TheWell BIOSCIENCE

3D Cell Culture and Beyond

VitroGel® NEURON

Xeno-free hydrogel for 3D and 2D neuronal culture

Catalog #: VHM07, VHM07S
Protocol, Data Analyses, and Case Studies

For research use only. Not intended for use in diagnostics or therapeutic procedures.

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VitroGel® NEURON

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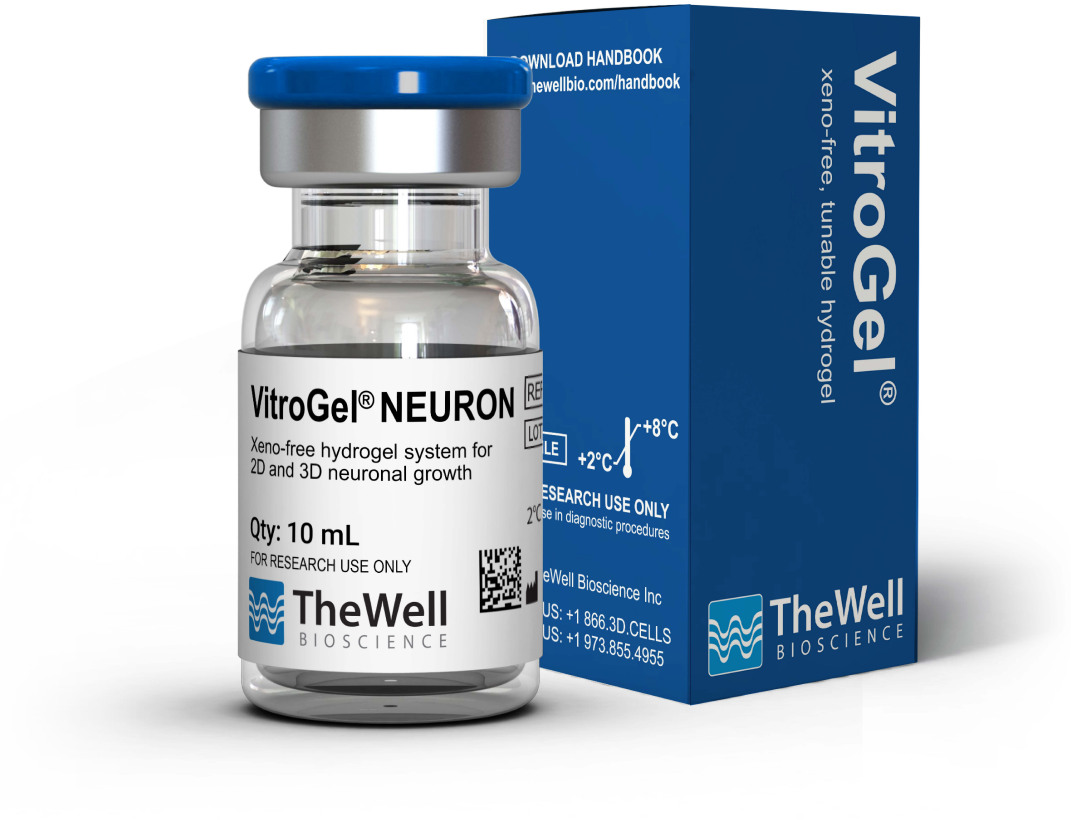
INTRODUCTION

VitroGel® NEURON hydrogel is a synthetic matrix with functional ligands that support the culture of neuronal neuroblasts, mature neurons, and iPSC-derived neural stem cell (NSC) maintenance and differentiation. The hydrogel can be used for 2D and 3D cell culture applications.

VitroGel® NEURON hydrogel is a ready-to-use, xeno-free, transparent, and room temperature stable system, compatible with imaging systems and suitable for laboratory automation and clinical applications. VitroGel® NEURON hydrogel polymerizes once the solution is combined with the medium. Growth factors and supplement systems can be mixed with the matrix or added on top of the gel to support NSC cultures.



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2D Cell Culture Protocol

2D thin coating protocol for Neural stem cells (NSCs)

VitroGel® NEURON is a defined synthetic hydrogel optimized for the growth and differentiation of cells of the neuron lineage. **Unlike animal-based ECM, VitroGel® NEURON coating saves both time and resources as it does not require removal or washing prior to use.** The cell culture plate can be used directly after a 30 minutes incubation with the RocketCell™ NSC Xeno-Free Medium diluted VitroGel® NEURON solution. **Generally, no or few minor changes to widely used cell culture maintenance protocols need to be made to use VitroGel® NEURON.**

MATERIALS

- **VitroGel® NEURON hydrogel (Catalog #: VHM07)**
- iPSC-derived NSCs
- Accutase (Innovative Cell Technologies, Inc., Catalog #: AT104)
- **RocketCell™ NSC Xeno-Free Medium***
- Rho-kinase (ROCK) inhibitor (10 µM of Y-27632 or 2 µM Thiazovivin)*
- **VitroPrime™ Spread-Attach Plates**
  - » 6-well (Catalog # VP-SA6W)
  - » 12-well (Catalog # VP-SA12W)
  - » 24-well (Catalog #: VP-SA24W)
  - » 48-well (Catalog # VP-SA48W)
  - » 96-well (Catalog # VP-SA96W)

* Medium is supplemented with penicillin/streptomycin and GlutaMAX™

PROTOCOL

1. Allow VitroGel® NEURON hydrogel and RocketCell™ NSC Xeno-Free Medium (supplemented with ROCK inhibitor) to reach room temperature (25°C).
2. Dilute VitroGel® NEURON hydrogel with RocketCell™ NSC Xeno-Free Medium (supplemented with ROCK inhibitor) at a 1:200 ratio and mix thoroughly (i.e., combine 10 µL of hydrogel and 2,000 µL of medium). Refer to Table 1 for suggested volumes of hydrogel mixture based on your cell culture plate well size.

   Note:
   - » For smaller volumes, homogenize the mixture 3-5 times using a micropipette.
   - » For larger volumes, use a serological pipette to mix several times to ensure a homogeneous mixing.
   - » Alternative method: Use a vortex at low speed to mix media while adding the required VitroGel® NEURON.

