TheWell BIOSCIENCE

3D Cell Culture and Beyond

Ready-To-Use VitroGel® Cell Invasion Assay Kit

Catalog #: IA-VHM01-1P, IA-VHM01-4P
Protocol, Data Analyses, and Case Studies

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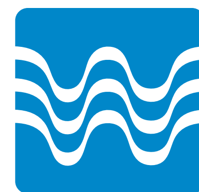
VitroGel® Cell Invasion Assay Kit (Ready-To-Use)



INTRODUCTION

Cell invasion is a dynamic process critical during embryonic development, immunosurveillance, and wound healing. Cell invasion is an orchestrated mechanism that occurs due to cell attachment to the extracellular matrix (ECM) followed by proteolytic degradation of the ECM, resulting in movement towards the newly invaded site. Cell invasion is crucial for physiological processes and for cancer cells to metastasize into local and distant regions within the body.

In vitro invasion assays have been developed throughout the years to better understand the processes underlying cell invasion. A method extensively performed is the traditional invasion assay, which requires the use of the Boyden chamber. The chamber comprises an insert coated with hydrogel matrices and then placed inside cell culture well plates. The insert contains a porous membrane, creating a physical barrier between the upper compartment and the outer well. The premise of this assay is that invasive cells degrade the hydrogel matrices in response to chemoattracts or other cell types placed in the outer well.

A significant challenge with the traditional invasion assay is the use of animal-based extracellular matrices (ECM): the components of animal-based ECM are not characterized and, as a result, their impact on cell invasion is unknown; the batch-to-batch variability of animal-based ECM can influence experimental findings and affect potential clinical applications; the temperature-sensitive operation protocols make the homogenous coating time-consuming and difficult for automated liquid handlers for high-throughput assays. These challenges can be circumvented using **VitroGel®**, a synthetic xeno-free, bio-functional hydrogel resembling the physiological ECM with tunable biophysical and biochemical properties. Unlike the traditional animal-based ECM, VitroGel® hydrogels can be adapted to evaluate how different mechanical strengths and functional ligands of hydrogel matrices, as well as chemokines, growth factors, cytokines, and serum within the hydrogel matrices or in the outer well affect cell mobility. This system offers a unique property that consists of embedding chemoattracts and chemical agents into the matrix to evaluate chemotaxis more closely. The VitroGel® hydrogel is easy-to-use at room temperature, shortening the operation time from hours to minutes, and supporting high-throughput operation. This powerful system is excellent for studying cell invasion and motility.

TheWell Bioscience's VitroGel®–Based Cell Invasion Assay Kits are powered by VitroGel® – a ground-breaking xeno-free, bio-functional hydrogel that closely mimics the physiological extracellular matrix and the premium quality VitroPrime™ Cell Culture Inserts. Both the **ready-to-use VitroGel® Hydrogel Matrix** and **tunable VitroGel® High-Concentration hydrogels** can be used for this cell invasion assay, providing versatility for cell mobility studies.

Ready-to-use VitroGel® Cell Invasion Assay Kit can be used for:

- **Traditional Invasion assay with chemoattraction from outer well:** Add cytokines, chemokines, growth factors, cells, serum, and pharmacological agents to the outer well to evaluate cell invasion.
- **Study the effect of cytokine/supplement of hydrogel matrix on cell mobility (Novel and unique assays offered by VitroGel®):** Incorporate cytokines, chemokines, growth factors, serum, and pharmacological agents inside the hydrogel to examine cell invasion.

VitroGel®-Based Cell Invasion Assay Kits are a robust and versatile tool for comprehensive cell invasion studies, offering the precision and control to uncover the mysteries of cell mobility.

Corporate Headquarters
TheWell Bioscience Inc.
1 Deerpark Dr, Ste C
Monmouth Junction, NJ 08852

www.thewellbio.com
US TF: +1 866.3D. CELLS
TEL: +1 973.855.4955
support@thewellbio.com

VitroGel® Cell Invasion Assay Kit (Ready-To-Use) Protocol

MATERIALS

- **VitroGel® Cell Invasion Assay Kit (Ready-to-use, Catalog #: IA-VHM01-1P or IA-VHM01-4P)**
  - » VitroGel® Hydrogel Matrix
  - » VitroPrime™ 24-well plate inserts (8 μm)
- Cells
- Basal cell culture medium (supplemented with penicillin, streptomycin, and L-glutamine)
- Micropipette; low retention pipette tips
- Centrifuge tubes or conical tubes
- Optional Supplement: serum, cytokines, growth factors, chemokines, or chemical agents

PROTOCOL

1. Allow VitroGel® Hydrogel Matrix and culture medium to reach room temperature.
2. Add 1 mL VitroGel® Hydrogel Matrix to 500 μL basal cell culture medium and gently pipette 5-10 times to homogenize the mixture. (Keep VitroGel® hydrogel solution and basal cell culture medium at 2:1 v/v mixing ratio).

   *If using cell culture medium with low salt concentration, such as RMPI 1640 medium, consider using 1:1 v/v mixing ratio (Example, 500 μL VitroGel® hydrogel solution to 500 μL cell culture medium).

   Note: If you need to add supplements such as cytokines, growth factors, chemokines, or chemical agents to the hydrogel matrix, add the 3X desired concentrations of supplement to the cell culture medium. The cell culture medium then can mix with VitroGel® hydrogel solution to get 1X final supplement concentration in hydrogel matrix. (Examples, Prepare medium with 30 ng/mL of the cytokine. Mix VitroGel® hydrogel solution with the medium at a 2:1 v/v mixing ratio to obtain a final concentration of 10 ng/mL cytokine inside the hydrogel matrix).

