Journal of Neuroscience Methods 339 (2020) 108699

Contents lists available at ScienceDirect

Journal of Neuroscience Methods

journal homepage: www.elsevier.com/locate/jneumeth

Adaptation of robust Z' factor for assay quality assessment in microelectrode array based screening using adult dorsal root ganglion neurons

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ARTICLE INFO

Keywords:
Microelectrode arrays
Dorsal root ganglion
Z'-factor
Assay quality
High content screening

ABSTRACT

Background: Cell-based assays comprising primary sensory neurons cultured *in vitro* are an emerging tool for the screening and identification of potential analgesic compounds and chronic pain treatments. High-content screening (HCS) platforms for drug screening are characterized by a measure of assay quality indicator, such as the Z'-factor, which considers the signal dynamic range and data variation using control compounds only. Although widely accepted as a quality metric in high throughput screening (HTS), standard Z'-factor are not well-suited to indicate the quality of complex cell-based assays.

New Method: The present study describes a method to assess assay quality in the context of extracellular recordings from dorsal root ganglion (DRG) sensory neurons cultured on multi-well microelectrode arrays. Data transformations are applied to electrophysiological parameters, such as electrode and well spike rates, for valid normality assumptions and suitability for use as a sample signal. Importantly, using transformed well-wide metrics, a robust version of the Z'-factor was applied, based on the median and median absolute deviation, to indicate assay quality and assess hit identification of putative pharmacological compounds.

Results: Application of appropriately scaled data and robust statistics ensured insensitivity to data variation and approximation of normal distribution. The use median and median absolute deviation of log transformed well spike rates in computing the Z'-factor revealed a value of 0.61, which is accepted as an "excellent assay." Known antagonists of nociceptor-specific voltage-gated sodium ion channels were identified as true hits in the present assay format under both spontaneous and thermally stimulated conditions.

Comparison with Existing Methods: The present approach demonstrated a large signal dynamic range and reduced sensitivity to data variation compared to standard Z'-factor used widely in HTS.

Conclusion: Overall, the present study provides a statistical basis for the implementation of a HCS platform utilizing adult DRG neurons on microelectrode arrays.

1. Introduction

The development of effective screening paradigms for chronic pain treatment and analgesic lead discovery is an active area of research (Black et al., 2019). Despite detailed understanding of several mechanistic pathways, the discovery of effective, non-addictive first-in-class analgesics remain limited (Taneja et al., 2017). This may be due to contemporary methods of drug discovery, which rely primarily on molecular target-based assays wherein a specific receptor or target is identified, modulated, and validated in an animal model. However, target-based strategies have resulted in limited yield for early drug discovery processes (Sams-Dodd, 2005, 2013; Woolf and Ma, 2007; Munos, 2009; Sapunar et al., 2012; Krames, 2014). An alternative approach, high-content phenotypic screening (HCS), leverages physiologically relevant functional and/or morphological endpoints, and is widely used in target validation and lead optimization; providing insight into complex biological processes underlying a specific disease phenotype (Giuliano et al. 1997; Arraste and Finkbeiner 2005; Dragunow 2008; Bickle 2010; Zanella et al. 2010; Jain et al. 2012). This type of approach is especially suitable for screening candidate therapeutics for chronic pain; where signaling complexity, due to a multitude of biomolecular pathways which act in concert, pose a significant challenge for therapeutics targeting specific molecular pathways. However, a significant challenge in translating HCS data is establishing the overall quality of a given assay (Azegrouz et al., 2013).

Our group is exploring the utility of cell-based assays using dorsal root ganglion (DRG) neurons *in vitro*. Nerve injury or inflammation may cause enhancement in DRG neuron excitability (Woolf and Ma, 2007;

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https://doi.org/10.1016/j.jneumeth.2020.108699
Received 6 January 2020; Received in revised form 4 March 2020; Accepted 23 March 2020
Available online 26 March 2020
0165-0270/

Sapunar et al., 2012; Krames, 2014). Importantly, DRG neuron hyperexcitability may be induced *in vitro* by incubating cells with proinflammatory cytokines and/or neurotrophic factors and be measured in the form of all-or-nothing action potentials using substrate-integrated microelectrode arrays (MEAs) (Sorkin et al., 1997; Junger and Sorkin, 2000; Ferrari et al., 2014; Black et al., 2018a). While recent pain-related HSC findings have been reported, assessment of assay quality has been limited in most cases.

