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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

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

VitroGel® NEURON

2D Coating ("blanket") Protocol

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MATERIALS

- **VitroGel® NEURON hydrogel (Catalog #: VHM07)**
- **VitroPrime™ Spread-Attach 96-well plates (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 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 the concentration of 1.3 x 10^5 cells/mL.
3. Add a 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. Perform a 7:1 mixing 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 5:1 to 10:1 range (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 1 below.
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 1.

   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 1: 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 cells inside a humidified chamber at 37°C.
6. Replace 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 a 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 15 minutes at 4°C*.
8. Dilute the primary antibody anti-beta-III-tubulin in blocking solution in a 1:100 ratio (v/v, antibody/blocking solution) and gently homogenize.
9. Remove blocking solution. Add a 100 µL of primary antibody dilution to each well and store plate 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 in 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 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 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 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

A. Materials required for hydrogel covering "blanket" method setup

- VitroGel® NEURON (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

B. Materials for immunofluorescence staining

- 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

B35 cells (1.3 x 10^5 cells/mL) were resuspended in DMEM basal medium with 10% FBS. A 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 in 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.

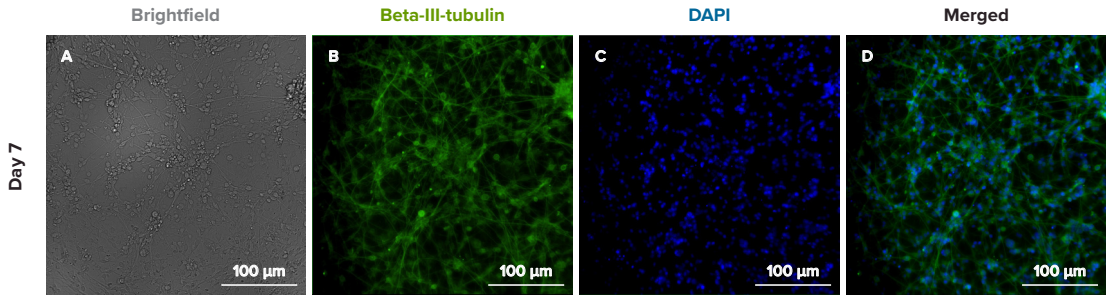

Figure 1: 2D "blanket" method using VitroGel® NEURON hydrogel promotes neuronal differentiation.

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 Image Xpress Nano system.

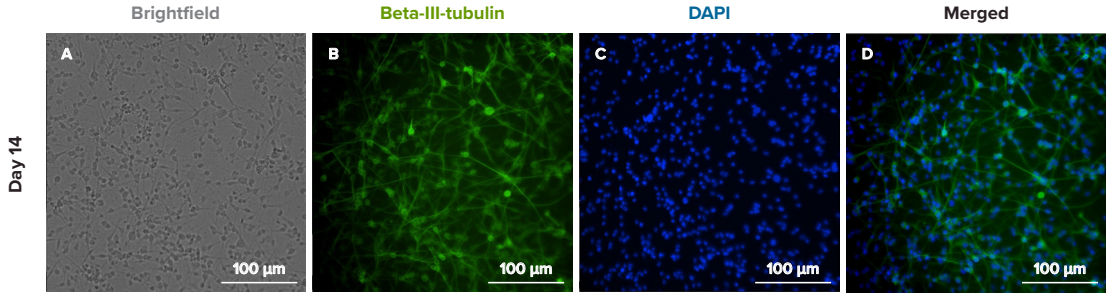

Figure 2: 2D "blanket" method using VitroGel® NEURON hydrogel prolongs *in vitro* neuronal differentiation.

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 Image Xpress Nano system.

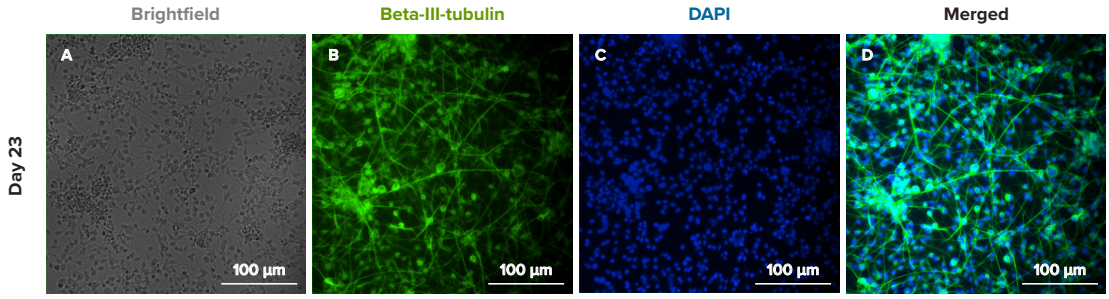

Figure 3: 2D "blanket" method using VitroGel® NEURON hydrogel sustains *in vitro* neuronal differentiation.