3. Add 100 μL of the hydrogel mixture to each insert and ensure there is an even hydrogel covering on the surface of each insert. Remove the fixation solution and wash 3 times with 100 μL DPBS. Wait 1 minute between washes.

   Note: If the experiment requires a thinner gel to evaluate invasion, adjust hydrogel volume to 50-100 μL per insert.

4. Allow hydrogel mixture to solidify for 20 minutes at room temperature before adding the cells on top of the hydrogel.
5. Prepare cell suspension in the desired culture medium (i.e., serum-free medium) at the concentration of 1-3 x 10^5 cells/mL and add 100 μL of cell suspension on top of the hydrogel.

   Optional Seeding Method: to ensure cells are seeded on the surface of the hydrogel, add 50% of the medium (without cells) on top of the hydrogel first. Wait 5-10 min then add the rest 50% of the medium with cells on top of the hydrogel. (For example, add 50 μL medium (without cells) first; wait 10-5 min; then add 50 μL medium with 2-6 x 10^5 cells/mL on top).

6. Prepare cell culture medium with factors of interest (i.e., chemokines, cytokines, or serum), and add 500 μL of cell culture medium to the outer wells.
7. Incubate cells in a humidified cell culture incubator at 37°C.

Crystal Violet Staining

Qualitative measurement of cell invasion-crystal violet staining

MATERIALS

- 1X Phosphate saline buffer (1X PBS)
- 4% formaldehyde
- Cotton swabs
- Forceps
- Methanol
- Crystal violet stain
- Micropipette; low retention pipette tips
- Microscope
- ImageJ software

PROTOCOL

1. Remove the medium from the inserts and the outer wells.
2. Use cotton swabs to remove the hydrogel coating from the inserts. Wash inserts and outer wells twice with 1X PBS (use 100 µL for each insert and 500 µL for each outer well).
3. Remove 1X PBS and add 50 µL (to each insert) and 500 µL (to each outer well) of 4% formaldehyde to fix the cells. Incubate for 10 minutes at room temperature.
4. Remove formaldehyde. Wash inserts and outer wells twice with 100 µL and 500 µL of 1X PBS, respectively.
5. Permeabilize cells by adding methanol to the inserts (50 µL for each insert) and outer wells (500 µL for each outer well). Incubate for 2 minutes at room temperature.
6. Remove methanol and wash inserts and outer wells twice with 100 µL and 500 µL of 1X PBS, respectively.
7. Add crystal violet stain to the inserts (100 µL for each insert) and outer wells (500 µL for each outer well). Incubate for 15 minutes at room temperature.
8. Remove the crystal violet stain from the inserts and outer wells and briefly wash the inserts and outer wells twice with 100 µL and 500 µL of 1X PBS, respectively [use a shorter time (30 seconds each time) for this last washing step].
9. Allow inserts and outer wells to dry at room temperature for 10 minutes.
10. Observe cells at the bottom of the insert by using a microscope. Take pictures of different fields within an insert.

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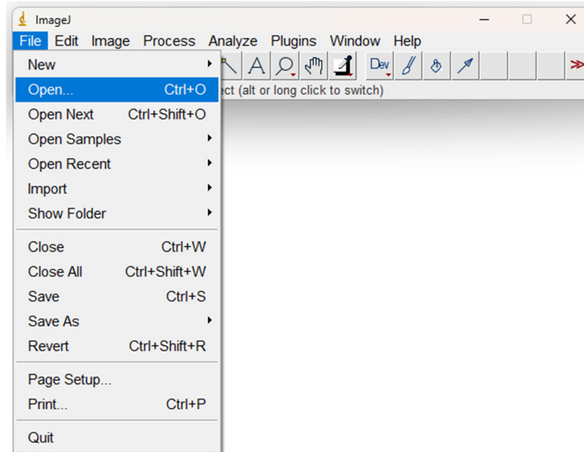
1 Deerpark Dr, Ste C
Monmouth Junction, NJ 08852

www.thewellbio.com
US TF: +1 866.3D. CELLS
TEL: +1 973.855.4955
support@thewellbio.com

Data Analyses

Quantification of cell invasion using ImageJ software

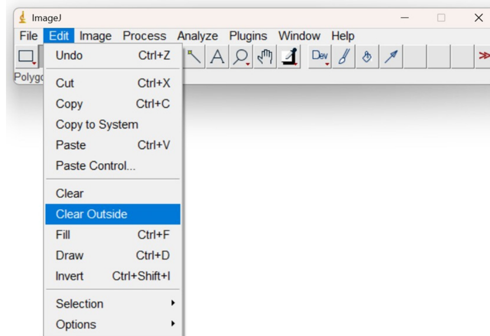
1. Remove the medium from the inserts and the outer wells.

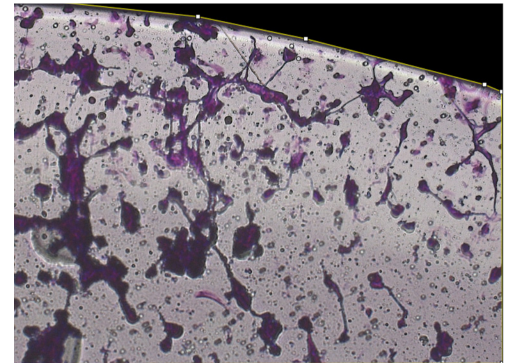


2. Select the regions of the image that will be analyzed by using the shape tools.
   Note: If no edges need to be excluded, proceed to step 4.