One measure of assay quality, defined by Zhang et al. (1999), is the Z'-factor, which has been widely adopted for high throughput screening (HTS). In the present study, we examined the Z'-factor metric for cell-based screening assays using adult DRG neurons cultured on MEAs. Normality assumptions, implicit in the use of the standard Z'-factor, are assessed for microelectrode-wise and well-wide spiking metrics and data transformation approaches are applied. We show a robust form of Z', based on median values and median absolute deviations, computed for background and temperature-evoked signal using lidocaine treatment as a negative control. A hit identification method is described using pharmacological compounds with a known mechanism of action under baseline and thermally-evoked conditions.

2. Experimental methods

2.1. Primary adult DRG culture

All surgical procedures were carried out in accordance with the University of Texas at Dallas's Institutional Animal Care and Use Committee. Institute for Cancer Research (CD-1) male mice (4–6 weeks old; Envigo) were utilized for DRG tissue extraction as described in detail previously (Sleigh, Weir, and Schiavo 2016). Briefly, mice were deeply anesthetized with 3% isoflurane and euthanized *via* cervical dislocation. Spinal columns were extracted, hemisected, and DRGs were isolated from central/peripheral roots and stored in ice-cold Hanks Balanced salt solution (HBSS). Tissue was dissociated as previously described with minor modifications (Black et al., 2018b). An enzyme buffer consisting of 2 mg/mL collagenase and 0.1 mg/mL DNase was added to pooled tissue and allowed to incubate at 37 °C for 40 min. After 40 min, 100 µL of 1X papain solution was added and the tissue was gently triturated using a fire polished pasture pipette. The cells were isolated *via* centrifugation (300 g for 10 min) and resuspended in fresh medium consisting of DMEM/F-12 + glutamax + 10 % fetal bovine serum (FBS) + 1% penicillin/streptomycin (PS) + 5 ng/mL GDNF. 10,000 viable neurons were plated on multi-well MEAs (Axion BioSystems, Atlanta, GA) pre-treated with 50 µg/mL poly-D-lysine (overnight) followed by surface coating of 20 µg/mL laminin (2 h). Cultures were maintained at 37 °C, 10 % CO2, and 95 % humidity and medium was exchanged every 48 h. After non-neuronal populations reached confluence of approximately 90 %, medium was supplemented with mitotic inhibitors uridine (17.5 µg/mL) and 5-fluoro-2′-deoxyuridine (7.5 µg/mL) for the remainder of the culture.

2.2. Spontaneous and Evoked extracellular recordings using in vitro microelectrode arrays

Spontaneous extracellular recordings were performed with 12-well plate MEAs (Axion Biosystems, Atlanta, GA) using the Axion Maestro MEA recording system (Axion Biosystems, Atlanta, GA) as described previously (Black et al., 2018b). Briefly, extracellular voltage recordings were carried out at 12.5 kHz sampling rate from a total of 768 available substrate integrated microelectrodes. Continuous data were filtered using a 1-pole Butterworth band pass filter (200 – 3000 Hz) and individual spikes were detected using a 5.5 σ adaptive threshold method. Thermally-evoked extracellular recordings were performed using the calibrated stage plate heater. Briefly, the temperature was increased from 37 °C to 42 °C at approximately 0.5 °C/min and held at 42 °C for an interval of 5 min while evoked activity was recorded.

2.3. Pharmacology and screening

All pharmacological compounds were reconstituted in either complete medium, water, or DMSO at a stock concentration of ≥100 × . Before addition of compounds, a baseline recording of 30 min was acquired. For exposure to IL-6 and NGF or vehicle (water), bolus volumes were added simultaneously to treatment groups (100 ng/mL IL-6 + 100 ng/mL NGF) for 48 h. To determine the signal of the negative control (background) data, > 50 µM lidocaine was administered to respective treatment groups. The negative control is defined as the control compound which produces the minimum signal (Zhang et al., 1999; Sui and Wu, 2007). To determine the pharmacological responsiveness to Nav1.7 (Huwentoxin-IV 30 nM), Nav1.8 (A-803,467 300 nM), and Nav1.1/1.3 (ICA-121,431 23 nM) blockers, respective compounds were added to treatment groups that were previously incubated with IL6 + NGF or vehicle for 48 h followed by a 10 min recording session.

