


One-Step Seeding of Neural Stem Cells with Vitronectin-Supplemented Medium for High-Throughput Screening Assays

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Sheng Dai^{1,2}, Rong Li¹, Yan Long^{1,3}, Steve Titus¹, Jinghua Zhao¹,
Ruili Huang¹, Menghang Xia¹, and Wei Zheng¹

Abstract

Human neuronal cells differentiated from induced pluripotent cells have emerged as a new model system for the study of disease pathophysiology and evaluation of drug efficacy. Differentiated neuronal cells are more similar in genetics and biological content to human brain cells than other animal disease models. However, culture of neuronal cells in assay plates requires a labor-intensive procedure of plate precoating, hampering its applications in high-throughput screening (HTS). We developed a simplified method with one-step seeding of neural stem cells in assay plates by supplementing the medium with a recombinant human vitronectin (VTN), thus avoiding plate precoating. Robust results were obtained from cell viability, calcium response, and neurite outgrowth assays using this new method. Our data demonstrate that this approach greatly simplifies high-throughput assays using neuronal cells differentiated from human stem cells for translational research.

Keywords

high-content screening, high-throughput screening, HTS, induced pluripotent stem cells, iPSC, neuronal cells, neural stem cells, plate coating, vitronectin

Introduction

Disease modeling using induced pluripotent stem cells (iPSCs) has recently been applied to a variety of diseases in the study of disease phenotype and pathophysiology.¹ The iPSCs are self-renewing and can be differentiated into various types of human cells, such as neurons, cardiomyocytes, and hepatocytes. Disease models play a critical role in evaluation of drug efficacy during drug development. However, animal models, particularly rodent models, may not mimic certain human diseases appropriately. Insufficient disease models for neurodegenerative and neuropsychiatric diseases have hindered the development of new therapeutics for these maladies in the last two decades.² Recent advancement in iPSC technology has enabled large-scale production of neuronal cells differentiated from patient iPSCs that models neurological disorders, including Parkinson's disease (PD),³ Alzheimer's disease (AD),³ amyotrophic lateral sclerosis (ALS),³ spinal muscular atrophy (SMA),⁴ and familial dysautonomia.⁵ Neuronal cells differentiated from patient iPSCs exhibited specific disease phenotypes, such as a decrease of mitochondrial function in PD dopaminergic neurons,⁶ accumulation of amyloid- β and p-tau/total tau in AD neurons,⁷ hyperexcitability in ALS motor neurons,⁸ apoptosis in SMA motor neurons,⁹ and cholesterol accumulation in Niemann–Pick disease type C (NPC).¹⁰ Different types of neuronal cells have been generated from iPSCs,

including neural stem cells (NSCs), astrocytes, oligodendrocytes, motor neurons, and dopaminergic neurons. These human neuronal cells, particularly patient-derived cells, can serve as cell-based disease models to evaluate compound efficacy and screen compound libraries for drug development, in addition to studying disease pathophysiology.

Practically, use of neuronal cells differentiated from iPSCs for various experiments involves labor-intensive laboratory work. Culturing neuronal cells in assay plates requires plate precoating with extracellular matrix (ECM)

¹National Center for Advancing Translational Sciences, National Institutes of Health, Bethesda, MD, USA

²Sir Run Run Shaw Hospital, Zhejiang University School of Medicine, Hangzhou, China

³Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou, China

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Corresponding Author:

Wei Zheng, PhD, National Center for Advancing Translational Sciences, National Institutes of Health, 9800 Medical Center Drive, Bethesda, MD 20892-3375, USA.

Email: wzheng@mail.nih.gov

proteins and/or positively charged polymers to support cell attachment and growth. The procedure of plate precoating involves multiple steps of reagent addition and plate washes, which not only reduces screening throughput but also yields large well-to-well and plate-to-plate variations.¹¹ Precoating of plates is also a bottleneck in high-throughput screening (HTS) using neuronal cells.¹¹

Here, we report development of a simple method of one-step seeding and culturing of neuronal cells in assay plates using medium containing a truncated recombinant human vitronectin (rhVTN-N), which has been used as a plate coating substrate for iPSC feeder-free culture.¹² Because plate precoating and plate washing are not needed in this method, it greatly simplifies experiments using neuronal cells differentiated from iPSCs (**Fig. 1A**). We have validated this method with several assays, including cell viability, calcium response, and neurite outgrowth. The results demonstrate that this method enables HTS using NSCs and neurons differentiated from stem cells. Therefore, this method of one-step seeding of NSCs in assay plates with the rhVTN-N-supplemented medium is useful for HTS employed to evaluate compound efficacy, to measure compound neural toxicity, and to identify new leads by screening of compound libraries.

Materials and Methods

Cell Lines and Cell Culture

Wild-type (WT) iPSCs generated from normal fibroblasts (GM23450, Coriell Cell Repositories) were cultured in Matrigel (catalog no. 354277, Corning, Corning, NY)-precoated six-well plates with Essential 8 Medium (A1517001, Life Technologies, Waltham, MA). After being cultured at least 10 passages, NSC induction (Supplemental Materials and Methods) was initiated using the Neural Induction Medium kit (Life Technologies). The resulting NSCs were cultured in the complete StemPro NSC Serum Free Medium (A1050901, Life Technologies) containing knockout DMEM/F-12, StemPro neural supplement, 20 ng/mL of basic fibroblast growth factor (bFGF), 20 ng/mL of epidermal growth factor (EGF), and $1 \times$ GlutaMAX on the Matrigel-precoated flasks.¹⁰ Immunohistochemistry staining with antibodies against nestin, SOX1, SOX2, PAX6, vimentin, and SSEA4 was performed for characterization of the NSCs induced from human iPSCs.

One-Step NSC Seeding Optimization in 1536-Well Plates

First, nine frequently used substrates (**Suppl. Table S1**) were tested in an effort to find a best substrate that could be supplemented to medium for directly seeding cells without plate precoating and plate wash steps. Each substrate was

handled according to the manufacturer's instructions and added to NSCs suspended in the complete StemPro NSC Serum Free Medium with the indicated concentration (**Suppl. Table S1**). In 1536-well regular tissue culture-treated plates, 5 μ L/well of the NSC suspension supplemented with a plate coating reagent was dispensed at a density of 3000 cells/well. The plates were incubated at 37 °C supplied with 5% CO₂ for 48 h; 5 μ L/well of the reagent mixture of ATP content assay or nuclear dye cell viability assay was added to the assay plates, followed by incubation for 30 min at room temperature and luminescence detection of assay plates. Cells seeded in the Matrigel-precoated plates were used as a control. After the initial test, a rhVTN-N (A14700, Life Technologies) was selected from these nine commonly used substrates for the one-step seeding of NSCs in 1536-well plates. Briefly, after being thawed on ice, rhVTN-N was added to the medium at a final concentration of 10 μ g/mL (5 μ g/mL of rhVTN-N also worked similarly). NSCs suspended in rhVTN-N-supplemented medium were seeded at 3000 cells/well in 5 μ L in tissue culture-treated 1536-well plates. After 4 h or overnight incubation, the cells were treated with compounds and incubated for indicated times, followed by plate detection. Immunofluorescence staining of NSCs with markers and karyotyping were performed to characterize NSCs grown in the rhVTN-N-supplemented medium (Supplemental Materials and Methods).

We also found that the neural expansion medium composed of Neurobasal medium, Advanced DMEM/F-12 medium, and GIBCO Neural Induction Supplement (21103-049, 12634010, and A16477-01, Life Technologies) could not be used in this method because the NSCs were not attached to the assay plates and unable to grow when they were seeded in this medium supplemented with rhVTN-N (**Suppl. Fig. S1F**). It is possible that the function of VTN may be interfered with by an unrevealed component in this medium.