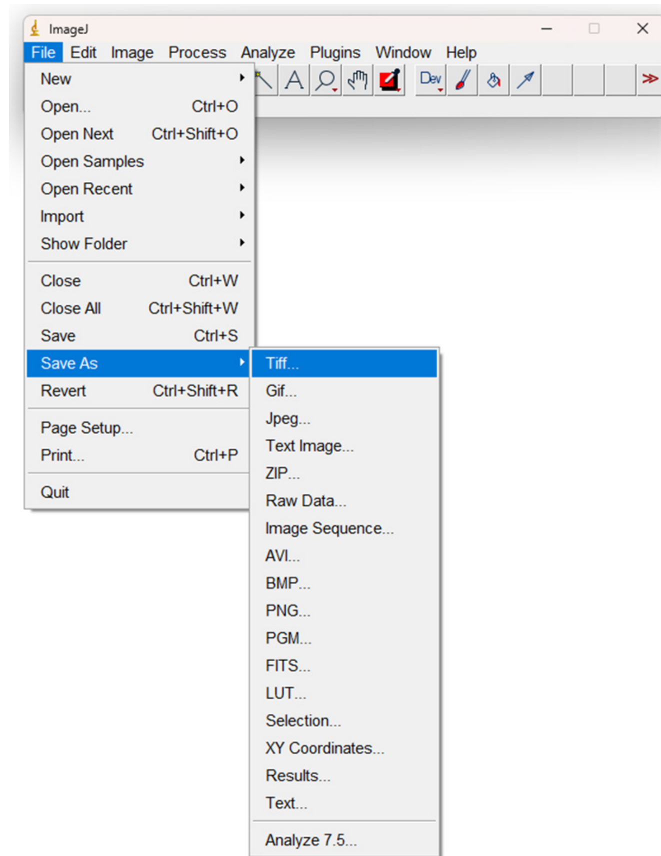
Tip: Exclude edges to avoid the software recognizing them as cell colonies.

3. Press *Edit* and select *Clear Outside*.

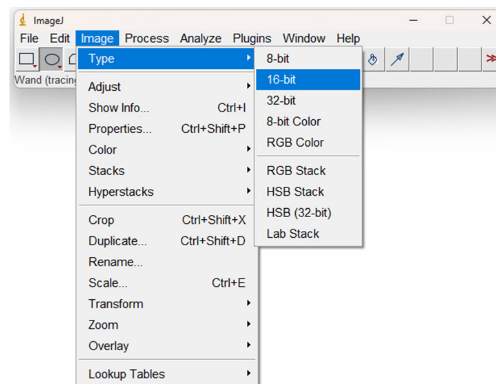


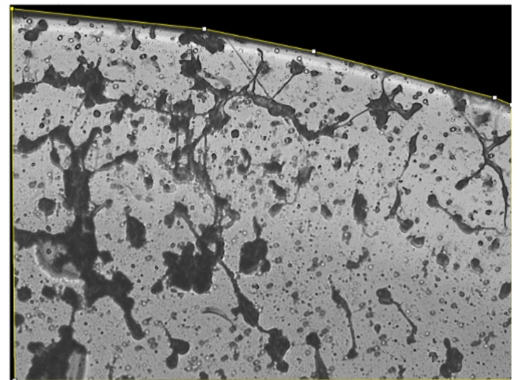


4. Save the image by going to ***File*** and then selecting ***Save As***.



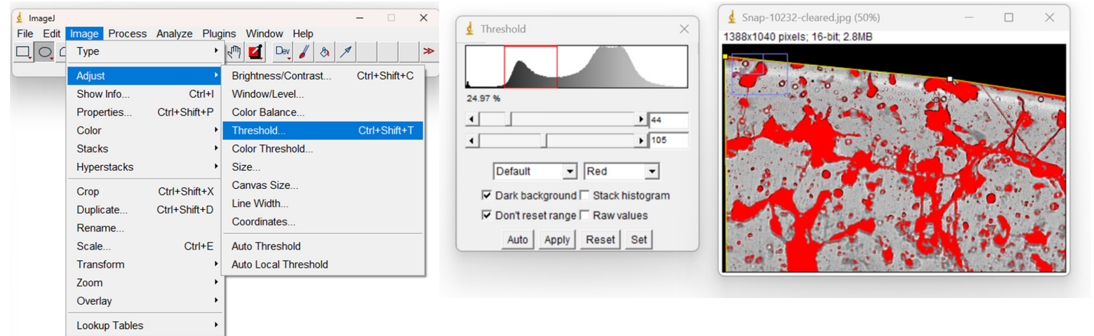
5. Change image format to grayscale by selecting ***Image, Type***, and then clicking ***16-bit***.