2.4. Data analysis

Microelectrodes were considered for analysis when a mean firing rate of at least 1 spike/min was recorded during a 30 min baseline recording. Mean firing rate (Hz) and total spikes/well were determined using NeuroExplorer (Nex Technologies, Madison, AL) and AxIS Metric (Axion Biosystems, Atlanta, GA) as described previously (Black et al., 2018b). All statistical tests and visualization of data sets were performed using OriginPro 2019 (OriginLab, Northampton, MA). A two-sample *t*-test was used to compare means of two groups to test the null hypothesis between treatment and vehicle groups.

3. Results

3.1. Normality assumption and log transformation

The design of a phenotypic, cell-based assay, requires consideration of the physiological end point which is relevant to pathology. Neuronal excitability is an especially important end point for chronic pain, since maladaptive changes, particularly in the context of neuropathic and inflammatory states, alter the bioelectrical properties of DRG neurons (Basbaum et al., 2009; Krames, 2014, 2015). Such alterations result in a decrease in the threshold and/or increase in the magnitude of responsiveness to sensory stimuli triggering increased generation of action potentials. Adult DRG neurons cultured on MEAs exhibit spontaneous extracellular action potentials that can be detected from individual microelectrode sites (Fig. 1(a)). Typically, recordings from embryonic DRG neurons exhibit stable baseline activity at DIV 11–14, consisting of at least 50 % active microelectrodes per well (16 microelectrodes/well – 48 wells) (Newberry et al., 2016). Similar observations have been made for adult DRG neurons, wherein peak activity is observed at DIV9 and stable for at least 21 days, with an active microelectrode yield of 25 % per well. (Black et al. 2018). Fig. 1(b) shows filtered continuous extracellular recordings from three representative microelectrodes. A data metric which is readily extractable from such recordings is the mean firing rate (MFR), defined as the number of spikes over a specified time period. However, the MFR of DRG neurons shows substantial variability when compared to electrical activity recorded from cortical networks (Charkhkar et al., 2014) and spinal motor neurons (Black et al., 2017), and may represent a skewed data set. A bias towards low MFR is consistent with previous *in vitro* observations made by Newberry et al. (2016) and Black et al. (2018), as well as with the observed spontaneous firing of nociceptors from the L4/L5 DRG *in vivo* (Djouhri 2006; Djouhri et al. 2015). This lack of normality may reflect the intrinsic variance associated with a heterogeneous cell population (Woolf and Ma, 2007).

Since the Z'-factor has been developed under the assumption of a normal distribution and best reflects assay quality for data sets which do not violate normality (Sui and Wu, 2007), we first sought to assess

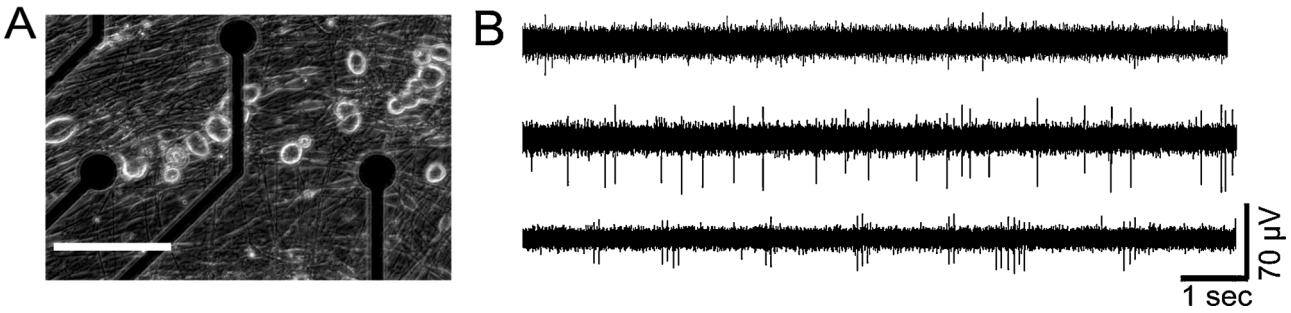

Fig. 1. Dorsal root ganglia neurons exhibit relatively low firing rates *in vitro*. (a) Representative phase image of dissociated DRG neurons cultured on microelectrode arrays (MEAs). Horizontal Scale bar represents 100 µm. (b) Raw trace of filtered continuous data recorded from three representative active microelectrodes.

the distribution of microelectrode MFR. The frequency distribution (n = 792 active microelectrodes (> 1 spike/min) from n = 5 plates) of adult DRG neuron cultures at day *in vitro* (DIV) 15–17 revealed an excessively right skewed distribution (skew = 5.85, kurtosis = 55.7) (p = 0, Shapiro-Wilk normality test) which significantly deviated from the standard normal distribution. On performing a log transformation of the microelectrode MFR, the data approximates a normal distribution on a log scale, however, the hypothesis that the sample was drawn from a normally distributed population was still rejected (p = 1.99 E^{-15} Shapiro-Wilk normality test), suggesting an overly-dispersed data set.