One-Step NSC Seeding with rhVTN-N-Supplemented Medium for Neuronal Differentiation in 384-Well Plates

Neuronal differentiation was conducted as previously described.¹⁰ The medium was composed of Neurobasal medium (21103-049, Life Technologies), B27, GlutaMAX, 10 ng/mL of brain-derived neurotrophic factor (BDNF), 10 ng/mL of glial cell-derived neurotrophic factor (GDNF), 1 μ M of cAMP, and 200 ng/mL of L-ascorbic. Briefly, 50 μ L of NSCs in the rhVTN-N-supplemented medium was seeded in poly-D-lysine (PDL)-coated 384-well plates (catalog no. 3845, Corning) or other plates as indicated at 3000 cells/well (cell density of 5×10^4 cells/cm²). As a control group, NSCs were seeded in the poly-L-ornithine (PLO) and laminin (LN) dual-precoated plates.¹³ Cells were cultured for 4 weeks in the neural

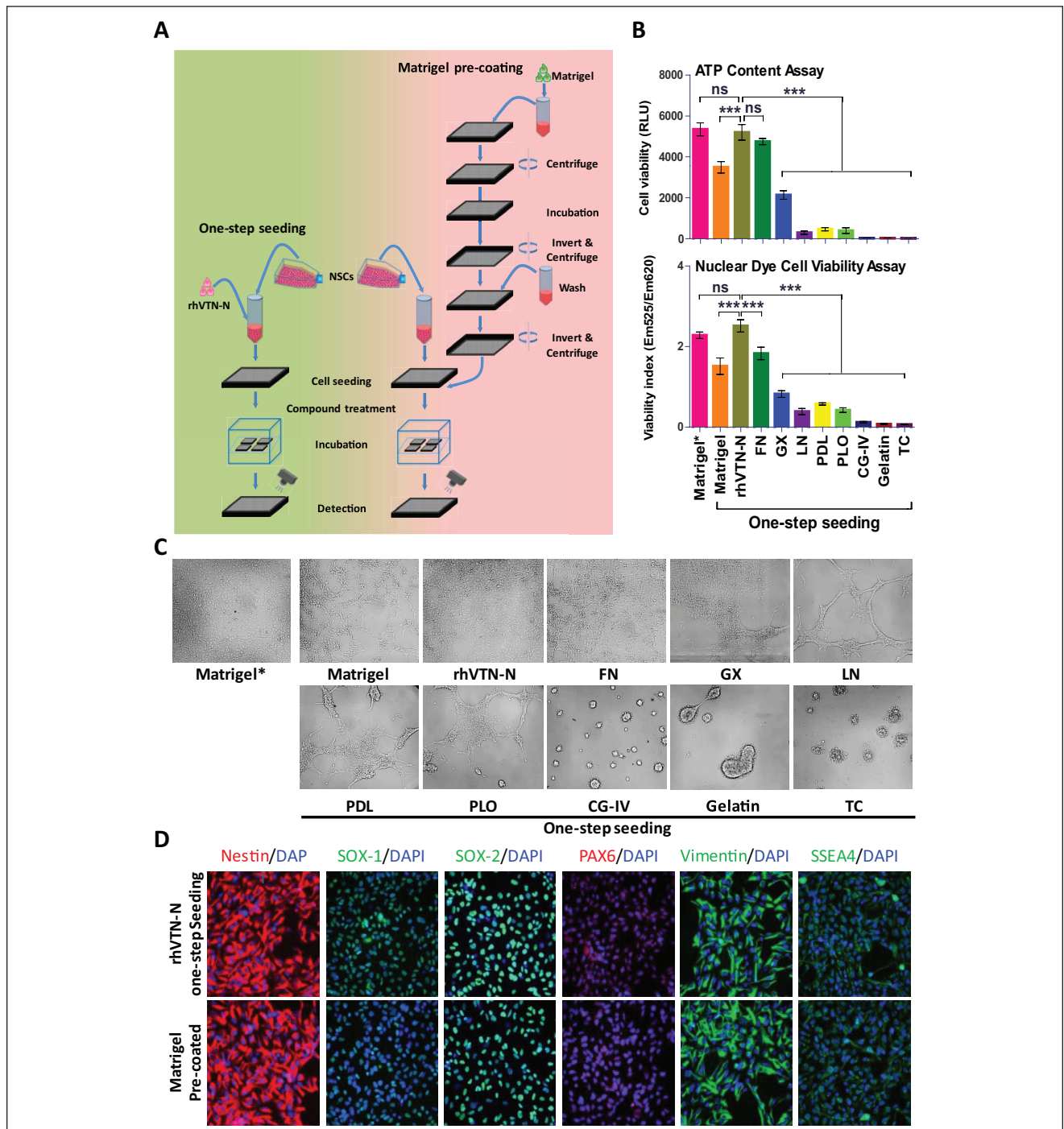


Figure 1. Development of the method for directly seeding NSCs without plate precoating in 1536-well plates. **(A)** Schematic comparison of a new method of directly plating NSCs suspended in the rhVTN-N-supplemented medium with the traditional method using Matrigel precoating plates for compound screening assays in 1536-well plates. This new method avoids plate pre-coating and plate washing steps and thus simplifies experiments that employ neuronal cells. **(B)** Results of cell viability determined in the ATP content assay and nuclear dye staining assay. NSCs were seeded in a medium containing one plate coating material. The cell viability in each substrate was compared with that obtained from the traditional Matrigel-precoated plate. The cell viability with the rhVTN-N-supplemented medium was similar to that of the control ($p > 0.5$). Data are represented as the mean \pm SEM of at least triplicates. **(C)** The bright-field images of NSCs in various media supplemented with different coating materials. Cells cultured in the rhVTN-N- or fibronectin (FN)-supplemented medium exhibited a healthy cell growth morphology similar to that of the cells cultured in the Matrigel-precoated plate. **(D)** Results of immunofluorescence profiles of NSC markers. Cells cultured in the rhVTN-N-supplemented medium showed a similar profile of NSC markers as the control (cells cultured in the Matrigel-precoated plate). Matrigel*, Matrigel precoated; GX, Geltrex; LN, laminin; PDL, poly-D-lysine; PLO, poly-L-ornithine; CG-IV, collagen type IV; TC, tissue culture treated.

differentiation medium, and half of the medium was replaced twice a week during the continuous culturing. Cell viability was measured by ATP content assay (CellTiter-Glo, Promega, Madison, WI) and nuclear dye cell viability assay (Cell Meter Kit, AAT Bioquest, Sunnyvale, CA) after 1, 2, and 4 weeks of neural differentiation.

Neurite Outgrowth Assay

NSCs were seeded in 384-well plates under the same conditions as described above. After 4 weeks of differentiation, neuronal cells were stained with anti- β -III tubulin antibody and Hoechst 33342 nuclear dye as described in the immunofluorescence staining section. Fluorescence imaging was performed in the IN Cell 2200 imaging system with a 40 \times objective lens using standard FITC and DAPI filter sets. Images were analyzed using IN Cell Developer v1.9.2 analysis software (GE Healthcare, Little Chalfont, UK). Briefly, the DAPI channel was used to identify nuclei using an intensity segmentation algorithm (objects greater than 650 RFU ranging in size from 5 to 75 μ m). β -III tubulin (FITC)-stained neurites were also identified by intensity segmentation (greater than 1150 RFU) with a postprocessing sieve to remove debris objects less than 2 μ m in size. The mean neurite length, measured in microns, was averaged to the number of nuclei per image.