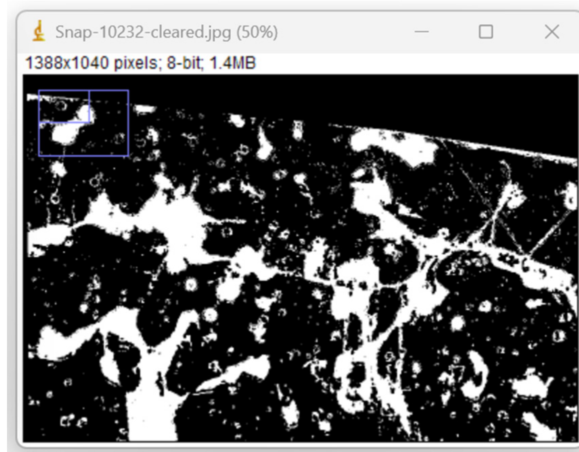




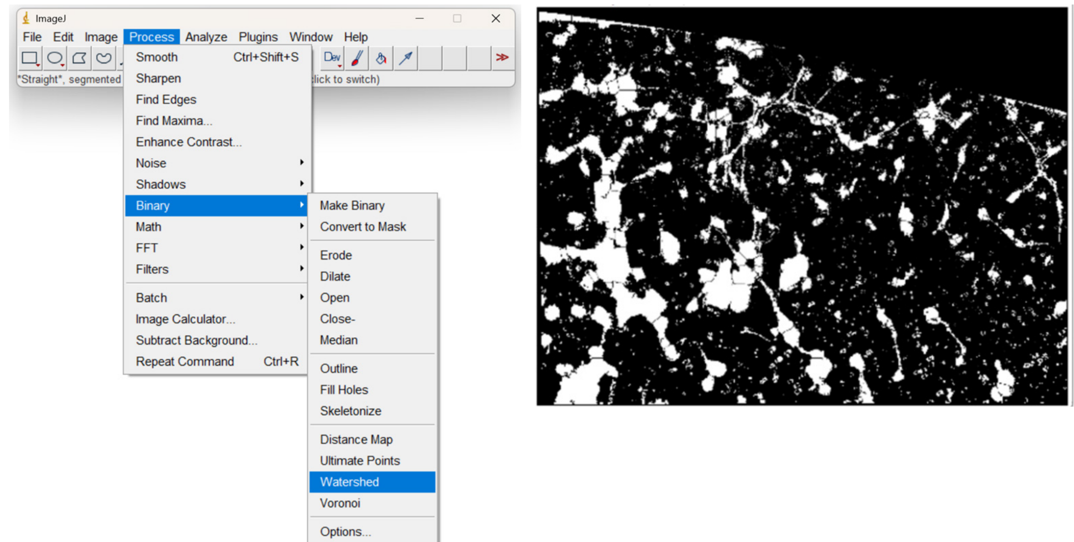
6. Select image icon, followed by adjust, threshold, and apply to select the cell colonies. The threshold can be adjusted to accurately select the cell colonies.

   Note: ImageJ might select the membrane pores as colonies. The pores can be excluded from quantification in the next step.

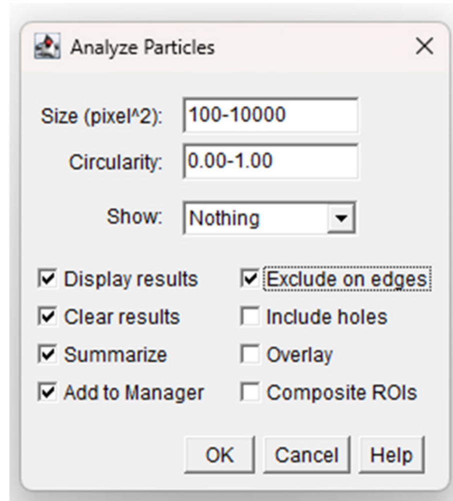




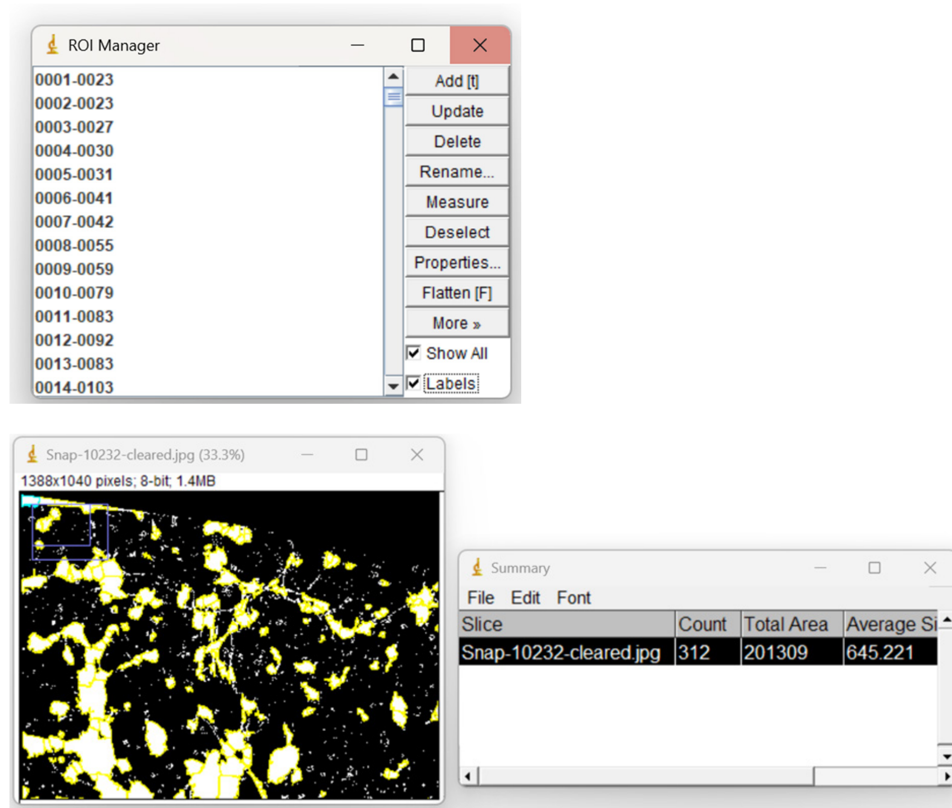
7. Divide clumped cell colonies into discrete ones by selecting *Process, Binary,* and *Watershed*.