Table 1: Recommended volume of diluted hydrogel to add based on well size.

	96-well plate	48-well plate	24-well plate	12-well plate	6-well plate
Volume of hydrogel-medium mixture per well	70 µL	180 µL	300 µL	600 µL	1200 µL

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3. Add the recommended volume (Table 1) of the diluted hydrogel to each well of the VitroPrime™ Spread-Attach Plate.
4. Incubate the diluted hydrogel in cultureware for 30 minutes at room temperature. **Optional:** If cells are not ready for plating, place cultureware with diluted hydrogel in the incubator for up to an additional 1.5 hours.
5. Harvest NSCs using Accutase based on standard cell culture procedures. Prepare cell suspension in RocketCell™ NSC Xeno-Free Medium (supplemented with ROCK inhibitor). Refer to Table 2 for recommended cell suspension density and volume based on well plate size.

   CRITICAL POINT: We highly recommend using Accutase, as it leads to efficient cell attachment compared to other enzymes.

   Table 2: Recommended volume and cell density based on plasticware well size.

	96-well plate	48-well plate	24-well plate	12-well plate	6-well plate
Volume/well	30 µL	120 µL	200 µL	400 µL	1000 µL
Number of cells/well	7500	30,000	50,000	100,000	250,000

   Note: Further titration of cell density may be needed to optimize for each individual IPSC-derived NSC line.

6. Add cell suspension to the wells containing the diluted hydrogel (check Table 2 for recommended volume and cell number).

   Note: No need to remove the coating hydrogel-medium mixture from the well. Add the cell suspension directly.

7. Incubate cells overnight at 37°C.

   CRITICAL POINT: We highly recommend that the plate and/or incubator not be disturbed for at least 2-4 hours post passaging to allow optimal adhesion. The most recommended procedure would be to passage at the end of the day leaving the incubator undisturbed overnight.

8. The following day, monitor the cultures using an inverted microscope.
9. Replace 70-75% of the medium in culture with RocketCell™ NSC Xeno-Free Medium without ROCK inhibitor every 2 days or 3 days (weekend; double the volume of fresh medium). Monitor the cultures using an inverted microscope.

2D "blanket" method for B35 cell line

MATERIALS

- **VitroGel® NEURON hydrogel (Catalog #: VHM07)**
- **VitroPrime™ Spread-Attach 96-well Plate (Catalog #: VP-SA96W)**
- Deionized water (DI H_2O)
- Cells
- Basal cell culture medium*
- Basal cell culture medium with 10% fetal bovine serum*
- Micropipette; low retention pipette tips
- Centrifuge tubes or conical tubes

* Media is supplemented with penicillin, streptomycin, and L-glutamine.

PROTOCOL

Day 1: Seeding cells

1. Allow the culture medium of choice supplemented with 10% FBS to reach room temperature.
2. Prepare cell suspension in culture medium of choice supplemented with 10% FBS, at a concentration of 1.3 x 10^5 cells/mL.
3. Add 100 µL of cell suspension to each well of the VitroPrime™ Spread-Attach 96-well Plate.
4. Incubate cells overnight in a humidified cell culture incubator at 37°C.

Day 2: Adding the hydrogel on-top of cells for 2D neuronal differentiation

Preparation of hydrogel mixture (Select from Option A or Option B method below)

Option A

1. Allow hydrogel and basal medium to reach room temperature.
2. Prepare a 7:1 mixture of hydrogel with basal medium (i.e., combine 70 µL of hydrogel with 10 µL of basal medium). Homogenize the mixture by pipetting gently 3-5 times. Avoid bubbles.

   Note: The mixing ratio between the hydrogel and basal medium should be within the range of 5:1 to 10:1 (v/v, gel/medium).

   IMPORTANT: The ionic molecules in the medium increase the hydrogel's viscosity and induce solidification. After preparing the hydrogel mixture, immediately proceed to the section after option B to prevent precipitated gelation of the sample. Do not wait.

Option B (Alternative method for Option A)

1. Keep hydrogel and basal medium at cold (4°C) temperature.

   Note: The reagents should be used from the refrigerator. Do not thaw.

2. Perform a 2:1 dilution of hydrogel and DI H_2O (i.e., combine 40 µL of hydrogel with 20 µL of DI H_2O). Pipette sample 3-5 times carefully to avoid bubbles. Refer to Table 3.
3. Mix hydrogel and basal medium in a 4:1 v/v ratio (i.e., combine the 60 µL of the previously diluted hydrogel with 15 µL of basal medium). Gently pipette the mixture 3-5 times to avoid bubbles. Refer to Table 3.

   IMPORTANT: The ionic molecules in the medium increase the hydrogel's viscosity and induce solidification. After preparing the hydrogel mixture, immediately proceed to the next section to prevent precipitated gelation of the sample. Do not wait.

Table 3: Preparation of hydrogel mixture for neuronal differentiation using the "blanket" hydrogel method - Option B

Volume of hydrogel	Volume of Deionized water	Volume of Basal medium
40 µL	20 µL	15 µL

Continuation of protocol after performing Option A or B

1. Remove medium from the wells.
2. Add 35 µL of hydrogel mixture on top of the cells.

   Note: Make sure to carefully add the hydrogel mixture by placing the pipette against the wall of the well.
3. Allow the hydrogel mixture to solidify for 30 minutes at room temperature.
4. Add 100 µL of basal medium on top of the hydrogel.
5. Incubate the cells inside a humidified chamber at 37°C.
6. Replace the basal medium every 2-3 days and perform cell imaging using a microscope to evaluate neuronal differentiation.

   Note: We recommend removing 70% of the medium to avoid disrupting the hydrogel.