High-Throughput Cytotoxicity Assays in iPSC NSCs

ATP content assay, nuclear dye cell viability assay, and caspase-3/7 assay (Caspase-Glo, Promega) were conducted according to the manufacturers' protocols using the one-step seeded NSCs in the rhVTN-N-supplemented medium in 1536-well plates. NSCs were plated at 3000 cells/well in 5 μ L of StemPro NSC Serum Free Medium complete culture medium with 10 μ g/mL of rhVTN-N in 1536-well plates (white, solid-bottom assay plates for ATP content assay and caspase-3/7 assay; black, clear-bottom plates for nuclear dye cell viability assay) and incubated for 4 h at 37 $^{\circ}$ C. Compounds were added to the assay plates at 23 nL/well using an NX-TR pintool station (WAKO Scientific Solutions, San Diego, CA). After 16 (caspase-3/7 assay) or 48h (ATP content assay and nuclear dye cell viability assay) of incubation at 37 $^{\circ}$ C with 5% CO₂, the assay reagents at 3 μ L/well were added to the assay plates. After incubation for the indicated times, the plates were detected by luminescence for the ATP content assay and caspase-3/7 assay or fluorescence for the nuclear dye cell viability assay in a plate reader.

Half maximal inhibitory (IC₅₀) or activating (EC₅₀) concentration of compound were calculated using Prism software (GraphPad Software, San Diego, CA). The percent

cell viability in the ATP content and nuclear dye cell viability assays was normalized using the positive control compound thapsigargin (270 μ M)-treated cells as 0% in cell viability, which has been shown to greatly reduce neuronal cell viability by depleting the intracellular calcium stores,¹⁴ and DMSO solvent-treated wells for the 100% viable cells. The percent activities of the caspase-3/7 assay were normalized using the positive control compound staurosporine (75 μ M)-treated cells as 100% in caspase activity, which has been shown to increase caspase-3/7 activities, and DMSO-treated wells for the 0% caspase activity. Signal-to-basal ratio (S/B), coefficient of variation (CV), and Z factor were calculated and used to evaluate the assay performance (Supplemental Materials and Methods). For cell viability evaluation of differentiated neuronal cells in 384-well plates, the cell viability measurement was conducted after the cells were cultured for 1, 2, and 4 weeks with ATP content assay and nuclear dye cell viability assay.

Homogenous LysoTracker Dye Staining Assay for Measurement of Enlarged Lysosomes in NPC Cells

Cells derived from patients with NPC exhibit the NPC disease phenotype of enlarged lysosome due to cholesterol accumulation in lysosomes that can be measured by the LysoTracker dye staining assay in a fluorescence imaging detection mode or in a fluorescence intensity mode by a plate reader.^{10,15,16} NSCs were plated at 1000 cells/well in 5 μ L of complete StemPro NSC Serum Free Medium supplemented with 10 μ g/mL of rhVTN-N in 1536-well, black, clear-bottom assay plates and incubated for 4 h at 37 $^{\circ}$ C and 5% CO₂. Columns 1 and 2 were used as the negative control with the WT NSCs, and columns 3–48 were NPC NSCs. Methyl- β -cyclodextrin (MBCD) and hydroxypropyl- β -cyclodextrin (HPBCD), which reduce cholesterol accumulation in NPC cells and thus decrease the enlarged lysosomes in NPC cells, were added to the assay plate at 23 nL/well as positive controls. After a 96 h incubation, 200 nM of LysoTracker Red dye (Life Technologies) was added to the assay plates to stain lysosomes. The fluorescence intensity (E_x = 570 nm, E_m = 590 nm) was measured with a bottom read mode in the Tecan plate reader. The data from the homogenous LysoTracker staining assay were normalized using the WT NSCs as a negative control and DMSO-treated NPC NSCs as a positive control to calculate IC₅₀ values, S/B ratio, CV, and Z factor.

High-Throughput Fluorescent Calcium Response Assay

ATP (a purinergic receptor agonist), carbachol (a muscarinic receptor agonist), and calcium ionophore ionomycin were

used as stimuli for intracellular calcium release (referred to as agonists). The purinergic receptor antagonists suramin hexasodium and AR-C 118925XX, and muscarinic acetylcholine receptor antagonists tolterodine and telenzepine, as well as calcium chelators tetra(acetoxymethyl ester) (EGTA-AM) and 1,2-bis(2-aminophenoxy)ethane- N,N,N',N' -tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM), were used as the antagonist compounds for the relevant stimulus (referred to as antagonist). The 25 μ L NSCs were plated at 10,000 cells/well in the 384-well, black, clear-bottom plates in StemPro NSC Serum Free Medium complete culture medium supplemented with 10 μ g/mL of rhVTN-N. The neurons were differentiated for 4 weeks as described above in the NSC one-step seeding with rhVTN-N-supplemented medium in commercial PDL-precoated 384-well plates. The fluorescence calcium dye (Cal-52-AM, AAT Bioquest) was prepared in DMSO at 2.5 mg/mL. The calcium dye loading solution was prepared by adding 10 μ L to a mixture of 9 mL of Hanks' balanced salt solution (HBSS) and 1 mL of 10 \times calcium assay buffer with quencher dye (Screen Quest 10 \times calcium assay buffer with Phenol Red Plus, AAT Bioquest). The loading solution was added at 25 μ L/well and incubated for 3 h at 37 $^{\circ}$ C with 5% CO $_2$, followed by a 30-min incubation at room temperature. For calcium agonist response experiments, agonists were prepared at 3 \times of the final concentration in HBSS buffer for a 16-concentration titration (1:2 dilution) in a 384-well compound plate in triplicate. The assay plate and compound plate were then placed into the FDSS μ CELL kinetic fluorescence plate reader (Hamamatsu, Japan) to measure fluorescence intensity kinetically for 5 min at 1 read/s (1 Hz). After the baseline fluorescent signals were measured for 15 s, 25 μ L/well of 3 \times agonist solutions in a titration were added to the assay plate from the compound plate of each well at the speed of 50 μ L/s, and the fluorescent intensity was continually recorded at 1 Hz, for 300 s in total. The ratio (F/F_0) of fluorescent intensity at each time point (F) after adding agonists to the average fluorescence intensity of baseline (F_0) was calculated. The maximum ratio (F_{\max}/F_0) of each well was used for calculation of EC $_{50}$ values.

For the antagonist experiments, the EC $_{80}$ concentration of each agonist was added as the stimulus to induce the calcium response. Prior to the calcium response experiment, the cells were treated with the antagonists for 30 min at 25 μ L/well with 16 concentrations of compound titration at a 1:2 ratio (see **Suppl. Fig. S3A**). The final DMSO concentration in both the antagonists and DMSO control well was 0.5%. The 4 \times agonist compound solutions (at their EC $_{80}$ values) were prepared in HBSS in 384-well compound plates. The dye loading procedure and detection method were the same as described above.

Single-Cell Calcium Response Recording

The NSCs and differentiated neurons were loaded with a calcium indicator as described above. The fluorescence images before and after addition of calcium releasing agonists were

recorded at 1 Hz in an IN Cell 2200 automated imaging station equipped with an integrated liquid handling system (GE Healthcare). All fluorescence images were acquired kinetically at 37 $^{\circ}$ C in the IN Cell 2200 automated imaging system using a 20 \times objective lens with standard DAPI and FITC excitation/emission filter sets. Cells were imaged at 1 Hz (1 frame per second) for a total of 180 s, with autofocus enabled at each time point. At time point 1, both DAPI (for nuclei) and FITC (for calcium response) images were acquired. But for the remaining 179 cycles, only FITC images were acquired. At time point 5, 25 μ L/well of agonist compound solution or control solution was added via an onboard liquid handling system. Images were collected at standard 1 \times 1 binning and analyzed with the IN Cell Workstation software v3.7.2 using the multi-target analysis algorithm. Briefly, Hoechst-stained nuclei (channel 1) were identified using top-hat segmentation looking for objects with a typical area of 75 μ m 2 , and green Cal-520 dye-stained cells (channel 2) were identified using multiscale top-hat segmentation looking for objects with a typical size of 200 μ m 2 . For the kinetic analysis, the nuclear images acquired at time point 1 were used as a unique cell ID for all subsequent time points and the Cal-520 images were analyzed at each time point for each cell. The average cellular fluorescence intensity ratio (F/F_0) for individual cells was plotted over the time. F_0 is the mean fluorescence intensity of 10 initial reads without addition of a stimulus, and F is the fluorescence intensity at a given time. The ratio of maximum fluorescence intensity (F_{\max}/F_0) collected from each cell was used for determination of positive responding cells. To define the positive calcium in a cell, an average of the maximal intensity ratio (F_{\max}/F_0) plus three times the standard deviation (mean + 3*SD) in the DMSO control was used as a threshold. The percent of cells with calcium response was calculated using the following equation: Calcium responding cells (%) = (Number of cells with their (F_{\max}/F_0) ratios \geq ($F_{\max(\text{control})}/F_0$) + 3 \times SD)/Total cell number in the wells that were imaged.