An alternative approach involves the use of the total spike count/well as a sample signal. Essentially, well-wide metrics represent the total combined signal from all available active microelectrodes in a given well (64 microelectrodes/well) and may be less sensitive to per microelectrode firing rate differences. Similar to the non-transformed microelectrode MFR described previously, the total spike count/well assumed a non-normal (p = -5.78E-8, Shapiro-Wilk Normality test) and an excessively right skewed distribution (skew = 1.71, kurtosis = 2.75) (Fig. 2(a), (b)). However, the transformed data assumed a normal distribution on the log scale (skew = - 0.30, kurtosis = - 0.07; p = 0.157, Shapiro-Wilk Normality Test) (Fig. 2(c)). This is readily apparent from the comparison of the standard normal distribution and the well-wide spike count distribution, wherein, the data values and the reference normal distribution have a linear relationship (Fig. 2(d)). The data suggest that transformed well-wide metrics may serve as more appropriately scaled data for assay characterization and hit identification.

3.2. Robust form of the Z'-factor

To assess the potential of MEA-based DRG recordings as a screening paradigm, the widely used Z'-factor (Zhang et al., 1999), based on mean and standard deviations (STD) was tested as an assay quality indicator. Both transformed microelectrode MFR and transformed spike count/well signals were compared to lidocaine-treated wells (> 50 µM) as the

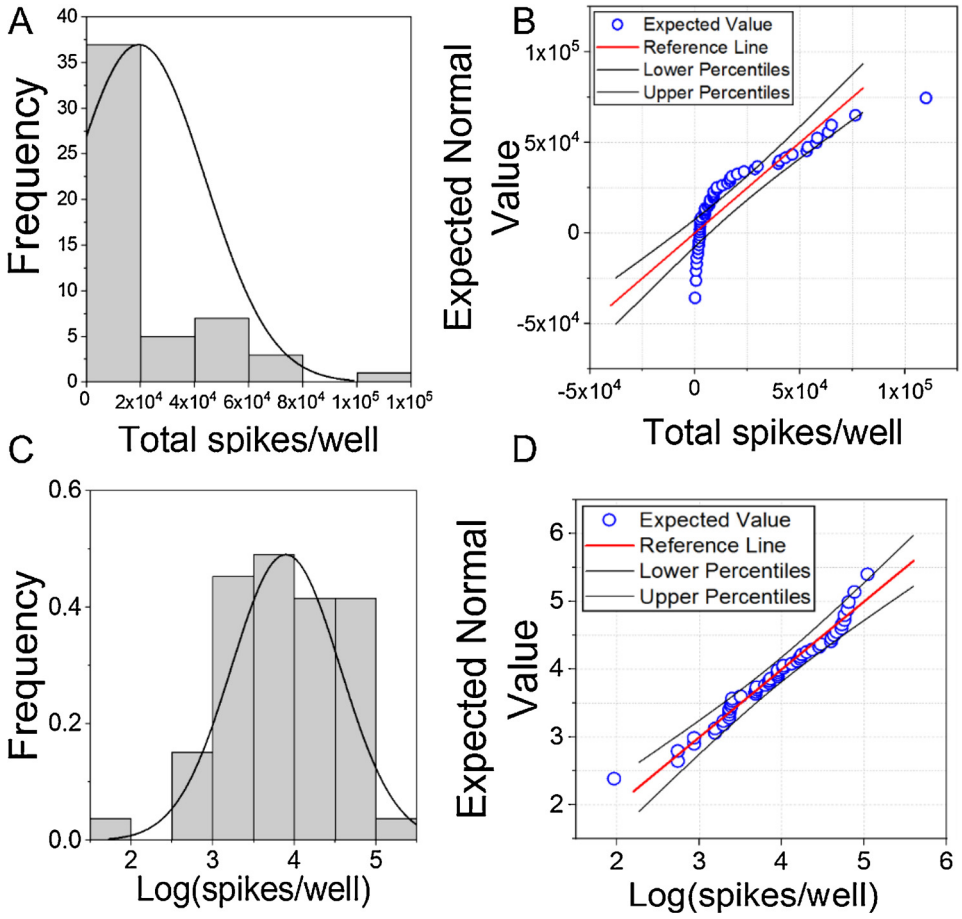

Fig. 2. Well-wide metrics assume a normal distribution on a log scale (a) Frequency histogram of total spike count/well from n = 5 separate cultures of adult DRG neurons consisting of n = 53 wells. Each well consists of a total of 64 available recording electrodes. (b) Normal Q-Q plot of total spike/count (c) Log transformed spikes/well (d) Normal Q-Q plot of the transformed well-wide metrics.