Immunofluorescence protocol performed after using VitroGel® NEURON hydrogel for culturing neurons via the "blanket" method

MATERIALS

- Cells cultured using VitroGel® NEURON hydrogel
- Dulbecco's Phosphate-Buffered Saline (DPBS), no calcium, no magnesium (Wash buffer)
- 4% formaldehyde solution (Fixation solution)
- 0.1% Triton X-100 (Permeabilization solution)
- 3% bovine serum albumin (BSA) in DPBS (Blocking solution)
- Beta-III-tubulin primary antibody (Thermo Fisher, catalog #: MA1-118)
- Alexa 488 secondary antibody (Thermo Fisher, catalog #: A32723)
- NucBlue™ Fixed Cell ReadyProbes™ Reagent (DAPI) (Thermo Fisher, catalog #: R37606)
- Parafilm sealing film
- Micropipette; Low retention pipette tips
- Fluorescence microscope

PROTOCOL

(Calculations are based on using a 96 well-plate, 35 µL gel/well as an example. Adjust accordingly for scale-up)

1. Carefully remove the cover medium on top of the hydrogel.
2. Add 100 µL of DPBS to wash the hydrogel and wait 1 minute before discarding. Wash for a total of 3 times.
3. Add 100 µL of 4% formaldehyde and incubate at 4°C for 30 minutes*.
4. Remove the fixation solution and wash 3 times with 100 µL of DPBS. Wait 1 minute between washes.

   Optional stopping point: After removing the fixative, add 100 µL of DPBS and seal the plate with parafilm. Store in the refrigerator at 4°C for up to 1 month.
5. Add 100 µL of permeabilization solution and incubate at 4°C for 15 minutes*.
6. Remove the permeabilization solution and carefully wash 3 times with 100 µL of DPBS. Wait 1 minute between washes.
7. Add blocking solution (3% BSA) and incubate for 15 minutes at 4°C*.
8. Dilute the primary antibody anti-beta-III-tubulin in blocking solution at a 1:100 ratio (v/v, antibody/blocking solution) and gently homogenize.
9. Remove the blocking solution. Add 100 µL of primary antibody dilution to each well and store the plate at 4°C overnight*.
10. Remove the primary antibody. Carefully wash with 100 µL of DPBS and wait 1 minute before discarding. Wash 3 times.
11. Dilute the secondary antibody (Alexa 488) in blocking solution at a 1:200 ratio (v/v, antibody/blocking solution). Add 100 µL of diluted secondary antibody to each well, cover the plate with aluminum foil, seal the plate edges with parafilm, and incubate in the dark for 4 hours at room temperature*.
12. Remove the secondary antibody and carefully wash 3 times with 100 µL of DPBS. Wait 1 minute for each wash.
13. Prepare DAPI solution by adding 2 drops of reagent to 1 mL of DPBS. Add 100 µL of DAPI solution to the wells, cover plate with aluminum foil, and incubate in the dark for 5 minutes at room temperature.
14. After incubation, the sample is ready for fluorescence imaging.

* Seal space between the lid and bottom part of the plate with parafilm.

CASE STUDY 1

Assessing neuronal differentiation using the hydrogel covering "blanket" method - Preparation of hydrogel mixture with Option A (recommended)

Materials

- **VitroGel® NEURON hydrogel (Catalog #: VHM07)**
- **VitroPrime™ Spread-Attach 96-well Plate (Catalog #: VP-SA96W)**
- B35 neuronal neuroblast cells
- DMEM 1X basal medium
- DMEM basal medium with 10% fetal bovine serum

B35 cells (1.3 x 10^5 cells/mL) were resuspended in DMEM basal medium with 10% FBS. One hundred microliters (100 µL) of cell suspension was added to the wells of the VitroPrime™ Spread-Attach 96-well Plate. The cultures were incubated overnight at 37°C. VitroGel® NEURON hydrogel was mixed with DMEM basal medium at a 7:1 ratio (v/v, gel/medium). Next, the medium from the wells was carefully removed. The hydrogel mixture (35 µL) was gently added on top of the cells and incubated for 30 minutes at room temperature for solidification. DMEM basal medium (100 µL) was added on top of the hydrogel, and the cultures were placed in the incubator at 37°C. The cultures were monitored with a microscope, and 70% of the medium was removed from the wells and replaced with fresh medium every 2-3 days.

Neuronal morphology and differentiation were evaluated by performing immunofluorescence staining on days 7, 14, and 23 (Figures 1-3). Cells were fixed and stained to assess the presence of the neuron-specific marker, beta-III-tubulin. The differentiated cells were observed by counterstaining with the secondary antibody Alexa 488, which generates a green-fluorescent signal once bound to the primary antibody targeting beta-III-tubulin (Figures 1B-3B). The nuclei were visualized with DAPI stain (Figures 1C-3C).

The findings illustrate that VitroGel® NEURON hydrogel sustains long-term neuronal differentiation, validated by the presence of beta-III-tubulin throughout multiple time-points. On day 7, we observed elongated axons and neural network formation, indicative of early neuronal differentiation (Figures 1, A-D). The data obtained in the subsequent time-points showed that VitroGel® NEURON hydrogel prolonged the culture of differentiated neurons, enhanced neuronal proliferation, and maintained viability (Figures 2-3, A-D). In conclusion, **VitroGel® NEURON hydrogel is a great tool for long-term neuronal culture and differentiation,** allowing researchers to evaluate a myriad of topics related to physiological and diseased states of the nervous system.

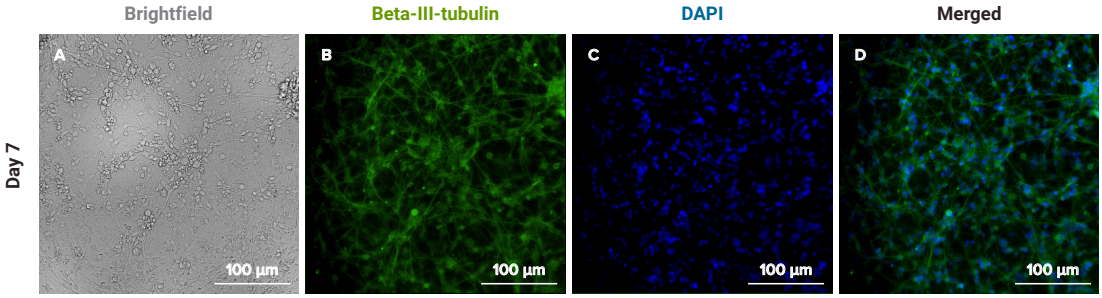

Figure 1: 2D "blanket" method using VitroGel® NEURON hydrogel promotes neuronal differentiation.