Data Analysis and Statistics

IC $_{50}$ or EC $_{50}$ concentration values of compound confirmation data were calculated using Prism software (GraphPad Software). Unless otherwise noted, all values are expressed as the mean \pm SD ($n = 3$). For statistical analysis, results were analyzed using one-way or two-way analysis of variance (ANOVA) and differences were considered statistically significant if $p < 0.05$.

Results

rhVTN-N-Supplemented Medium for One-Step Seeding of Neural Stem Cells

Plate precoating with positively charged synthetic polymers and/or biological reagents containing ECM proteins such as Matrigel, fibronectin (FN), and LN is required for culture

and growth of neuronal cells in assay plates. The plate pre-coating process involves multiple steps of reagent addition, incubation, and plate washing, which are labor-intensive and time-consuming, as well as a possibility of microbe contamination. Although synthetic polymers such as PDL and PLO can be coated on plates in advance, the ECM proteins must be freshly coated in a thin layer to assay plates prior to the experiments because the coated proteins cannot be dried in the plates. To simplify the experiments using neuronal cells, we designed a one-step seeding of neuronal cells by adding ECM proteins to cell culture medium without a need for plate pre-coating (**Fig. 1A**). We first tested the addition of one of nine commonly used coating reagents in cell culture medium for one-step seeding of NSCs in tissue culture-treated plates without plate pre-coating (**Suppl. Table S1, Fig. S1A–C**). We found that the direct seeding of cells suspended in the medium supplemented with rhVTN-N or FN exhibited the highest cell viability as measured by a homogenous ATP content assay (**Fig. 1B**). The results were comparable to those obtained with Matrigel-precoated plates (positive control). The cells seeded and cultured in the rhVTN-N-supplemented medium showed a better live-to-dead cell fluorescent intensity ratio than those in the FN-supplemented medium as determined by a nuclei dye assay (**Fig. 1B**). The NSC spreading and growth patterns observed from cells seeded in the rhVTN-N-supplemented medium were similar to those cultured in the Matrigel-precoated control plate, in contrast to cells cultured in FN-supplemented medium, which did not spread as completely (**Fig. 1C**). The cells seeded in the rhVTN-N-supplemented medium also exhibited similar positive NSC markers compared with the cells cultured in the Matrigel-precoated plate (**Fig. 1D**). We found that the NSCs seeded in rhVTN-N-supplemented medium exhibited normal attachment, spreading morphology, and a profile of NSC markers after 10 passages (**Suppl. Fig. S1D**), as well as normal chromosome morphology (**Suppl. Fig. 1E**). Therefore, 10 $\mu\text{g/mL}$ of rhVTN-N in medium for one-step seeding of NSCs in assay plates was selected as an optimal condition and used in the subsequent experiments.

One-Step Seeding of Neural Stem Cells for Neuron Differentiation

In order to determine if a one-step seeding method could be applied to neuron differentiation in assay plates, we plated NSCs in 384-well assay plates. We initiated the neuron differentiation during one-step seeding by adding the rhVTN-N-supplemented neuronal differentiation medium to commercial PDL-coated plates or tissue culture-treated plates. Neurons differentiated in the LN and PLO doubly precoated plates were used as a control.¹⁰ For neurons cultured in the rhVTN-N-supplemented medium in commercial PDL-coated plates, the cell growth rate and cell viability were similar to those cultured

in the control plates precoated with LN and PLO. However, most neuronal cells died after 2 weeks in culture in the rhVTN-N-supplemented medium in plates without PDL coatings (**Fig. 2A**). After 4 weeks of differentiation in the assay plates, the cells showed positive staining of β -III tubulin, microtubule-associated protein (MAP2), neurofilament-L (NF-L) with negative nestin, sex-determining region Y-box 2 (SOX-2), and glial fibrillary acidic protein (GFAP) (**Fig. 2B**), indicating relatively mature neurons. The neurite outgrowth rate of these neurons was also similar to that of the control (**Fig. 2C**), as measured by a mean length of neurite projections stained with β -III tubulin. The results indicate that neuronal cell differentiation in plates requires both rhVTN-N-supplemented medium and the use of commercial PDL-coated plates.

One-Step Seeding of Neural Stem Cells in rhVTN-N-Supplemented Medium for High-Throughput Cell Viability Assays

To validate this method, we first determined the viability of NSCs directly seeded in 1536-well plates with the rhVTN-N-supplemented medium. A set of parameters for HTS assays were first determined with cell viability experiments, including the ATP content assay, nuclear dye cell viability assay, and cell apoptosis assay for caspase-3/7 activity. The scatter plots of these three assays exhibited robust assay profiles (**Fig. 3A**). The S/B ratios of ATP content, nuclear dye, and caspase-3/7 assays were 123.8-, 34.3-, and 1.6-fold; CV values were 8.6%, 7.2%, and 4.4%; and Z factors were 0.74, 0.73, and 0.57, respectively. The results indicated that these assays are robust and suitable for HTS.

We also selected 24 neuronal toxic compounds from the literature (**Suppl. Table S2**) to determine the cytotoxicity in these assays using NSCs. These compounds exhibited cytotoxic effects concentration-dependently in NSCs (**Suppl. Fig. S2**). The IC_{50} values of these 24 compounds correlated well: $R^2 = 0.98$ for ATP and nuclear dye assays, $R^2 = 0.93$ for ATP and caspase-3/7 assays, and $R^2 = 0.92$ for nuclear dye and caspase-3/7 assays (**Fig. 3B**). Together, the results indicate that the method of NSCs directly seeded in the rhVTN-N-supplemented medium in 1536-well plates produced high-quality results and is useful for high-throughput cell viability assays.

Homogenous LysoTracker Staining Assay for Measurement of Enlarged Lysosomes in NPC Cells

We also applied this method to a phenotypic compound screening assay using NSCs differentiated from iPSCs of an NPC patient. NPC NSCs exhibit an NPC disease phenotype of enlarged lysosome due to cholesterol accumulation; this can be detected by the LysoTracker dye staining assay.^{10,15} The fluorescence signal of LysoTracker dye staining in