8. Press the *Analyze* icon and choose *Analyze* particles to calculate how many cell colonies are on the image. Specify the following parameters:
    - Size pixel^=100-10000. This range can be customized to exclude the membrane pores from being counted as cell colonies.
    - Circularity=do not alter
    - Mark *Display results, Clear results, Add to Manager, Summarize,* and exclude on edges.



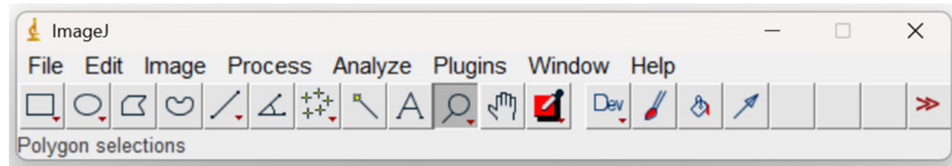
9. Unclick the labels option on the ROI manager to view discrete cell colonies instead of visualizing the cell count. The total colony count can be obtained from the summary tab.



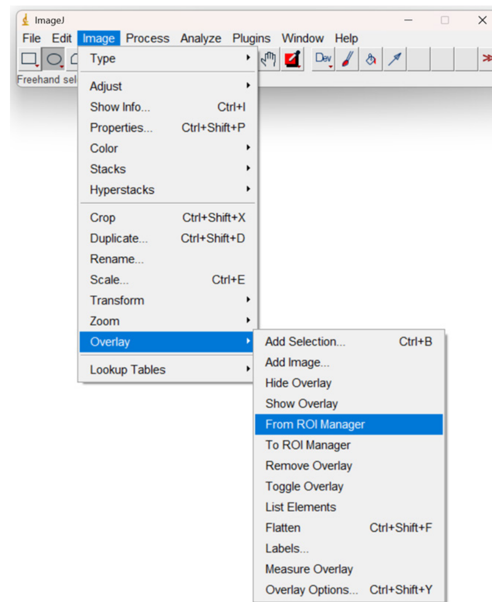
Corporate Headquarters
TheWell Bioscience Inc.
1 Deerpark Dr, Ste C
Monmouth Junction, NJ 08852

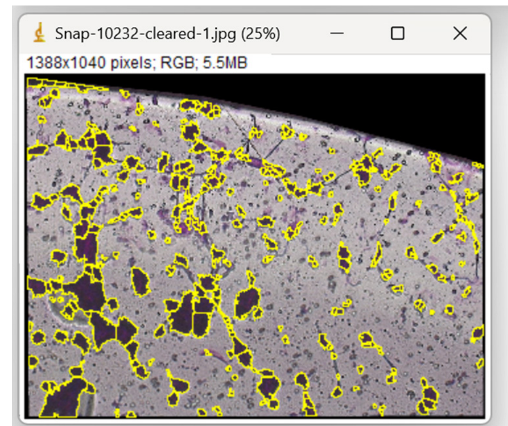
www.thewellbio.com
US TF: +1 866.3D. CELLS
TEL: +1 973.855.4955
support@thewellbio.com

10. Use the magnifying glass to verify if ImageJ counted membrane pores or regions within the edges as cell colonies.



11. If ImageJ counted image edges as cell colonies, repeat steps 2 and 3. Then, repeat step 8.
12. Open the saved image from step 4.
13. Press ***Image, Overlay,*** and select ***From ROI Manager***. This step will overlay the analysis with the image.





CASE STUDY 1

Traditional Invasion assay by chemoattraction from outer well (Invasion of U87-MG glioblastoma cells towards an FBS gradient)

Materials

- **VitroGel® Cell Invasion Assay Kit (Ready-to-use, Catalog #: IV-VHM01-1P)**
- **Insert: VitroGel® Hydrogel Matrix mixed with MEM basal medium at 2:1 v/v mixing ratio**
- Outer well: MEM basal medium (without serum) or MEM with 20% FBS
- Cells: U87-MG cells (3.8×10^4 cells per insert)
- Cell incubation time: 48 hrs.

To perform the traditional invasion assay, VitroGel® Hydrogel Matrix was mixed with MEM basal medium at 2:1 v/v ratio. The hydrogel mixture (100 µL) was added to each insert followed by a 20-minute incubation at room temperature for hydrogel solidification. U87-MG cells (3.8×10^4 cells per insert) were then resuspended in MEM basal medium and placed on top of the coated inserts. The outer wells were replenished with MEM basal medium or MEM medium supplemented with 20% FBS (500 µL per well, Figure 1A). The cultures were incubated for 48 hours at 37°C. Following incubation of the cells, we performed crystal violet staining to visualize cell invasion (Figures 1 B, C).