Immunofluorescence staining of neuronal cultures evaluating the presence of the neuron-associated marker beta-III-tubulin after 7 days post-differentiation induction. **A.** Light microscopy image showing neuronal cultures. **B.** Green-fluorescent image illustrating the presence of beta-III-tubulin. **C.** The nuclei were observed using DAPI (blue) staining. **D.** Combination of C and D images. The cultures were visualized using Molecular Devices ImageXpress Nano system.

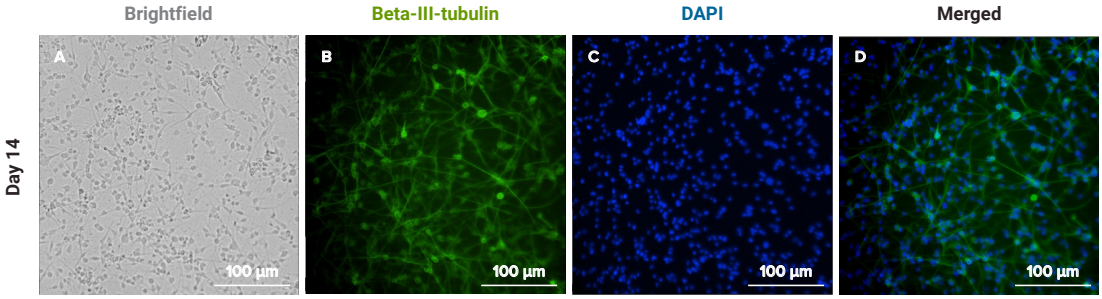

Figure 2: 2D "blanket" method using VitroGel® NEURON hydrogel prolongs *in vitro* neuronal differentiation.

Immunofluorescence staining of neuronal cultures was performed to evaluate the presence of the neuron-associated marker beta-III-tubulin 14 days post-differentiation induction. **A.** Light microscopy image showing neuronal cultures. **B.** Green-fluorescent image illustrating the presence of beta-III-tubulin. **C.** The nuclei were observed using DAPI (blue) staining. **D.** Combination of B and C images. The cultures were visualized using Molecular Devices ImageXpress Nano system.

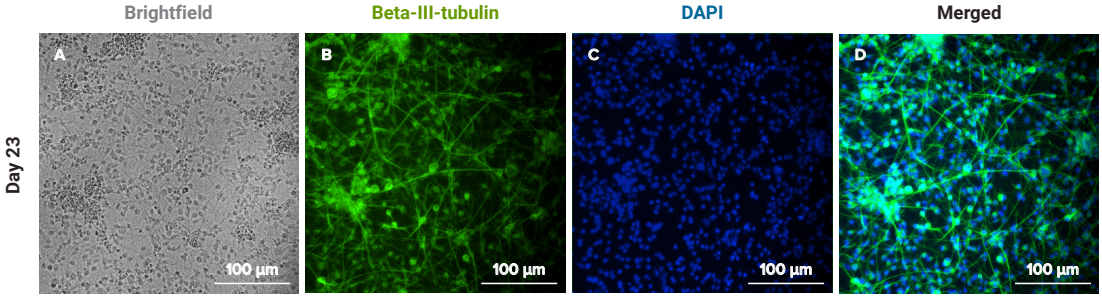

Figure 3: 2D "blanket" method using VitroGel® NEURON hydrogel sustains *in vitro* neuronal differentiation.

Immunofluorescence staining of neuronal cultures was performed to evaluate the presence of the neuron-associated marker beta-III-tubulin 23 days post-differentiation induction. **A.** Light microscopy image showing neuronal cultures. **B.** Green-fluorescent image illustrating the presence of beta-III-tubulin. **C.** The nuclei were observed using DAPI (blue) staining. **D.** Combination of B and C images. The cultures were visualized using Molecular Devices ImageXpress Nano system.

CASE STUDY 2

Evaluating neuronal differentiation using the hydrogel covering "blanket" method - Preparation of hydrogel mixture with Option B

Materials

- **VitroGel® NEURON** diluted in a 2:1 ratio with DI H_2O (v/v, gel/ DI H_2O) and mixed in a 4:1 ratio with DMEM basal medium (v/v, gel/medium)
- **VitroPrime™ Spread-Attach 96-well Plate (Catalog #: VP-SA96W)**
- B35 neuronal neuroblast cells
- DMEM 1X basal medium
- DMEM basal medium with 10% fetal bovine serum

Similar to the previous experiment, B35 cells (1.3 x 10^5 cells/mL) were resuspended in DMEM basal medium with 10% FBS, followed by the addition of a 100 µL of the cell suspension to the wells of the VitroPrime™ Spread-Attach 96-well Plate. The cells were incubated overnight at 37°C. The following day, VitroGel® NEURON hydrogel was diluted with DI H_2O in a 2:1 ratio (v/v, gel/ DI H_2O). The diluted hydrogel was combined with DMEM basal medium in a 4:1 ratio (v/v, gel/medium) and gently homogenized. Then, the medium was removed from the wells and 35 µL of hydrogel mixture was added on top of the cells. The hydrogel mixture was incubated for 30 minutes at room temperature, allowing it to solidify. After the incubation, 100 µL of DMEM basal medium was added on top of the hydrogel to induce differentiation, and the cultures were placed inside the incubator. The cultures were observed under the microscope on days 1, 3, and 7 (Figures 4-6).