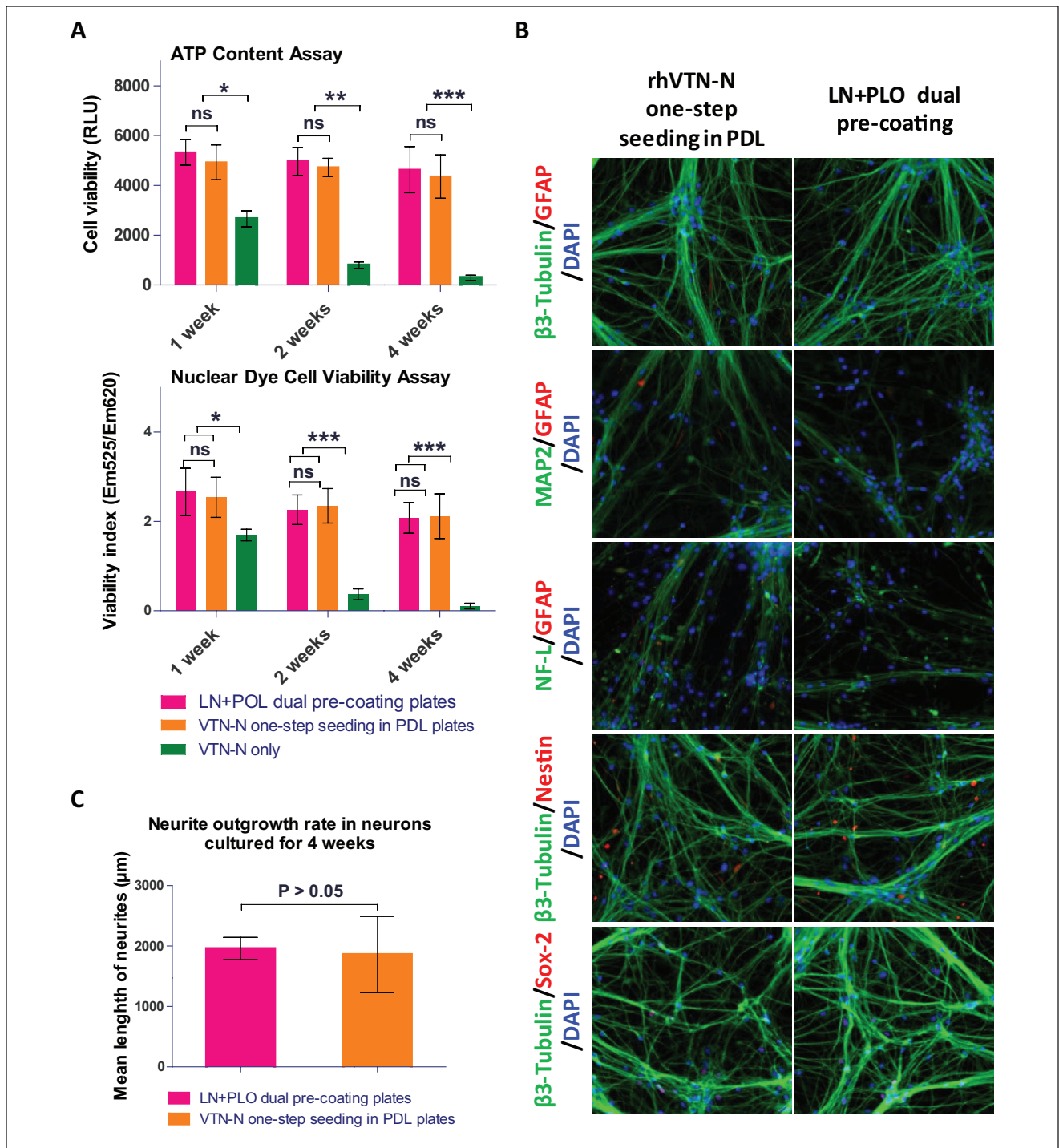


Figure 2. Results of direct seeding of NSCs for neuron differentiation. **(A)** Results of cell viability determined in the ATP content and nuclear dye assays in neurons differentiated from NSCs. Cells directly seeded in commercial 384-well PDL-coated plates with the rhVTN-N-supplemented medium showed cell viability similar to that of the control cells plated in the traditional LN + PLO dual-coated plates ($p > 0.05$). However, neurons differentiated from NSCs directly seeded in regular (noncoated) 384-well plates with the rhVTN-N-supplemented medium exhibited low cell viability ($***p < 0.05$). Data are represented as the mean \pm SEM from three replicates. **(B)** Images of neurons differentiated from the direct seeding of NSCs in rhVTN-N-supplemented medium compared with the control condition of LN + POL plate precoating neurons. The NSCs differentiated to neurons with similar positive staining of the β -III tubulin, MAP2, and NF-L neuronal markers as the control cells cultured in LN + PLO dual-coated plates. The neurons also exhibited positive SOX-2 staining; they were negative for the markers nestin and GFAP (an astrocyte marker). **(C)** rhVTN-N. The β -III tubulin-stained neurites were measured after 4 weeks of differentiation in assay plates. The neurite outgrowth rate of neurons differentiated from NSCs directly seeded in commercial 384-well PDL plates with rhVTN-N-supplemented medium was similar to that of NSCs seeded in LN + PLO dual-coated plates ($p > 0.05$). Data are represented as the mean \pm SEM from three replicates.

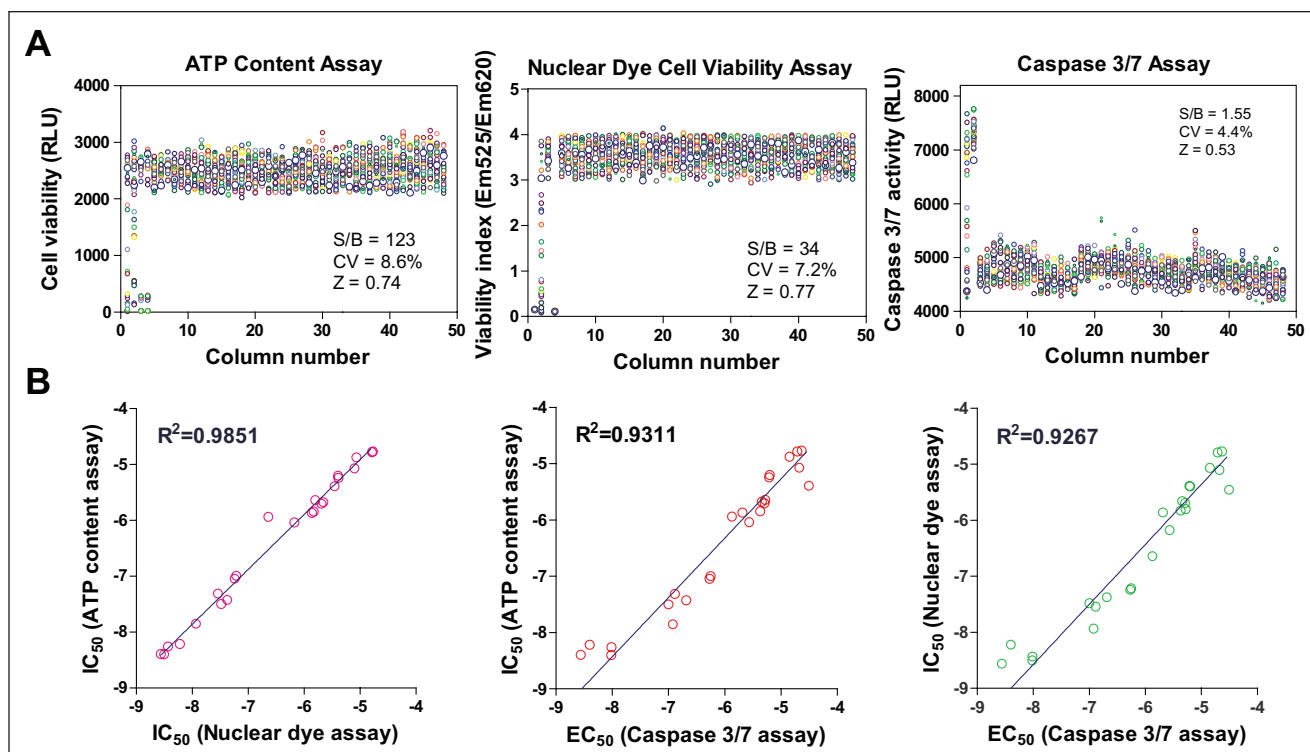


Figure 3. Assay performances of one-step seeding of NSCs in 1536-well plates. **(A)** Scatter plots of DMSO plate tests in the ATP content, nuclear dye, and caspase-3/7 assays using NSCs directly seeded in rhVTN-N-supplemented medium in 1536-well plates. The results of HTS assays (Z score > 0.5) indicated statistically robust performances in all three assays. Each circle was the data from a single well in assay plates. The first column was a concentration titration of the control compound, and the next column was the highest concentration of control compound. The rest of the columns were added with DMSO, a solvent control for compounds. **(B)** Correlation cytotoxicity activities (IC_{50} or EC_{50} values) of 24 reported neurotoxic compounds determined in the ATP content, nuclear dye, and caspase-3/7 assays. The activities of these compounds correlated well in all three assays, with R^2 values from 0.93 to 0.98.