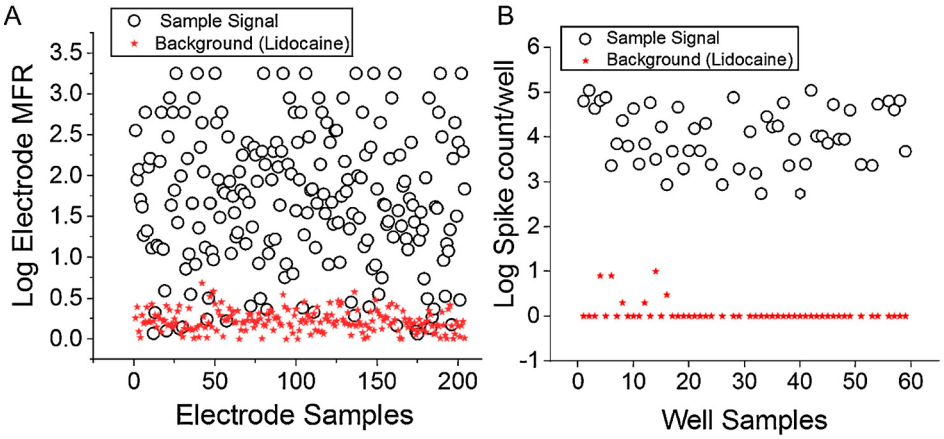

Fig. 3. The application of Z'-factor as an indicator of assay quality to sample signals using transformed electrode MFR and transformed well-wide metrics (a) Sample and background (lidocaine > 50 μM) signal of log transformed electrode MFR (n = 204 active electrodes). (b) Sample and background signal of log transformed spike count/well (n = 53 wells, 64 electrodes/well).

negative control. In Fig. 3(a), representative MFR of active microelectrodes (n > 200 active microelectrodes) are plotted and compared against lidocaine treated (background) MFRs. The Z'-factor computed for the transformed microelectrode MFR sample signal revealed a Z'-factor value of -1.7. A Z' value < 0 indicates an assay which cannot be used for any deterministic identification of compounds, since there is no separation band and the distributions of the sample and background overlap (Fig. 3(a)). In contrast, well-based metrics revealed a value of 0.23. The use of well-wide metrics result in increased signal dynamic range and lowered data variation (Fig. 3(b)). However, this represents a "double assay" (0.5 < Z < 0, separation band is too small) and may be ineffective for use in a screening paradigm.

Although the well-wide sample and negative controls illustrate a large signal dynamic range as observed previously, the Z'-factor is extremely sensitive to data variability. One approach is the use of robust statistics which include measures such as median and the median average deviation (MAD) and is commonly used in HTS data analysis for its insensitivity to outliers. To this end, a robust form of Z'-factor was applied for well-wide metrics using the median and MAD of the transformed spike count/well:

$$Z'_M = 1 - \frac{3MADc^+ + 3MADc^-}{|Mc^+ - Mc^-|}$$

Where $MADc^+$ and $MADc^-$ refers to the median average deviation of the positive and negative controls and Mc^+ and Mc^- refer to the median of the positive and negative controls, respectively. The robust Z' (Z'_M) revealed a value of 0.61 for well-based metrics, which is accepted as an "excellent assay" (Zhang et al., 1999).