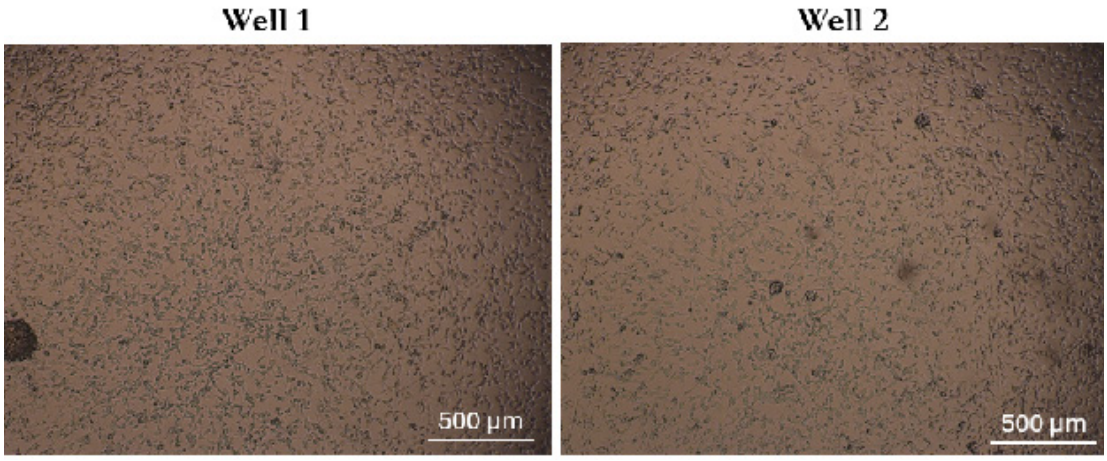

Figure 4: Neuronal differentiation of B35 cells using the "blanket" method by diluting VitroGel® NEURON with DI H_2O (Option B).

Light microscopy images of neuronal cultures on day 1 without serum. Images were obtained with Zeiss microscope at a 4X magnification.

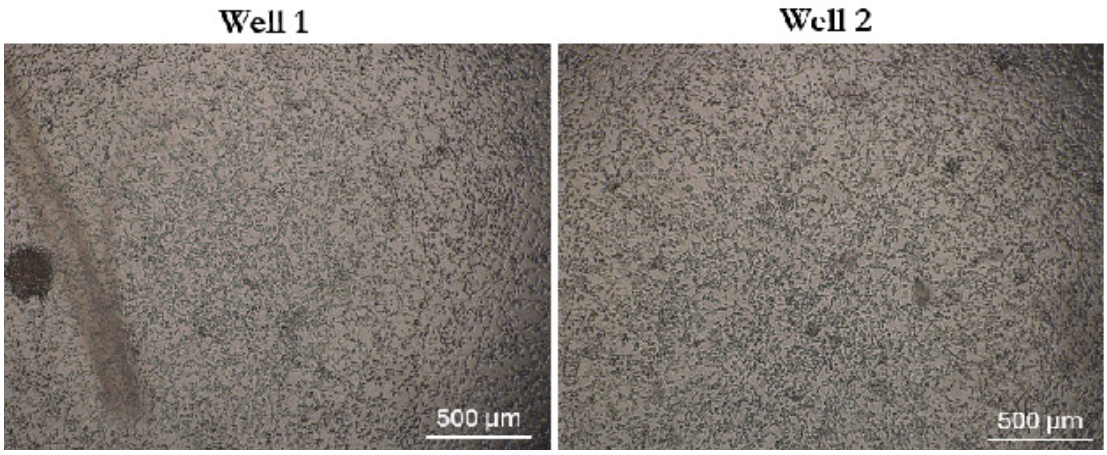

Figure 5: Neuronal differentiation of B35 cells using the "blanket" method by diluting VitroGel® NEURON with DI H_2O.

Light microscopy images of neuronal cultures on day 3 without serum. Images were obtained with Zeiss microscope at a 4X magnification.

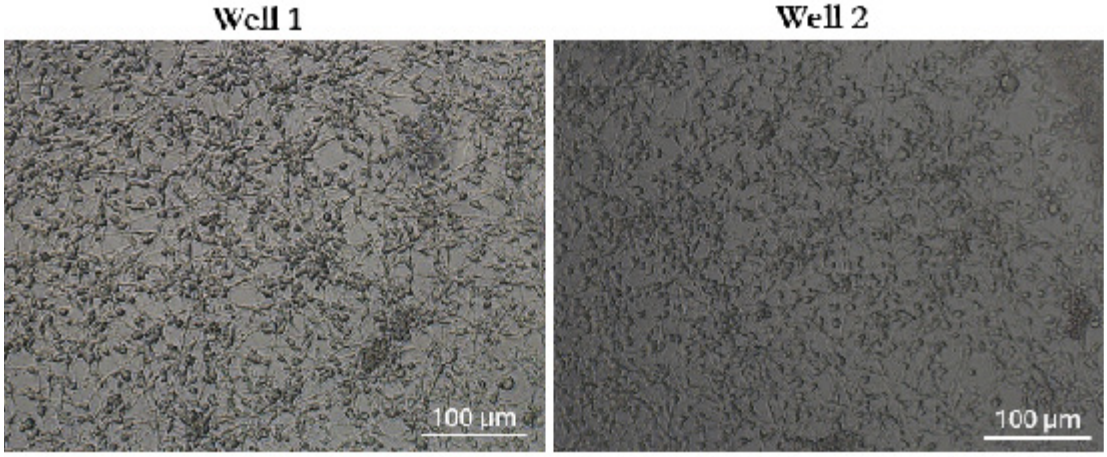

Figure 6: Neuronal differentiation of B35 cells using the "blanket" method by diluting VitroGel® NEURON with DI H_2O.

Light microscopy images of neuronal cultures on day 7 without serum. Images were obtained with Zeiss microscope at a 10X magnification.

3D Cell Culture Protocol

3D Cell Culture Protocol #1 (example: Neural Stem Cells)

MATERIALS

- **VitroGel® NEURON hydrogel (Catalog #: VHM07)**
- iPSC-derived NSCs
- Accutase (Innovative Cell Technologies, Inc., Catalog #: AT104)
- **RocketCell™ NSC 1X Xeno-Free Medium**
- **RocketCell™ NSC Supplement at 50X**
- Rho-kinase (ROCK) inhibitor (final concentration in medium at 10 µM Y-27632 or 2 µM Thiazovivin)*
- **VitroPrime™ Spread-Attach Plates**
  - » 6-well (Catalog # VP-SA6W)
  - » 12-well (Catalog # VP-SA12W)
  - » 24-well (Catalog #: VP-SA24W)
  - » 48-well (Catalog # VP-SA48W)
  - » 96-well (Catalog # VP-SA96W)

* Medium was supplemented with penicillin/streptomycin and GlutaMAX™

PROTOCOL

1. Equilibrate VitroGel® NEURON hydrogel and RocketCell™ NSC Xeno-Free Medium (supplemented with ROCK inhibitor) to room temperature (25°C).
2. Prepare the cell suspension with RocketCell™ NSC Xeno-Free Medium (supplemented with ROCK inhibitor) and 3-10X of RocketCell™ NSC Supplement.
3. Harvest NSCs using Accutase.
4. Prepare cell suspension in NSC cell suspension medium at a concentration of 0.6-1 x10^6 cells/mL.

