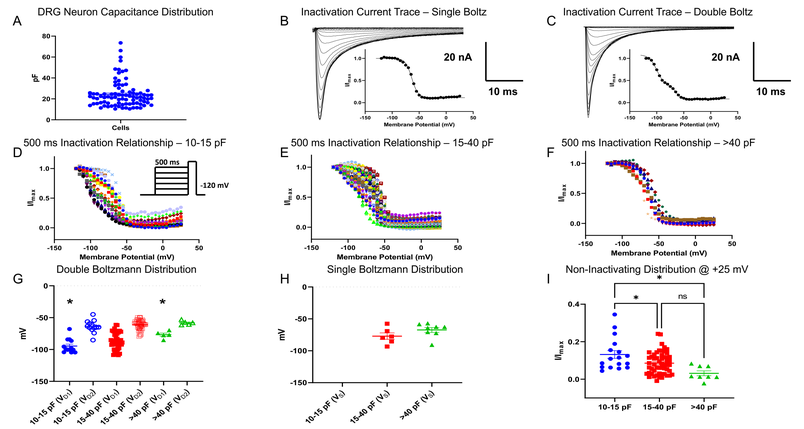

Figure 3 – Steady-state inactivation of Nav channels using a 500 ms pre-pulse
A) The distribution of the capacitance. B-C) Samples of single vs. double Boltzmann traces/curves. D-F) The current-voltage relationships. G) Cells that fit better with double Boltzmann function. H) Cells that were better fit with single Boltzmann. I) Plots of non-inactivating current (persistent current) in bins. p<0.0001 for G, p=0.0406 (small vs. mid), p=0.0226 (small vs. large) for I.

Recovery From Inactivation of DRG Neurons

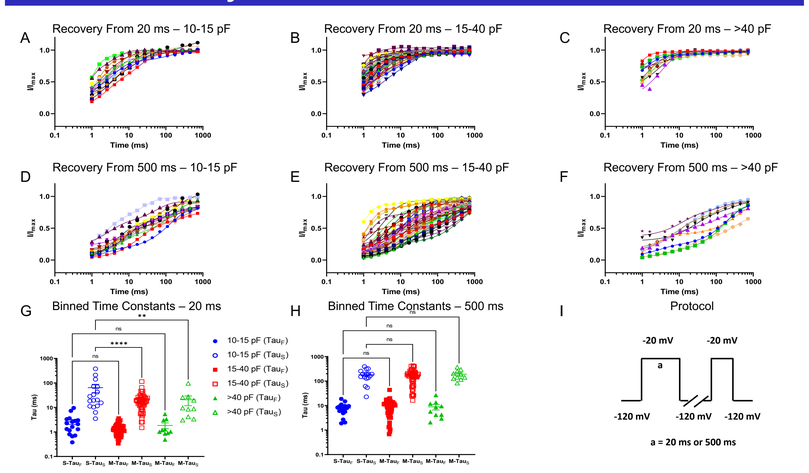

Figure 4 – Recovery from 20 ms (fast) and 500 ms (intermediate) inactivation
A-C) Data divided up into bins as above for 20 ms, and D-F) for 500 ms. G) Distribution of Tau_{Fast} and Tau_{Slow} from 20 ms across bins. ** p=0.0054, *** p<0.0001. H) at 500 ms, there were no significant differences across bins. I) The protocols that were used. The S, M, and L designations on the X-axis (G, H) refer to small (10-15 pF), medium (15-40 pF), and large (>40 pF) neurons.

Sensitivity to TTX in DRG Neurons

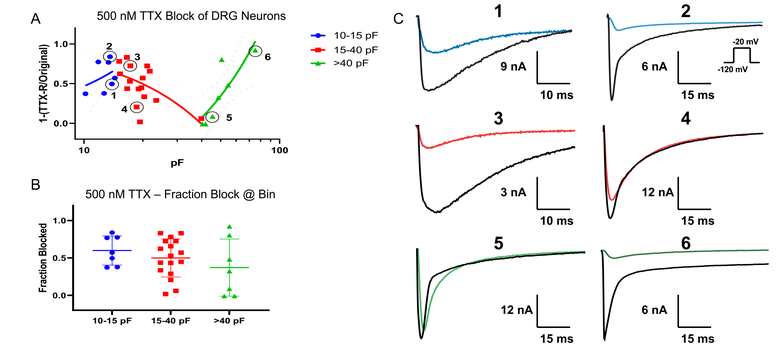

Figure 5 – Pharmacological block by 500 nM TTX
A) The fraction of Nav current inhibited by TTX vs. capacitance. The dotted lines display the 95% confidence intervals. B) The variable distribution of fraction of the current blocked across each bin, and C) sample traces from cells that are circled in panel A.