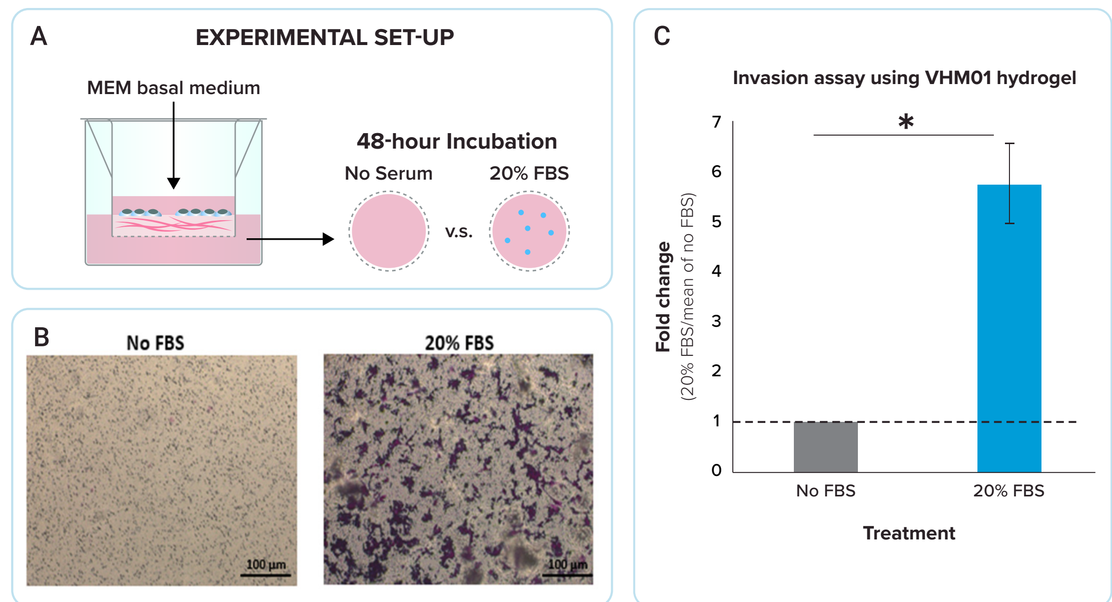


Figure 1. Invasion of U87-MG glioblastoma cells through VitroGel® Hydrogel Matrix caused by a serum gradient.

A. Schematic representation demonstrating the invasion assay cell culture set-up. **B.** U-87 MG cell invasion was visualized by performing crystal violet staining followed by light microscopy. The images show the membrane inserts from control group (No FBS) and 20% FBS condi-tions. Images were obtained with a Zeiss microscope at a 10X magnification. **C.** Fold change of U87-MG cell invasion between con-trol and 20% FBS groups. The control group was normalized to 1. The asterisk (*) stands for $p<0.05$.

Corporate Headquarters
TheWell Bioscience Inc.
1 Deerpark Dr, Ste C
Monmouth Junction, NJ 08852

www.thewellbio.com
US TF: +1 866.3D. CELLS
TEL: +1 973.855.4955
support@thewellbio.com

CASE STUDY 2

Evaluating chemotaxis by adjusting the growth factors compositions within VitroGel® Hydrogel Matrix

Materials

- **VitroGel® Cell Invasion Assay Kit (Ready-to-use, Catalog #: IV-VHM01-1P)**
- **Insert: VitroGel® Hydrogel Matrix mix with MEM with or without TGF-β1**
- Outer well: MEM basal medium (without serum) or MEM with 20% FBS
- Cells: U87-MG cells (3 x 10^4 cells per insert)
- Cell incubation time: 24 hrs.

Unlike the traditional invasion assay which is frequently employed to examine cell invasion towards chemokines or cytokines located in the outer well, we manipulated the hydrogel composition by embedding the cytokine, transforming growth factor (TGF-β1), into the hydrogel matrix. VitroGel® Hydrogel Matrix was mixed with MEM basal medium or MEM medium supplemented with 30 ng/mL of TGF-β1 at a 2:1 ratio to get a final concentration of 10 ng/mL of TGF-β1 in hydrogel matrix (Figure 2A). The hydrogel mixture (100 μL) was added homogeneously to each insert and allowed to solidify for 20 minutes at room temperature. U87-MG cells (3 x 10^4 cells per insert) were prepared in MEM basal medium and added on top of the hydrogel. The outer wells were filled with MEM basal medium or MEM medium supplemented with 20% FBS (500 μL per well). The cultures were incubated for 24 hours at 37°C and then subjected to crystal violet staining to assess cell invasion (Figure 2B, C).