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 Image Xpress 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 plates (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).

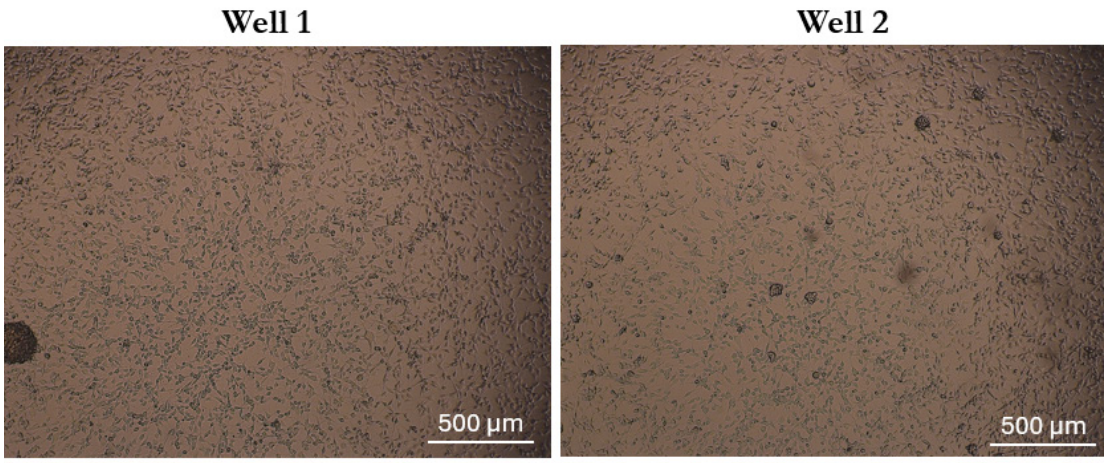

Figure 4: Neuronal differentiation of B35 cells using the "blanket" method by diluting VitroGel® NEURON with DI H_2O (Option B).

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

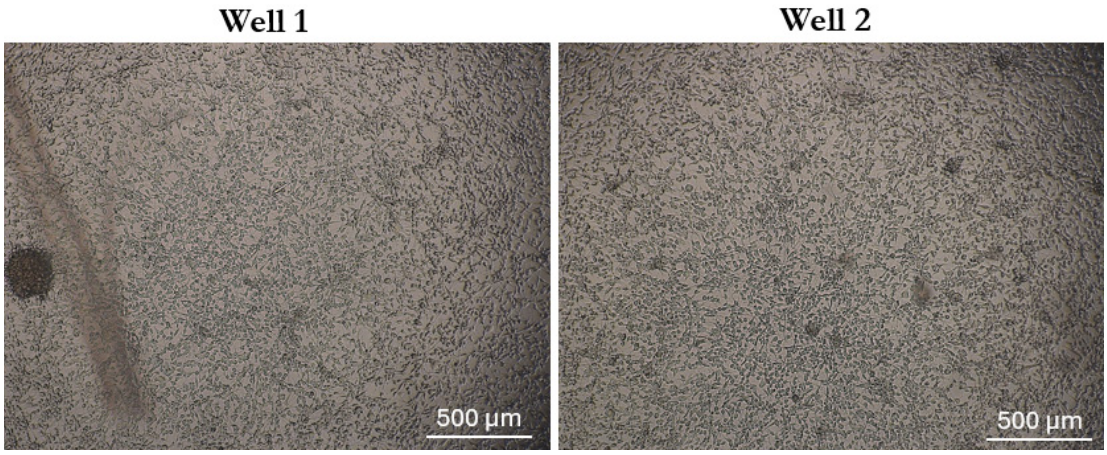

Figure 5: Neuronal differentiation of B35 cells using the "blanket" method by diluting VitroGel® NEURON with DI H_2O.