VC & CC in the Same DRG Neurons

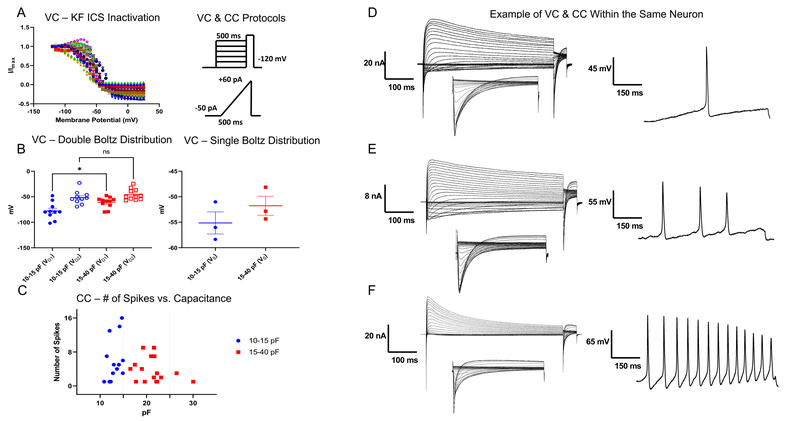

Figure 6 – VC and CC in the same neurons.
A) Inactivating current-voltage (IV) relationship of Nav channels in KF internal solution. Protocols are on the right. B) Comparison of double vs. single Boltzmann approach, as before (the biggest cell we got in these experiments was 30 pF). * p=0.0104. C) Number of AP spikes that were elicited using a standardized ramp CC protocol. D-F) Sample VC/CC traces for high and low spiking cells/

Critical Numbers Associated with New Method

Type	Cells recorded/day	Perfusion	VC/CC in same neuron	Cell selection	Data analysis	Overnight coverslip incubation	Diverse neuron, head-to-head comparison
Current method	~1-10	Yes	No	Experimenter bias	Subconscious bias	Yes	No
Novel assay	Up to ~200 (Assuming 2 preps/day)	Yes	Yes	Blinded	Unbiased	No	Yes

Table 1 – Comparison of our assay to traditional manual patch-clamp, with respect to freshly isolated DRG neurons.

Number of adult mice per prep	3-4 mice
Number of preps tested	7: (5 for VC), (2 for CC + VC)
Number of cells patched at the beginning of experiment per prep	Up to ~80-100 cells
Activation – number of cells that passed filters	27 cells
Inactivation – number of cells that passed filters	87 cells
Recovery from 20 ms inactivation – number of cells that passed filters	83 cells
Recovery from 500 ms inactivation – number of cells that passed filters	82 cells
CC + VC – number of cells that passed filters	29 cells
Number of runs/prep	2-4
Viability of cells post dissection and dissociation for automated patch-clamp	Up to ~4 hours
Lifespan of cells while being patched	Up to ~40-50 minutes

Table 2 – Characteristic features and output of the assay. The cell counts include all the cells that passed all our extensive quality and mathematical filtration steps.

CONCLUSIONS

- High throughput electrophysiological method to investigate freshly isolated neurons
- Our method enables fast, unbiased, and head-to-head analysis of diverse neurons
- Enables analysis of neurons without requiring overnight culture on glass coverslips
- Enables high-throughput voltage- and current-clamp within the same neurons

References: 1) Dib-Hajj et al., (2009) *Nature Prot.* 2) Cummins et al., (2009) *Nat. Prot.* 3) Neher & Sakmann, (1976) *Nature.* 4) Ghovanloo et al., (2022) *BJP*

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