3.3. The effect of well-exclusion criteria on Z'-factor

Considerations regarding the number of active electrodes per well are imperative, since the collection of microelectrode derived firing rates directly contribute to the total spike count per well, and therefore, the acquired sample signal. To determine the minimum number of active electrodes required per well or an active electrode criterion, first a frequency histogram of the number of active microelectrodes as a function of well percentages was determined (n = 5 cultures, n = 52 wells) (Fig. 4). On further analysis on the relationship of an exclusion criteria and the effect on the Z'_M factor, a score of > 0.5 ("excellent assay") was observed at a minimum requirement of 2 active electrodes/well (Fig. 4). While the Z'_M factor continually increases and trends towards an "ideal assay" with more active microelectrodes, the number of available wells drops significantly to impact assay practicality (Fig. 4). Based on prior studies of DRG cultures on MEAs, where stable baseline activity typically comprises of 25–50 % of active microelectrodes (Newberry et al., 2016; Black et al., 2018a), the data suggest that 4 active microelectrodes/well is suitable as the sample signal, provides a

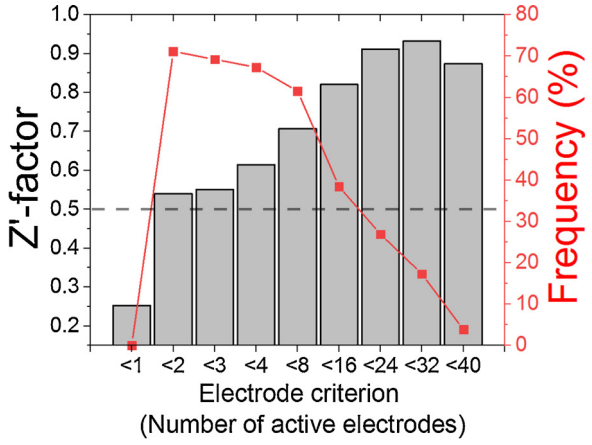

Fig. 4. Assessment of well exclusion criteria and the effect on the quality of the assay. Z'_M factor is plotted as a function of the electrode exclusion criteria and the frequency of encountering a given number of active electrodes per well.

Z'_M factor of > 0.5, and may forecast the use of 48-well plate formats, with 16 microelectrodes/well for assay implementation.

3.4. Hit detection and identification methodology

The main goal of screening assays is to be able to identify putative "hits". Such hits in a screen represent outliers which have large differences from the average sample signal and have a desired physiologically relevant mode of action to the pathology under investigation. Typically, conventional cut-offs for assays configured for inhibition/antagonist screening set thresholds of 3 STD below the mean sample signal or the robust version of 3 MAD for hit detection which represents a type I error rate (false positive) of 0.0013 (Wu et al., 2008; Goktug et al., 2013). Since the Z'_M leverages the use of the median as an estimate of the signal, intuitively, a hit threshold can be defined as a compound with a signal below 3 MAD for an inhibition/antagonist type assay which aims to detect compounds that reduce the observed hyperexcitability of DRG neurons. In addition to the selection of a hit limit, the quality of the assay also affects confirmation rates. We have previously discussed the use of exclusion criteria to improve the Z'_M factor which directly increases the median value and lowers the absolute deviation. Another complementary approach is to introduce an external stimulus to induce neuronal excitability which may improve signal intensity compared to stochastic spontaneous activity. Adult DRG neurons have been previously demonstrated to undergo IL6-mediated increase in thermally evoked responses at noxious temperature range, an effect mediated by vanilloid receptor, TRPV1(Basbaum et al., 2009; Black et al., 2018b). Therefore, we sought to determine the hit quality