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

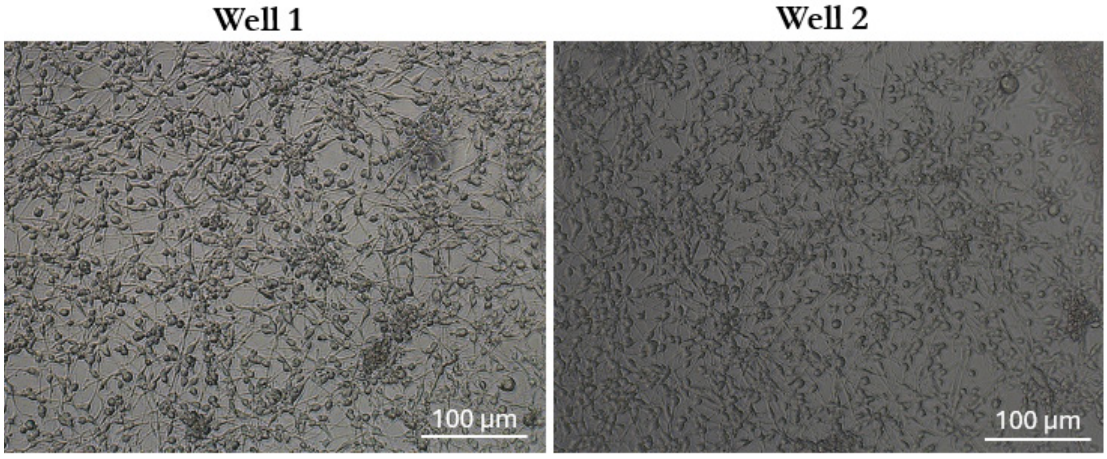

Figure 6: Neuronal differentiation of B35 cells using the "blanket" method by diluting VitroGel® NEURON with DI H_2O.

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

VitroGel® NEURON

3D Cell Culture Protocol

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MATERIALS

- **VitroGel® NEURON hydrogel (Catalog #: VHM07)**
- **VitroGel® 3D High Concentration Hydrogel Kit with Dilution Solution TYPE 2 (Catalog #: TWG001-2)**
- **VitroPrime™ Spread-Attach 96-well plates (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

Step 1: Well coating with VitroGel® 3D High Concentration Kit

(Recommended but optional. These protocol steps prevent cell migration to the bottom of well plate and attachment as 2D on the surface).

1. Allow VitroGel® 3D hydrogel to reach room temperature.
2. Dilute hydrogel with VitroGel® Dilution Solution TYPE 2 in a 1:1 ratio. (i.e., combine 50 µL of VitroGel® 3D hydrogel with 50 µL of VitroGel® Dilution Solution TYPE 2). Homogenize the mixture by pipetting 3-5 times. Avoid bubbles by pipetting gently.
3. Add 40 µL of the mixture to each well of the VitroPrime™ Spread-Attach 96-well plate. Incubate for 15 minutes at room temperature.
4. Seal the edges of the plate with parafilm and place inside the refrigerator at 4°C. Incubate overnight.

Step 2: Preparation of VitroGel® NEURON and cell mixture for 3D neuronal culture

1. Remove the coated plate from the refrigerator and allow it to reach room temperature.
2. Prepare cell suspension in cold culture medium of choice supplemented with 10% FBS at the concentration of 1.2 x 10^6 cells/mL.

   IMPORTANT: The medium must be cold (i.e., 4°C).
3. Take the hydrogel from the refrigerator and perform a 1:1 mixing of cold hydrogel with cell suspension by combining 50 µL of hydrogel with 50 µL of cell suspension. Homogenize the mixture by pipetting gently 3-5 times.

   IMPORTANT: The hydrogel must be cold (i.e., 4°C)

4. Add 50 µL of the hydrogel mixture on top of the wells coated with VitroGel® 3D hydrogel to achieve a final concentration of 3 x 10^4 cells inside each well.
5. Allow the hydrogel mixture to solidify for 30 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 80 µL of basal medium with 10% FBS on top of the hydrogel.

   Note: Make sure to add the medium by placing the pipette against the walls of the well.
7. Incubate cells in a humidified cell culture incubator at 37°C for 24 hours.
8. After 24 hours, observe the cells and perform imaging using a microscope.
9. Remove the medium from the wells carefully and replace it with basal medium without serum.

   Optional: Remove 90% of the medium without disrupting the hydrogel.
10. Replace the basal medium every 2-3 days and perform imaging to observe neuronal differentiation.