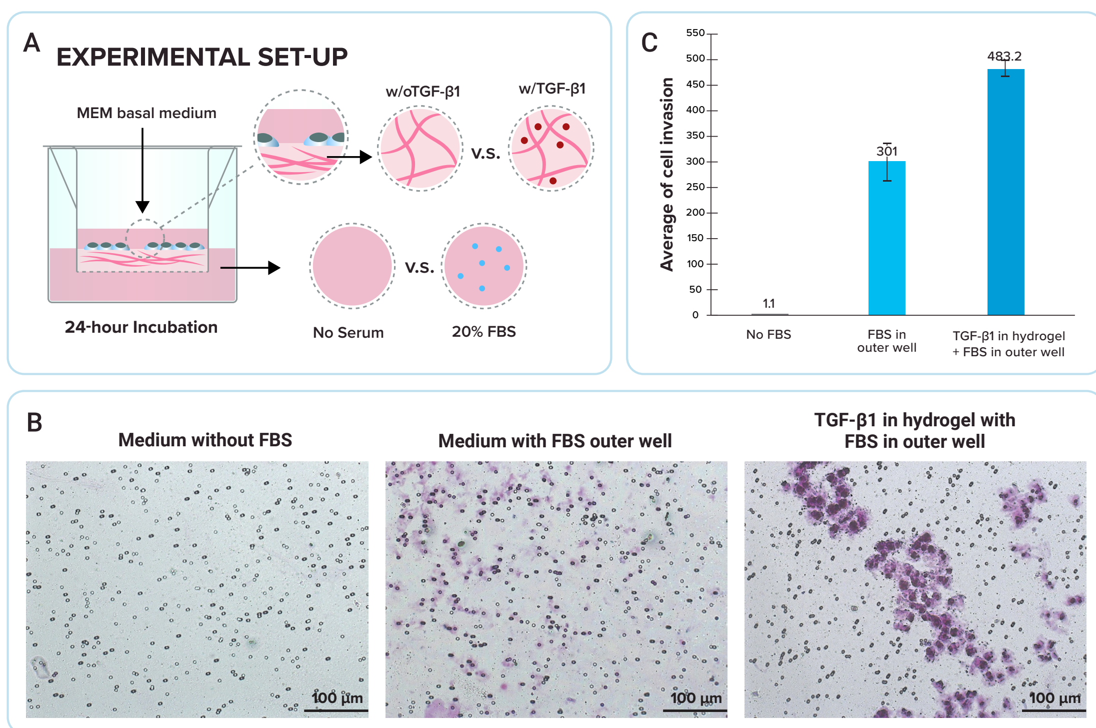


Figure 2. TGF-β1 inside of VitroGel® Hydrogel Matrix induces invasion of U87-MG glioblastoma cells.

A. Visual representation of invasion assay setup. **B.** Light microscopy images demonstrating cell invasion in the different groups after crystal violet staining. Images were obtained with a Zeiss microscope at a 10X magnification. **C.** Mean of U87-MG cell invasion for each of the experimental conditions.

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1 Deerpark Dr, Ste C
Monmouth Junction, NJ 08852

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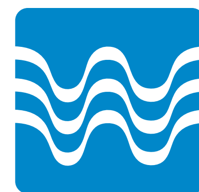
US TF: +1 866.3D. CELLS
TEL: +1 973.855.4955
support@thewellbio.com

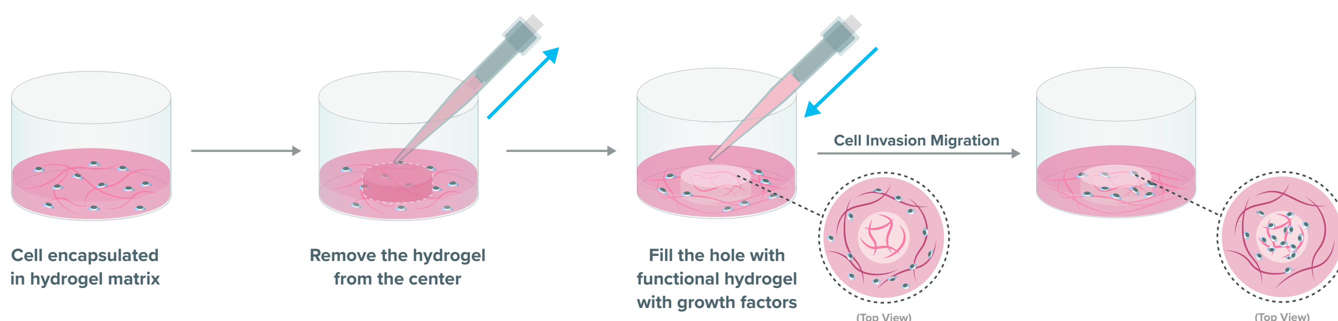
Other ways to use VitroGel® hydrogel for invasion assay

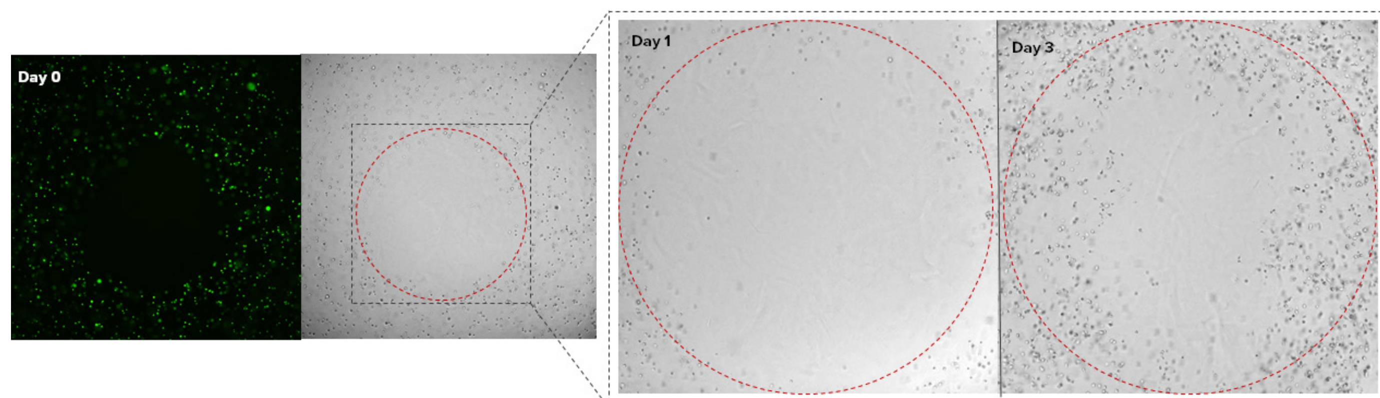


Horizontal Movement

Study the effects of both cytokine and hydrogel functional ligands on cell mobility

The 3D horizontal invasion can be studied by encapsulating cells in a hydrogel matrix. Scientists can adjust the properties of the hydrogel in the same well and observe the cell movement within the hydrogel matrix.