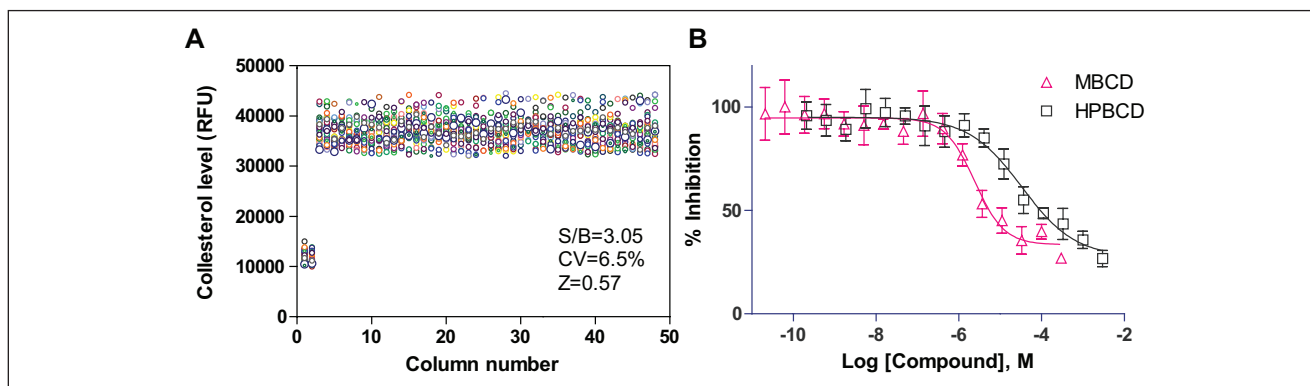


Figure 4. Results of the homogenous LysoTracker dye staining assay using NPC patient NSCs directly seeded in 1536-well plates with rhVTN-N-supplemented medium. **(A)** Scatter plot of results from a DMSO plate test. Columns 1 and 2 were WT NSCs and columns 3–48 were NPC NSCs that showed increased LysoTracker dye staining (enlarged lysosomes) due to the lysosomal cholesterol accumulation in the patient cells. **(B)** Concentration–response curves of two positive control compounds: MBDC and HPBCD. The IC_{50} values of MBDC and HPBCD were 2.4 and 33.7 μ M in the LysoTracker assay in the NPC NSCs, respectively. Data are represented as mean \pm SEM from three biological replicates.

NPC cells can be measured by fluorescence microscopy and also by a fluorescence plate reader.¹⁵ We found that the S/B ratio was 3.05-fold and the Z factor was

0.57, as determined by a LysoTracker dye staining assay in 1536-well plates when measured using a plate reader (**Fig. 4A**). The IC_{50} values of two control compounds, MBDC

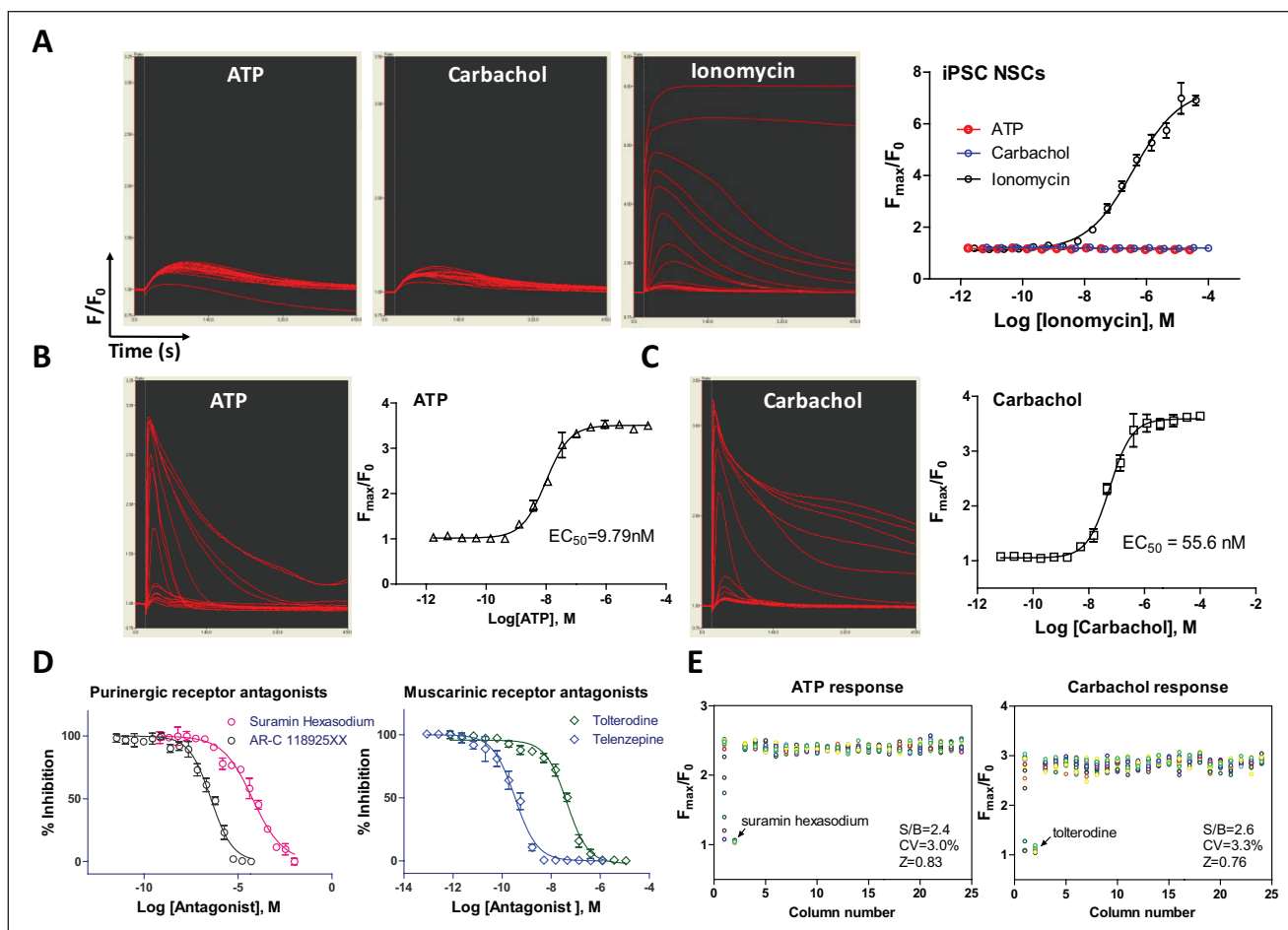


Figure 5. Results of calcium response assay using NSCs directly seeded in rhVTN-N-supplemented medium in 384 plates. **(A)** Kinetic fluorescence intensity tracers of intracellular calcium responses of NSCs stimulated by ATP (a purinergic receptor agonist), carbachol (an acetylcholine muscarinic receptor agonist), and ionomycin (calcium ionophore). In the NSCs, only small and insignificant responses to ATP and carbachol were observed, while ionomycin showed a robust signal with an EC_{50} of 0.39 μ M. **(B,C)** Kinetic fluorescence intensity tracers and concentration-response curves of intracellular calcium determined in neurons differentiated from NSCs stimulated by ATP (EC_{50} = 9.8 nM) and carbachol (EC_{50} = 55.7 nM). **(D)** Inhibitory concentration-response curves of two purinergic receptor antagonists and two muscarinic acetylcholine receptor antagonists determined in neurons differentiated from NSCs. Data are represented as mean \pm SEM of at least three biological replicates. **(E)** Scatter plots of ATP-induced and carbachol-induced intracellular calcium responses. The S/B ratio, CV, and Z factor determined in a DMSO plate test exhibited robust assay parameters (S/B ratio > two fold, Z score > 0.5). Wells in column 1 were antagonist titration and in column 2 were the highest concentration of antagonist.

and HPBCD, were 2.4 and 33.7 μ M, respectively (Fig. 4B), similar to a previous report.¹⁰ The results demonstrated that the one-step seeding method can also be used with patient-derived NSCs in a HTS context.