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

Immunofluorescence protocol performed after culturing neurons in 3D with VitroGel® NEURON

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 for a total of 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 in 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 in 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

Evaluating 3D Neuronal Differentiation Using VitroGel® NEURON

Materials

- **VitroGel® NEURON (Catalog #: VHM07)**
- **VitroGel® 3D High Concentration Hydrogel Kit with Dilution Solution TYPE 2 (Catalog #: TWG001-2)**
- **VitroPrime™ Spread-Attach 96-well plates (Catalog #: VP-SA96W)**
- B35 neuronal neuroblast cells
- DMEM 1X basal medium
- DMEM basal medium with 10% fetal bovine serum (FBS)
- Parafilm sealant

Before performing 3D neuronal differentiation, the wells of the VitroPrime™ Spread Attach plate were coated with 40 µL of VitroGel®3D hydrogel previously diluted in a 1:1 ratio with VitroGel® Dilution Solution TYPE 2 (v/v, gel/dilution solution). The hydrogel was incubated for 15 minutes at room temperature. Then, the plates were sealed with parafilm and stored in a refrigerator at 4°C for 24 hours. Coating the wells with diluted VitroGel® 3D High Concentration hydrogel prevents cells from migrating to the bottom of the well. The following day, the plate was removed from the refrigerator and allowed to reach room temperature.

To perform 3D neuronal differentiation, B35 cells (1.2 x 10^6 cells/mL) were resuspended in cold DMEM basal medium supplemented with 10% FBS. The next step consisted of mixing the VitroGel® NEURON with the cell suspension in a 1:1 ratio (v/v, gel/medium). 50 µL of hydrogel mixture was added on top of the wells coated with VitroGel® 3D hydrogel and incubated for 30 minutes at room temperature. After the incubation, 80 µL of DMEM basal medium with 10% FBS was gently added on top of the hydrogel. The cultures were placed inside the incubator at 37°C for 24 hours. Subsequently, the medium was carefully removed, and the cultures were replenished with DMEM basal medium to induce differentiation. The cultures were imaged under the microscope and stored in the incubator at 37°C. Every 2-3 days, the medium was removed and replaced with fresh medium.

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.

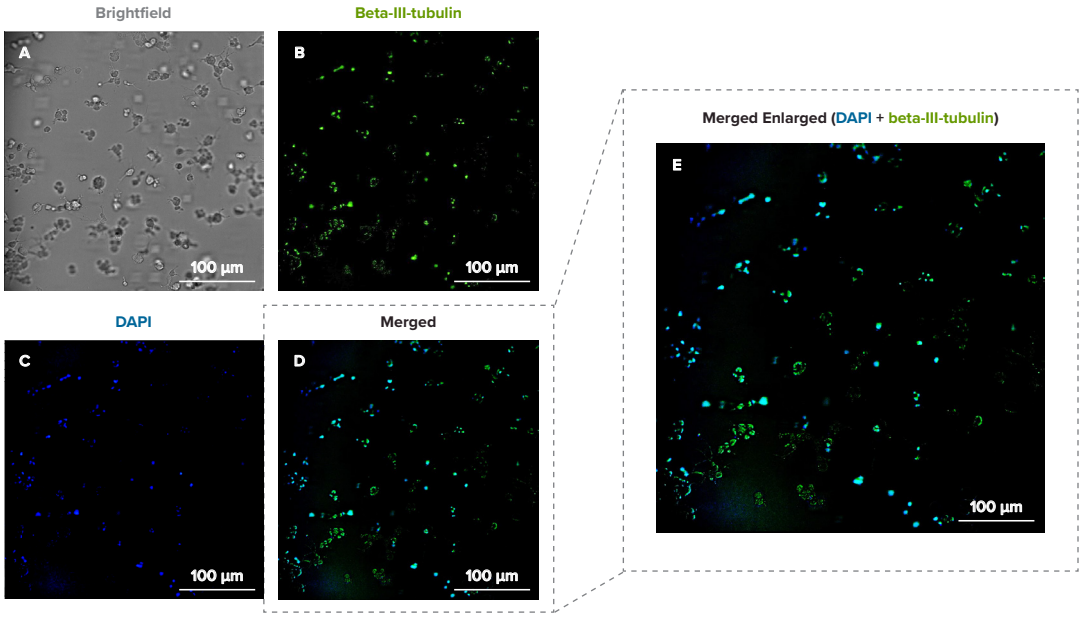

Figure 7: 3D neuronal differentiation of B35 cells using VitroGel® NEURON.