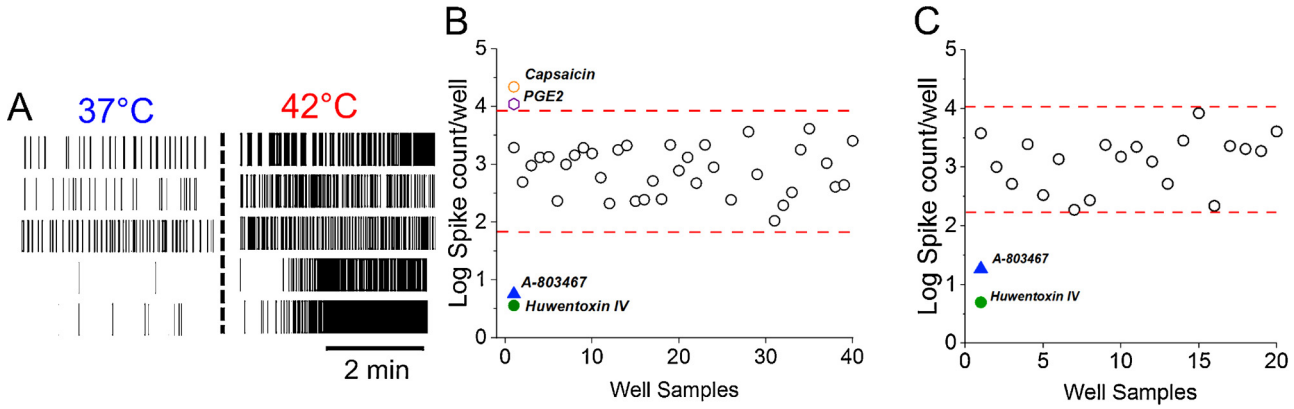

Fig. 5. Identification of putative hits with known mechanism of action in spontaneous and thermally stimulated adult DRG cultures. (a) Representative raster plot from 4 active electrodes at 37 °C (spontaneous activity) and 42 °C (thermally evoked). Detection of hits in the assay at (b) baseline spontaneous activity (37 °C) and (c) thermally evoked (42 °C). Agonist/activation type assay configuration was tested using 1 μM PGE2 and 10 nM capsaicin. Antagonist/inhibition type assay configuration was tested using 30 nM Huwentoxin IV and 300 nM A-803467. Red dashed lines indicate hit detection thresholds. The upper limit corresponds to median + 3MAD (3.87) and lower limit corresponds to median – 3MAD (2.02).

under spontaneous and thermally-stimulated conditions.

Preferentially expressed voltage gated sodium ion channel subtype Nav1.7 and Nav1.8 were inhibited with known ion channel blockers in sensitized adult DRG cultures. Briefly, DRG neurons pre-incubated for 72 h with pro-inflammatory cytokines, IL-6 and NGF, were treated with 30 nM Huwentoxin IV (HWTX-IV) (Nav1.7 blocker) or 300 nM A-803,467 (Nav1.8 blocker) under baseline (37 °C) and noxious (42 °C) temperature range. Calibrated stage plate heating to 42 °C induced reliable and reproducible increase in the firing rates of the available active microelectrodes (Fig. 5(a)). At nanomolar concentrations, HWTX-IV and A-803467 were potent at inhibiting spontaneous DRG activity (HWTX-IX: 83 ± 9% inhibition, n = 6 wells; A-803467: 65 ± 9%, n = 6 wells, $p < 0.05$ compared to negative control (DMSO)) and identified as positive hits in the assay under spontaneous and thermally-evoked formats (Fig. 5(b), (c)), as the median spike count/well for both compounds were reduced beyond the defined lower cut-off from the median sample signal (37 °C: HWTX M = 0.48, A-803,467 M = 1.72, 42 °C: HWTX M = 0.69, A-803,467 = 1.27). Although both spontaneous and thermally-evoked assay formats accurately identified hits, it is important to note the latter resulted in a $Z'_M = 0.74$ above that which was observed at 37 degrees C ($Z'_M = 0.61$). Interestingly, we observed a decrease in the MAD in thermally-stimulated conditions which may be due to a greater contribution of nociceptive-specific sub-types which are primarily temperature sensitive. To further explore the utility of the present assay to include conditions of hyperexcitability, we investigated the detection of putative compounds known to increase DRG neuronal excitability using an activation/agonist assay format. To this end, 1 μM PGE2 and 10 nM capsaicin were tested in DIV 13-DIV15 cultures of adult DRG neurons. Both PGE2 and capsaicin were identified as positive hits in the assay under spontaneous baseline activity (Fig. 5(b)) with the median spike count/well exceeding the upper limit or activation threshold defined 3 MAD above the median sample signal (capsaicin: 4.36, n = 6 wells, PGE2: 4.04, n = 4 wells). Taken together, the present assay configuration accurately detects compounds which modulate DRG neuron excitability under both activation and inhibition assay format types.

4. Discussion

Primary DRG neuron-based assays may have a significant impact in identifying novel therapeutic compounds for ameliorating chronic pain conditions (Melli, 2014). Under normal physiological conditions, DRG neurons exhibit limited spontaneous activity *in vitro* and *in vivo*. However, nerve-injury and inflammation cause cells to become hyperexcitable; playing a major role in the generation and maintenance of chronic pain. Therefore, *in vitro* phenotypic assays using peripheral sensory neurons which leverage cellular excitability as an endpoint are a useful tool for identifying compounds which attenuate DRG hyperexcitability. However, a significant challenge in the development of phenotypic-screening assays using DRG neurons is the lack of quantitative statistical approaches to validate the quality of the assay which is an indispensable step in assay validation (Chai et al., 2015). In the present study, we describe a robust statistical approach to determine the assay quality of a MEA-based adult DRG neuron screening platform.