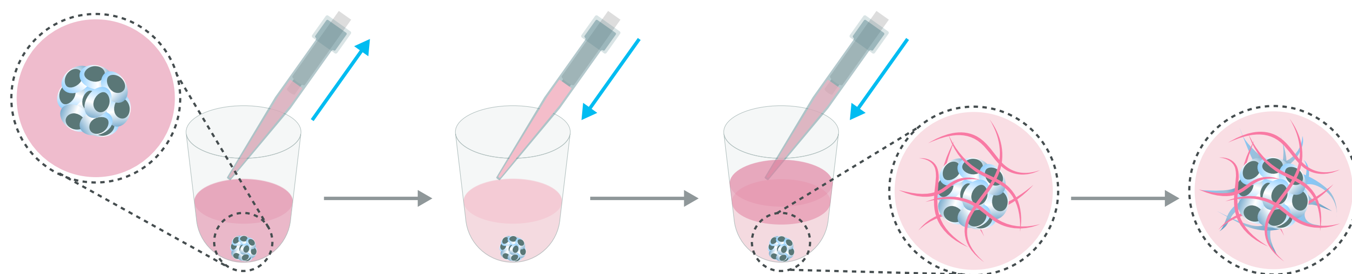


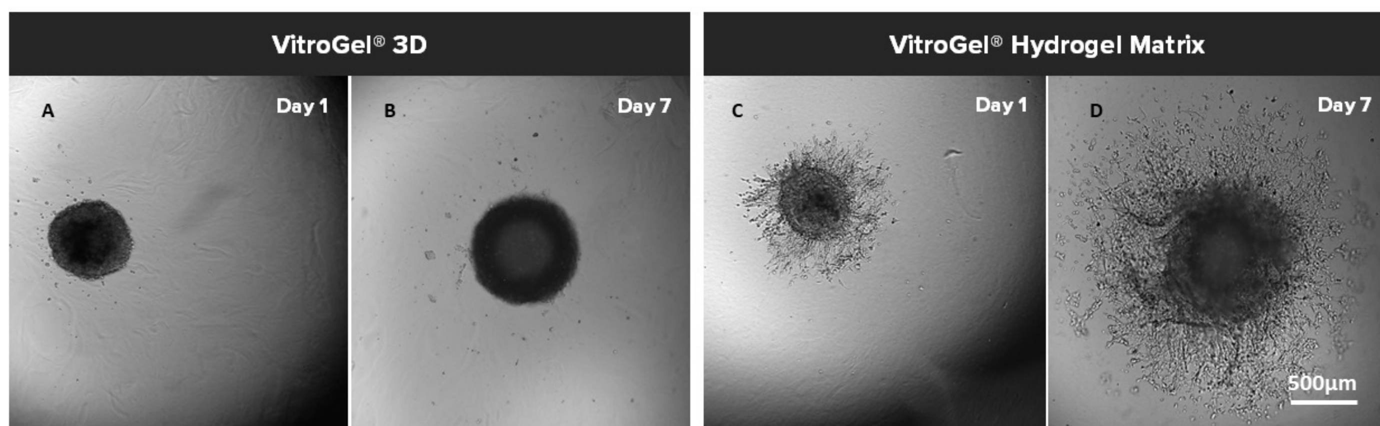
3D Invasion of U-87 MG Glioblastoma Cells in VitroGel® system. U-87 MG cells were encapsulated in VitroGel® RGD with 2% FBS and seed to a cell culture plate. After gel stable (30-60 min), use a micropipette to create a 5-10 μL hole by sucking the cell/hydrogel mixture out. Fill the hole with VitroGel® RGD with 20% of FBS without cells. Add the cover medium with 10% of FBS on top of the hydrogel. The different concentrations of FBS in the hydrogel matrix would induce cell invasion/migration within the hydrogel matrix. The cells movement was observed under a microscope with Z-stack function. The projecting imag-es were created with different z levels.

Please read the application note "3D Invasion of Glioblastoma Cells in VitroGel® Hydrogel System" for more details: https://www.thewellbio.com/application-notes/3d-invasion-glioblastoma-cells/

3D Spheroid Invasion Assay

Spheroid culture allows better preservation of the interactions between cells and/or between cells and the extracellular matrix. The spheroid invasion can be established by adding the cell spheroid directly on top of a layer of hydrogel or encapsulating inside of the hydrogel matrix for 3D cell mobility study.





3D spheroid invasion assay of U-87 MG cells in VitroGel® 3D and VitroGel® Hydrogel Matrix. Growth of cell spheroids over time in the VitroGel® 3D (A and B) and the VitroGel® Hydrogel Matrix (C and D). The spheroid in VitroGel® 3D maintained the spheroid morphology with an expansion in size from day 1 to day 7 without developing epithelial extensions characteristics. However, spheroids grown in VitroGel® Hydrogel Matrix produced not only significantly larger spheroids by day 7 but invading epithelial structures, demonstrating the clear cell penetration into the hydrogel matrix.

Please read the application note "3D Spheroid Invasion Assay With the Xeno-free, Bio-Functional VitroGel® Hydrogel Matrix" for more details: https://www.thewellbio.com/application-notes/3d-spheroid-invasion-assay-xeno-free-vitrogel-hydrogel-matrix