High-Throughput Fluorescent Calcium Response Assays Using Neural Stem Cells and Neurons

To further validate this method, we tested it with a high-throughput calcium response assay in a 384-well assay plate (Suppl. Fig. S3A). We first measured the total calcium response using a fluorescence kinetic plate reader in a homogeneous format without the plate washing steps. In the

NSCs, ATP (a purinergic receptor agonist) and carbachol (a muscarinic receptor agonist) did not stimulate significant intracellular calcium responses, whereas the calcium ionophore ionomycin concentration dependently induced a calcium response with an EC_{50} of 0.39 μ M (Fig. 5A, Suppl. Fig. S3B), similar to that previously reported.¹⁷ The calcium response of ionomycin in these cells was blocked by calcium chelators EGTA-AM and BAPTA-AM with IC_{50} values of 3.3 and 2.8 μ M, respectively (Suppl. Fig. S3C). The S/B ratio, CV, and Z factor with the ionomycin-stimulated calcium response and EGTA blocked were 4.68-fold, 6.32%, and 0.75, respectively (Suppl. Fig. S3D), indicating a robust HTS assay.

While NSCs only express low levels of receptors and ion channels,¹⁸ the differentiated neurons express much higher levels of ion channels and receptors, including calcium channels, purinergic receptors, glutamate receptors, and ryanodine receptors.¹⁹ We then measured the intracellular calcium responses to ATP and carbachol in differentiated neurons plated in commercial PDL-coated 384-well plates with the rhVTN-N-supplemented medium. The EC₅₀ values of the ATP and carbachol were 9.8 and 55.7 nM, respectively (**Fig. 5B,C**). The calcium responses stimulated by ATP and carbachol were concentration dependently inhibited by the purinergic receptor antagonists suramin hexasodium (IC₅₀ = 59.5 μ M) and AR-C 118925XX (IC₅₀ = 359.8 nM) and the muscarinic acetylcholine receptor antagonists tolterodine (IC₅₀ = 42.8 nM) and telenzepine (IC₅₀ = 0.3 nM), respectively (**Fig. 5D**). These calcium responses in neurons were robust with the favorable HTS assay parameters (**Fig. 5E, Suppl. Fig. S4**). Together, the results demonstrate the excellence of the high-throughput calcium response using our new method with the rhVTN-N-supplemented medium.

Single-Cell Calcium Response in Neural Stem Cells and Neurons

Because not all of the cells in a population respond equally to a stimulus, a single-cell recording of calcium response offers a much more sensitive detection system that distinguishes the responding cells from nonresponding cells in a cell population. Using a kinetic fluorescence imaging system, we measured the single-cell calcium response in NSCs and neurons, in addition to the above whole-well calcium response. In NSCs, only a small portion of cells responded to the ATP or carbachol stimulation (**Fig. 6A**), while the majority of cells responded to ionomycin (**Suppl. Fig. S5**). The percentage of positive responding NSCs was 26% for ATP and 18% for carbachol. The results suggested that the weak calcium response in NSCs is more distinguishable by the single-cell calcium response than that which is otherwise not detectable by the total calcium response in a well of assay plates, as described above. In differentiated neurons, ATP and carbachol induced extensive calcium responses measured at the single-cell level (**Fig. 6B**). On the contrary, the majority of neurons responded to agonists: 97% responded to ATP and 86% responded to carbachol stimulation ($p < 0.01$) (**Fig. 6C**). The results indicated that the neurons differentiated from NSCs in the rhVTN-N-supplemented medium directly seeded in commercial PDL-coated plates responded well to ATP and carbachol stimulations.

Discussion

We described here a simple method (**Suppl. Fig. S6**) for directly seeding NSCs in assay plates without the need for precoating. By addition of a recombinant VTN protein to

the medium, the labor-intensive plate precoating and plate wash steps are eliminated from the workflow. NSCs can be directly seeded in regular tissue culture-treated plates with the VNT-N-supplemented medium. Neuronal cells can be differentiated from NSCs that were seeded in commercial PDL-coated plates with the VNT-N-supplemented neuronal differentiation medium. Because no additional reagent and plate wash steps are needed, this new method simplifies the experimental procedure, improves assay throughput, and increases reproducibility.

Additional surface treatment is often needed for culturing primary cells and neurons, although tissue culture-treated plasticware prepared by gas plasma is generally used.²⁰ The additional surface treatment provides an environment similar to that of the ECM that the cells experience in vivo. ECM consists of secreted proteins that mediate proper attachment, spreading, growth, and differentiation of neuronal cells.²¹ In many types of cell culture, fetal bovine serum (FBS) supplementation provides ECM proteins and various growth factors to cells. But FBS is usually not added to culture medium for stem cells and neurons differentiated from iPSCs, because only a few specific growth factors can be added in the medium to prevent uncontrolled differentiation. Therefore, precoating of dishes and plates with ECM proteins and polymers is a common practice for culturing stem cells and neurons, although the process is labor-intensive with a high reagent cost. Neuronal cell culture requires freshly precoated plates, covered with thin layers of both ECM proteins and positively charged polymers. Because an ECM protein like LN is very easily absorbed by plastic and tends to form aggregates in plates at room temperature, precautions such as keeping the coating material on ice are usually needed for the plate precoating.²²

Positively charged synthetic polymers such as PDL, polyethyleneimine, and PLO are commercially available and commonly used to coat dishes and plates. They provide electrostatic interaction with the negatively charged cell membrane, supporting cell attachment.²³ These materials are usually stable and can be precoated in assay plates in advance or can be obtained from commercial vendors. On the other hand, extracellular proteins such as Matrigel, FN, LN, and VTN have to be freshly coated onto assay plates. A dual precoating with LN and PLO in assay plates is commonly used for neuronal cell culture and neuronal cell differentiation. The process involves dispensing of a coating reagent to plates, incubation, and multiple plate wash steps.^{24,25} A recent protocol for using the rhVTN-N to precoat plates describes that it is unnecessary to wash plates after the plate coating, but it still requires removal of VTN (http://tools.thermofisher.com/content/sfs/manuals/vitronectin_man.pdf).