   CRITICAL POINT: Prepare cell suspension in the medium with 3-10X supplement concentration to ensure the cell-hydrogel mixture has enough supplement in the next step.
5. Mix VitroGel® NEURON hydrogel with the cell suspension at a 2:1 ratio (i.e., 100 µL of hydrogel and 50 µL of cell suspension). Refer to Table 4 for recommended volumes based on your cell culture well size.

Table 4: Volume of hydrogel mixture based on plasticware well size.

	96-well plate	48-well plate	24-well plate	12-well plate	6-well plate
Volume of hydrogel	50 µL	150 µL	300 µL	600 µL	1200 µL

6. Add hydrogel mixture to the VitroPrime™ Spread-Attach Plate of choice.
7. Incubate the hydrogel mixture for 20 minutes at room temperature.
8. Carefully add RocketCell™ NSC 1X Xeno-Free Medium without ROCK inhibitor on top of the hydrogel. See Table 5 for recommended medium volume.

Table 5: Recommended volume of NSC 1X growth medium to add on top of hydrogel.

	96-well plate	48-well plate	24-well plate	12-well plate	6-well plate
Volume of hydrogel	100 µL	200 µL	300 µL	600 µL	1200 µL

9. Place cultures in the incubator at 37°C. Replace 50-60% of the medium every 2-3 days with RocketCell™ NSC 1X Xeno-Free Medium. Monitor the cultures using an inverted microscope.

3D Cell Culture Protocol #2 (example: B35 Cell Line)

MATERIALS

- **VitroGel® NEURON hydrogel (Catalog# VHM07)**
- **VitroPrime™ Spread-Attach Plates**
  - » 6-well (Catalog # VP-SA6W)
  - » 12-well (Catalog # VP-SA12W)
  - » 24-well (Catalog #: VP-SA24W)
  - » 48-well (Catalog # VP-SA48W)
  - » 96-well (Catalog # VP-SA96W)
- Cells
- Basal cell culture medium*
- Basal cell culture medium with 10% fetal bovine serum*
- Micropipette; low retention pipette tips
- Centrifuge tubes or conical tubes
- Parafilm sealant

*Media is supplemented with penicillin, streptomycin, and L-glutamine.

PROTOCOL

1. Equilibrate VitroGel® NEURON hydrogel and cell culture medium of choice with 10% FBS to room temperature (25°C).
2. Prepare cell suspension in culture medium of choice supplemented with 10% FBS at concentration of 0.6-1.2 x 10^6 cells/mL.
3. Mix VitroGel® NEURON hydrogel with the cell suspension at a 2:1 ratio (i.e., 100 µL of hydrogel and 50 µL of cell suspension). Homogenize the mixture by pipetting gently 3-5 times. Refer to Table 6 for recommended volumes based on well size.

Table 6: Volume of hydrogel mixture based on plasticware well size.

	96-well plate	48-well plate	24-well plate	12-well plate	6-well plate
Volume of hydrogel	50 µL	150 µL	300 µL	600 µL	1200 µL

4. Add hydrogel mixture to the VitroPrime™ Spread-Attach Plate of choice.
5. Allow the hydrogel mixture to solidify for 20 minutes at room temperature

   Note: Do not place the plate inside the incubator at 37°C, as higher temperatures reduce the hydrogel's viscosity and gelation rate.
6. Carefully add the cover medium (i.e., basal medium with 10% FBS) on top of the hydrogel. See Table 7 for recommended volume of medium to add to the VitroPrime™ Spread-Attach Plate of choice.

   Note: Make sure to add the medium by placing the pipette against the walls of the well.

Table 7: Recommended volume of cover medium to add on top of hydrogel.

	96-well plate	48-well plate	24-well plate	12-well plate	6-well plate
Volume of hydrogel	100 µL	200 µL	300 µL	600 µL	1200 µL

7. Incubate the cells in a humidified cell culture incubator at 37°C for 24 hours.
8. After 24 hours, observe the cells using an inverted microscope.
9. Remove the medium from the wells carefully and replace it with basal medium without serum.

   Optional: Remove 90% of the medium to avoid disrupting the hydrogel.

   Important: Use basal medium without serum to induce cell differentiation and axonal projections. If serum is added, the neuronal neuroblasts will not mature into neurons.
10. Replace the basal medium every 2-3 days. Monitor the cultures using an inverted microscope.

    Note: Avoid disrupting the hydrogel and remove 70% of the medium.

Immunofluorescence protocol for 3D cultures (example: B35 cell line)

MATERIALS

- Cells cultured using VitroGel® NEURON hydrogel
- Dulbecco's Phosphate-Buffered Saline (DPBS), no calcium, no magnesium (Wash buffer)
- 4% formaldehyde solution (Fixation solution)
- 0.1% Triton X-100 (Permeabilization solution)
- 3% bovine serum albumin (BSA) in DPBS (Blocking solution)
- Beta-III-tubulin primary antibody (Thermo Fisher, catalog #: MA1-118)
- Alexa 488 secondary antibody (Thermo Fisher, catalog #: A32723)
- NucBlue™ Fixed Cell ReadyProbes™ Reagent (DAPI) (Thermo Fisher, catalog #: R37606)
- Parafilm sealant
- Micropipette; Low retention pipette tips
- Fluorescent microscope

PROTOCOL

(Calculations are based on using a 96 well-plate, 35 µL gel/well as an example. Adjust accordingly for scale-up)

1. Carefully remove the cover medium on top of the hydrogel.
2. Add 100 µL of DPBS to wash the hydrogel and wait 1 minute before discarding it. Wash 3 times.
3. Add 100 µL of 4% formaldehyde and incubate in the refrigerator at 4 °C for 30 minutes*.
4. Remove the fixation solution and wash 3 times with 100 µL of DPBS. Wait 1 minute between washes.