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

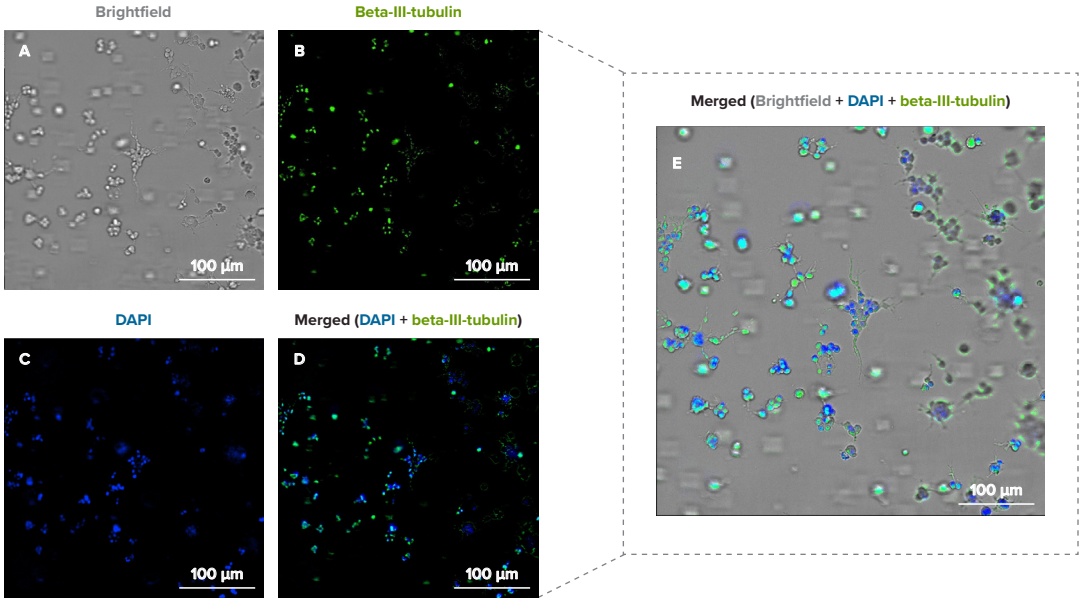

Figure 8: VitroGel® NEURON sustains 3D neuronal cultures and differentiation

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 Image Xpress Nano Imaging System from Molecular Devices.

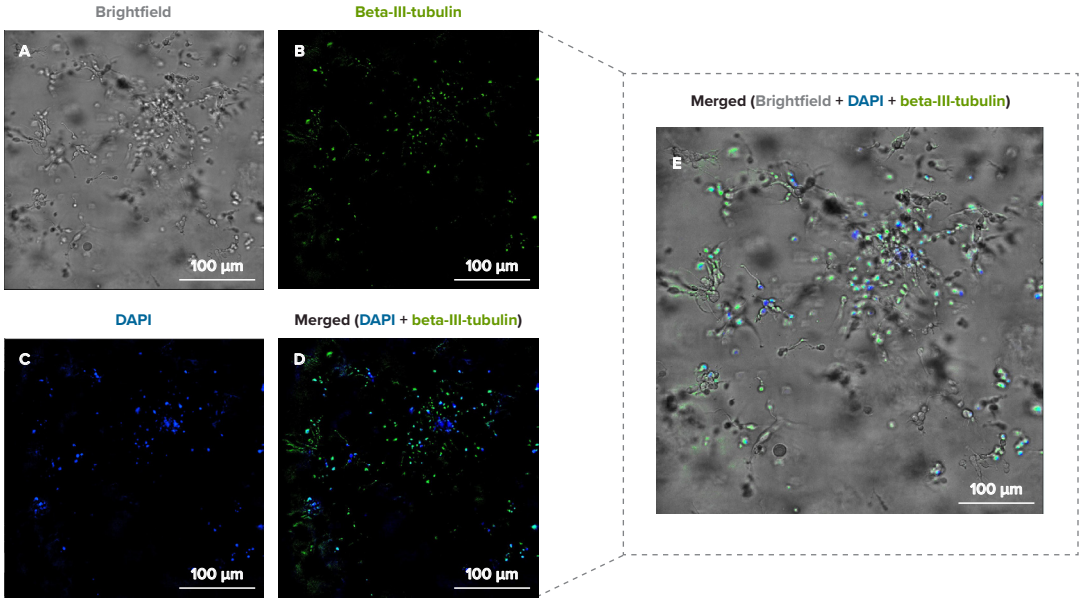

Figure 9: VitroGel® NEURON sustains long-term 3D neuronal differentiation

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 Image Xpress Nano Imaging System from Molecular Devices.