First, we assessed the distribution of the microelectrode mean firing rates and well spike counts as sample signals to determine whether the normality assumption, implicit in the widely used and accepted assay quality indicator Z'-factor, was maintained. Both raw sample values of microelectrode-wise and well-wide metrics assumed excessively right skewed distributions. Data transformation schemes, based on log transformations, generated a normal distribution for well-wide metrics which was further used as an endpoint for computing the Z'-factor, as described previously by Zhang et al. (1999). Standard Z'-factor, based on the mean and STD, produced a poor Z'- factor value (< 0.5). In contrast, a more robust version, consisting of the median and MAD parameters of the signal, revealed a Z'-factor of 0.6, a range suggesting an excellent assay; described as having a large signal dynamic range and low variance.

To further establish a standard for DRG/MEA-based screening platforms, we investigated the dependency of Z' factors on minimum active microelectrode criteria. In short, we asked the questions "How many active microelectrodes did we typically observe per well given a set of culture conditions?" and "How does pooling data from wells with different active microelectrode numbers effect our assay quality?" A minimum of 2 active microelectrodes per well were required to produce a Z'-factor > 0.5, and therefore, only 30 % of the data acquired were omitted (where a well showed < 2 active microelectrodes). This is especially important, since it allows the use of scaled-up multi-well MEA plates; for example, 48-well plates which consist of 16 microelectrodes per well. The MEA-based assay was robust and reproducible at detecting nociceptive-specific sodium ion channel antagonists as true hits under stable baseline (37 °C) and thermally-evoked stimuli (42 °C). Prior studies developing sensory neuron-based phenotypic screens have used chemical and physical antagonists to evoke membrane depolarization when screening chemical libraries (Rana et al., 2017; Stacey et al., 2018; Martínez et al., 2019). For example, Stacey et al. (2018) employed veratridine, a non-specific sodium channel agonist to evoke neuronal depolarization in iPSC-derived sensory neurons. However, large concentrations of veratridine were required to induce neuronal excitability which directly affected the tested potency of sodium

channel blockers in the assay and, therefore, limited the classes of nociceptor-specific compounds which may be detected. Electrical field stimulation has been used as a an alternative approach to induce cellular excitability and record intracellular calcium transients, demonstrating stable assays with Z' > 0.5 (Jägervall et al., 2015). However, these studies used calcium transient imaging as a surrogate measure of action potential generation. Moreover, the ability to resolve the effects on neuronal subpopulations may be contaminated due to calcium elevation in adjacent glia (Suadicani et al., 2010). In the present study, temperature sensitivity was used as a stimuli to evoke cellular excitability which improved the Z' factor compared to those computed under baseline spontaneous activity. More importantly, since nociceptors are known to respond noxious thermal stimuli, temperature-based conditions may be more physiologically relevant while screening compounds. Notably, the approach presented here does not preclude the extension to assess the quality of cell-based assays leveraging other sources of excitable tissue such as cortical neurons (Zwartsen et al., 2018; Hyvärinen et al., 2019), spinal motor neurons(Black et al., 2017), and cardiomyocytes (Carlson et al., 2013; Woo et al., 2020) for moderate-to-high throughput screening.

Author contributions

R.A., B.J.B, and J.J.P, conceived and designed the study; R.A. and B.J.B performed the experiments; R.A. analyzed the data; R.A., B.J.B, and J.J.P interpreted the results of the experiments; R.A. drafted the manuscript, R.A., B.J.B, and J.J.P edited and revised the manuscript, R.A., B.J.B, and J.J.P approved the final version of the manuscript.

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CRediT authorship contribution statement

Rahul Atmaramani: Conceptualization, Methodology, Formal analysis, Writing - original draft, Writing - review & editing, Visualization. **Joseph J. Pancrazio :** Conceptualization, Writing - review & editing, Supervision. **Bryan J. Black:** Conceptualization, Methodology, Validation, Formal analysis, Writing - original draft, Writing - review & editing, Visualization, Supervision.

Declaration of Competing Interest

The authors have no competing interests to declare.

Acknowledgements

The authors thank Dr. Zachary T. Campbell (Biological Sciences, University of Texas at Dallas) for providing multi-well MEA plates. Research reported in this publication was supported by National Center for Advancing Translational Sciences of the National Institutes of Health under award number UG3TR003149.

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