To eliminate the need for plate precoating, we first tested the addition of ECM proteins and polymers into cell culture medium for directly seeding cells onto plates without plate

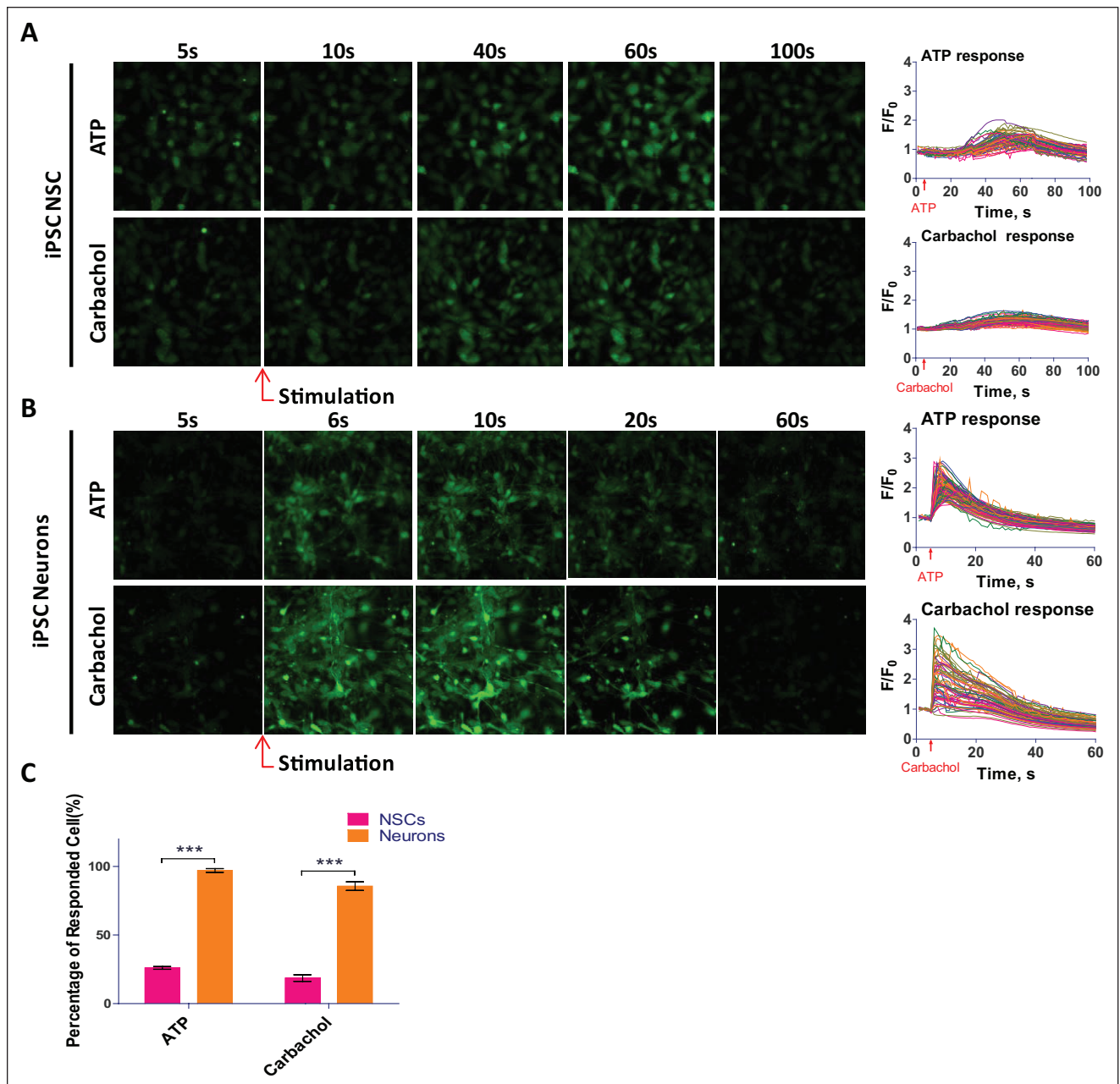


Figure 6. Results of single-cell calcium responses in NSCs and neurons with NSCs directly seeded in rhVTN-N-supplemented medium in 384-well plates. **(A)** Serial fluorescence images and kinetic tracers of NSCs recorded in a single-cell calcium response assay. **(B)** Serial fluorescence images and kinetic tracers of neurons recorded in a single-cell calcium response assay. **(C)** Comparison of percentage of responding cells determined from the single-cell calcium response in NSCs and neurons. A positive responding cell was defined by a cell responding to a stimulus with a $>3 \times SD$ increase in mean calcium signal of the control cells. The positive responding NSCs were 26% for ATP stimulation and 18% for carbachol stimulation, whereas positive neurons were 97% for ATP and 86% for carbachol. Data are represented as mean \pm SEM of at least three biological replicates.

precoating. Nine reagents were examined, including rhVTN-N, Matrigel, FN, Geltrex, LN, collagen type IV, gelatin, and two polymers, PDL and PLO. (Suppl. Table S1). We found that NSCs seeded in rhVTN-N-supplemented medium exhibited a similar viability, attachment, and spreading morphology when compared with those cultured

in the traditional Matrigel-precoated plates (Fig. 1B,C). The cells displayed a normal profile of NSC markers and normal chromosome morphology. The results measured in the cell viability and calcium response assays were robust with high reproducibility (Figs. 2–4). Therefore, this method is useful for HTS assays used to identify lead

compounds using NSCs for the cell-based disease model. It also can be applied to cell viability assays and cytotoxicity assays that employ NSCs to determine compound neurotoxicity.²⁶ In addition, we utilized it in a few assays with neurons differentiated from NSCs (Fig. 2A). We found that only the addition of rhVTN-N to the medium is not enough for a proper neuron differentiation. A combination of rhVTN-N-supplemented medium and PDL-coated plates is needed. PDL has been commonly used for plate coating. PDL-coated plates can be sterilized and stored for over a year. These coated plates are also available from commercial vendors. On the other hand, we found cytotoxic effect of two polymer-supplemented mediums, suggesting that the polymer-supplemented medium is not suitable for seeding of cells without plate washes (Fig. 1B).

NSCs,²⁷ neural progenitor cells,²⁸ and neurons differentiated from stem cells¹³ have recently been employed for compound screens, but the screening throughput of these reported experiments was limited due to the requirement of plate pre-coating to support cell attachment and growth. Matrigel, FN, LN, VTN, PLO, and PDL have been used as substrates for plate pre-coating in neuronal cell culture (Suppl. Table S3). Matrigel is a protein mixture secreted by Engelbreth-Holm-Swarm mouse sarcoma cells that supports cell attachment on plasticware. As a mixture, Matrigel suffers from large batch-to-batch variations and potential xenogeneic contamination. Both FN and LN are ECM glycoproteins with high molecular weight. However, human VTN is a relatively small (75 KDa) glycoprotein that is present in serum and supports cell adhesion and spreading.²⁹ It has also been reported that VTN-supported human embryonic stem cell self-renewal via an interaction with $\alpha V\beta 5$ integrin and stem cells maintained normal morphology and functions in the presence of VTN.^{29,30} rhVTN-N is a truncated recombinant VTN corresponding to the amino acid fragment 62–478 of human VTN expressed in *Escherichia coli*.¹² rhVTN-N has recently been used in iPSC culture medium for supporting human iPSC attachment and growth.¹² The size of rhVTN-N is smaller than that of other ECM proteins, and its cost is relatively low. Therefore, rhVTN-N is a good choice as a supplement to cell culture medium for one-step seeding of NSCs compared with other ECM proteins.

Although the plate pre-coating can be handled in these experiments for testing a few compounds, it is a burden for HTS with a large amount of compounds. Reductions of robotic steps in a screening assay can significantly increase compound screening throughput, reduce the chances of interruptions in robotic operation due to errors and malfunctions of liquid handlers, and increase data quality by decreasing well-to-well and plate-to-plate variations. This method of one-step seeding of NSCs significantly reduces assay steps, which is specifically useful for robotic screening of large compound collections using neuronal cells.

In conclusion, we have developed a simple method for one-step seeding of NSCs in assay plates with an rhVTN-N-supplemented medium without the need for plate pre-coating. This method greatly increases the throughput and reproducibility of experiments using neuronal cells derived from human iPSCs for translational research. Additionally, the principle of this method has the potential to be extended to seed iPSCs and iPSC-derived progenitor cells, which in turn can be differentiated into other cell types for various experiments.

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Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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