   Optional stopping point: After removing the fixative, add 100 µL of DPBS and seal the plate with parafilm. Store in the refrigerator at 4 °C for up to 1 month.
5. Add 100 µL of permeabilization solution and incubate in the refrigerator at 4 °C for 30 minutes*.
6. Remove the permeabilization solution and carefully wash 3 times with 100 µL of DPBS. Wait 1 minute between washes.
7. Add blocking solution (3% BSA) and incubate for 1 hour in the refrigerator at 4 °C*.
8. Dilute the primary antibody anti-beta-III-tubulin in blocking solution at a 1:100 ratio (v/v, antibody/blocking solution) and gently homogenize.
9. Remove blocking solution. Add a 100 µL of antibody dilution to each well and store plate in the refrigerator at 4 °C overnight*.
10. Remove the primary antibody. Carefully wash with 100 µL of DPBS and wait 1 minute before discarding. Wash for a total of 3 times.

11. Dilute the secondary antibody (Alexa 488) in blocking solution at a 1:200 ratio. Add 100 µL of diluted secondary antibody to each well. Cover the plate with aluminum foil, wrap the plate edges with parafilm sealant, and incubate overnight at 4 °C*.
12. Remove the secondary antibody and carefully wash 3 times with 100 µL of DPBS. Wait 1 minute between each wash.
13. Prepare DAPI solution by adding 2 drops of reagent to 1 mL of DPBS. Add a 100 µL of DAPI solution to the wells. Cover plate with aluminum foil and incubate in the dark for 5 minutes at room temperature.
14. After incubation, the sample is ready for fluorescence imaging.

***Note:** Seal space between lid and bottom part of the plate with parafilm sealant.

CASE STUDY

3D neuronal differentiation using VitroGel® NEURON (example: B35 cell line)

Materials

- **VitroGel® NEURON hydrogel (Catalog #: VHM07)**
- **VitroPrime™ Spread-Attach 96-well Plate (Catalog #: VP-SA96W)**
- B35 neuronal neuroblast cells
- DMEM 1X basal medium
- DMEM basal medium with 10% fetal bovine serum (FBS)
- Parafilm sealant

To perform 3D neuronal differentiation, B35 cells (1.2 x 10^6 cells/mL) were resuspended in DMEM basal medium supplemented with 10% FBS. VitroGel® NEURON hydrogel was mixed with the cell suspension in a 2:1 ratio (v/v, gel/medium). Fifty microliters (50 uL) of hydrogel mixture were added to the VitroPrime™ Spread-Attach, 96-well Plate, and incubated for 20 minutes at room temperature. After the incubation, a 100 µL of DMEM basal medium with 10% FBS was gently added on top of the hydrogel. The cultures were placed in the incubator at 37°C for 24 hours. The following day, the culture medium was replaced with DMEM basal medium without serum to induce differentiation. The cultures were imaged using an inverted microscope and placed the incubator. Every 2-3 days, the culture medium was removed and replaced with DMEM basal medium without serum.

Neuronal morphology, growth, and differentiation were assessed by immunofluorescence staining on days 7, 14, and 21. We evaluated the presence of the neuron-specific marker beta-III-tubulin to determine whether VitroGel® NEURON hydrogel can support neuron differentiation and axonal projections. Indeed, we observed that VitroGel® NEURON hydrogel promotes axonal projection and neuronal differentiation by day 7 post-differentiation induction (Figure 7). Interestingly, on days 14 and 21, we showed that VitroGel® NEURON hydrogel allowed cells to form elongated axonal projections and enhanced long-term neuronal differentiation (Figures 8-9). Altogether, the findings demonstrate that VitroGel® NEURON hydrogel supports 3D neuronal culture, differentiation, and axonal projections.

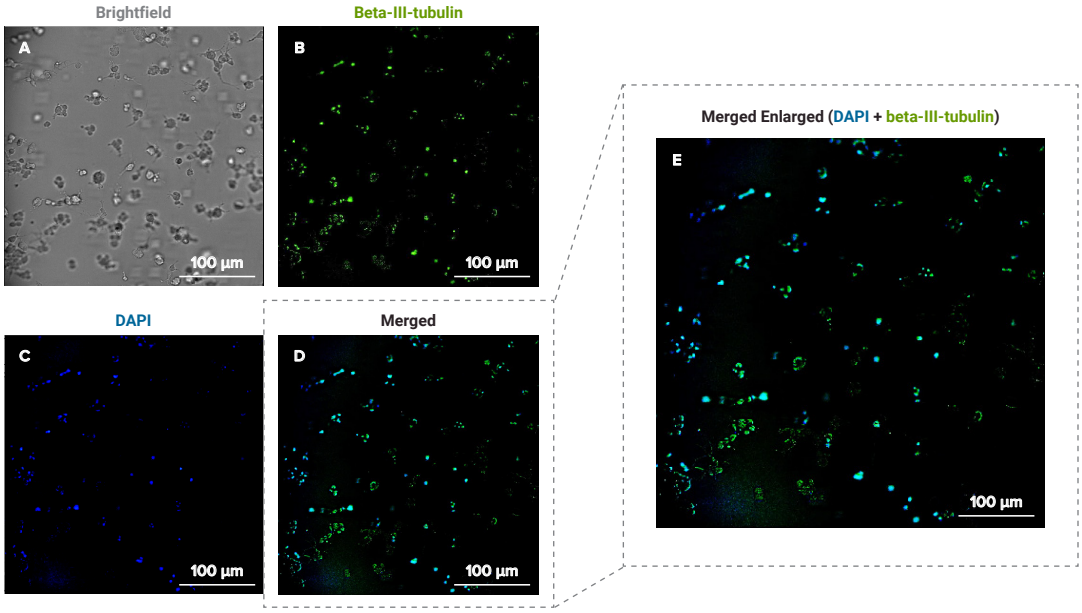

Figure 7: 3D neuronal differentiation of B35 cells using VitroGel® NEURON.

Immunofluorescence staining of neuronal cultures for the neuron-specific marker beta-III-tubulin on day 7 post-differentiation induction. Images represent the following: **A.** Light microscopy image of neuronal cultures. **B.** Beta-III-tubulin presence shown in green. **C.** Nuclei staining using DAPI (blue). **D.** Merged images of B and C. **E.** Enlarged merged image of B and C. Images were obtained using Image Xpress Nano Imaging System from Molecular Devices

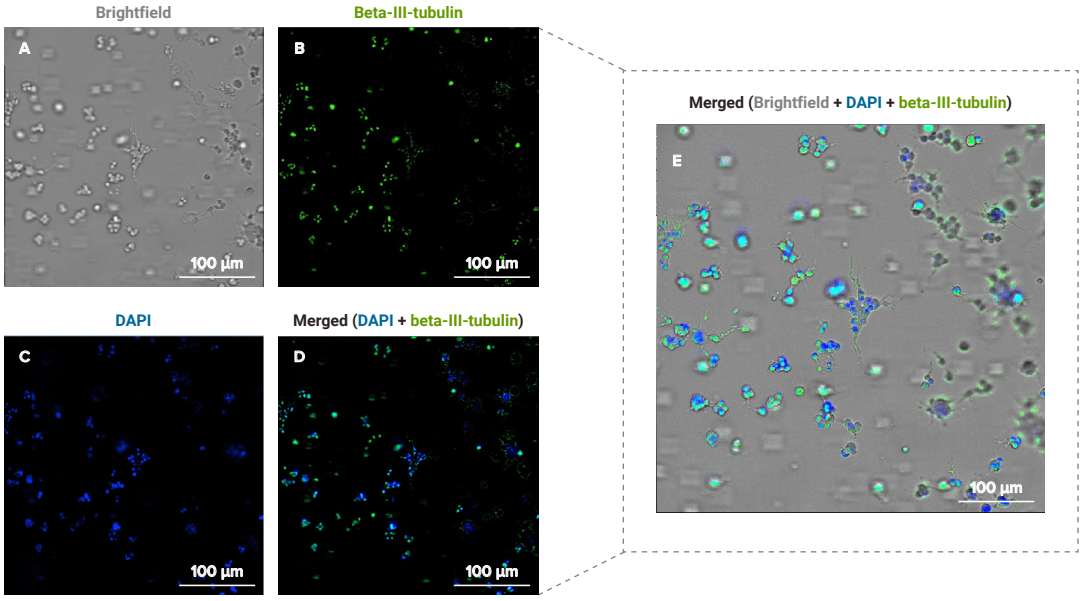

Figure 8: VitroGel® NEURON sustains 3D neuronal cultures and differentiation

Immunofluorescence staining of neuronal cultures for the neuron-specific marker beta-III-tubulin on day 14 post-differentiation induction. Images represent the following: **A.** Light microscopy image of neuronal cultures. **B.** Beta-III-tubulin presence, indicative of positive neuronal differentiation. **C.** Nuclei staining using DAPI (blue). **D.** Merged images of B and C. **E.** Merged images of A, B, and C. Images were obtained using ImageXpress Nano Imaging System from Molecular Devices.

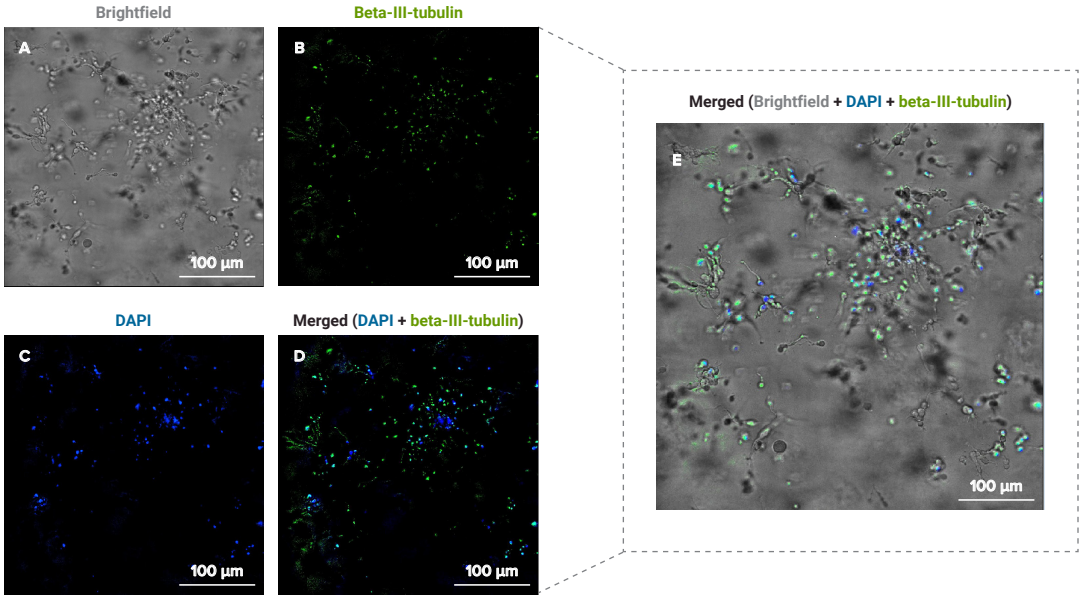

Figure 9: VitroGel® NEURON sustains long-term 3D neuronal differentiation

Immunofluorescence staining of neuronal cultures for the neuron-specific marker beta-III-tubulin on day 21 post-differentiation induction. Images represent the following: **A.** Light microscopy image of neuronal cultures. **B.** Beta-III-tubulin presence, indicative of positive neuronal differentiation. **C.** Nuclei staining using DAPI (blue). **D.** Merged images of B and C. **E.** Merged images of A, B, and C. Images were obtained using ImageXpress Nano Imaging System from Molecular Devices.