TheWell BIOSCIENCE

3D Cell Culture and Beyond

Tunable VitroGel® High-Concentration Cell Invasion Assay Kits

Catalog #:
IA-HC001-1P, IA-HC001-4P, IA-HC003-1P, IA-HC003-4P
IA-HC007-1P, IA-HC007-4P, IA-HC008-1P, IA-HC008-4P
IA-HC009-1P, IA-HC009-4P, IA-HC010-1P, IA-HC010-4P

Protocol, Data Analyses, and Case Studies

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VitroGel® High-Concentration Cell Invasion Assay Kits (Tunable)

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.

Tunable VitroGel® High-Concentration Cell Invasion Assay Kits 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 different hydrogel mechanical strengths on cell mobility**
  (Novel and unique assays offered by VitroGel®): VitroGel® High-Concentration hydrogels allow researchers to adjust the mechanical strength of the hydrogel matrix by changing the dilution ratio of the hydrogel solutions in order to understand its effects on cell mobility.

- **Study the effect of functional ligands in hydrogel matrix on cell migration**
  (Novel and unique assays offered by VitroGel®): VitroGel® High-Concentration hydrogels are modified with biofunctional ligands from fibronectin, collagen, and laminin that can be leveraged to assess their effect on cell invasion.

- **Study the effect of hydrogel degradability on cell mobility**
  (Novel and unique assays offered by VitroGel®): VitroGel® MMP is the high-concentration VitroGel® modified with matrix metallo-proteinases (MMPs) sensitive ligands to manipulate the hydrogel degradability for different cell mobilities.

- **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.

VitroGel® High-Concentration Cell Invasion Assay Kit (Tunable) Protocol

MATERIALS

- **VitroGel® High-Concentration Cell Invasion Assay Kits** of choice:
  - » VitroGel® 3D Cell Invasion Assay Kit (Catalog #: IA-HC001-1P or IA-HC001-4P)
  - » VitroGel® RGD Cell Invasion Assay Kit (Catalog #: IA-HC003-1P or IA-HC003-4P)
  - » VitroGel® IKVAV Cell Invasion Assay Kit (Catalog #: IA-HC007-1P or IA-HC007-4P)
  - » VitroGel® YIGSR Cell Invasion Assay Kit (Catalog #: IA-HC008-1P or IA-HC008-4P)
  - » VitroGel® COL Cell Invasion Assay Kit (Catalog #: IA-HC009-1P or IA-HC009-4P)
  - » VitroGel® MMP Cell Invasion Assay Kit (Catalog #: IA-HC010-1P or IA-HC010-4P)
  - » VitroGel® Dilution Solution, TYPE 2
  - » 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

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PROTOCOL

The protocol below is suitable for all versions of the tunable VitroGel® High-Concentration Cell Invasion Assay Kits. VitroGel® RGD Cell Invasion Assay Kit is used as an example below. Replace VitroGel® RGD Cell Invasion Assay Kit with other versions of choice.

1. Allow VitroGel® hydrogel and culture medium to reach room temperature.
2. Dilute VitroGel® RGD hydrogel by mixing with VitroGel® Dilution Solution for desire concentration. (Refer to Table 1 for recommended volumes for different ratios.)
3. Mix the diluted hydrogel solution with basal medium at a 4:1 ratio. (Refer to Table 1 for recommended volumes for mixing.)

Table 1:

Dilution Ratio (VitroGel®/Dilution Solution)	VitroGel®	Dilution Solution Medium	Basal Medium
1:0	1 mL	0 μL	250 μL
1:1	500 μL	500 μL	250 μL
1:2	300 μL	600 μL	225 μL
1:3	250 μL	750 μL	250 μL
1:4	200 μL	800 μL	250 μL
1:5	200 μL	1 mL	300 μL

Note: If you need to add supplements such as cytokines, growth factors, chemokines, or chemical agents to the hydrogel matrix, add the 5X desired concentrations of supplement to the cell culture medium. The cell culture medium can then mix with the diluted VitroGel® hydrogel solution at a 4:1 ratio to get 1X final supplement concentration in hydrogel matrix. (Examples: Prepare medium with 50 ng/mL of the cytokine. Mix diluted VitroGel® hydrogel solution with the medium at a 4:1 v/v mixing ratio to obtain a final concentration of 10 ng/mL cytokine inside the hydrogel matrix).

4. Add 100 μL of the hydrogel mixture to each insert and ensure there is an even hydrogel covering on the surface of each insert.

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

5. Allow the hydrogel mixture to solidify for 20 minutes at room temperature before adding the cells on top of the hydrogel.
6. Prepare cell suspension in 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 remaining 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 10^6 cells/mL on top).

7. 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.
8. 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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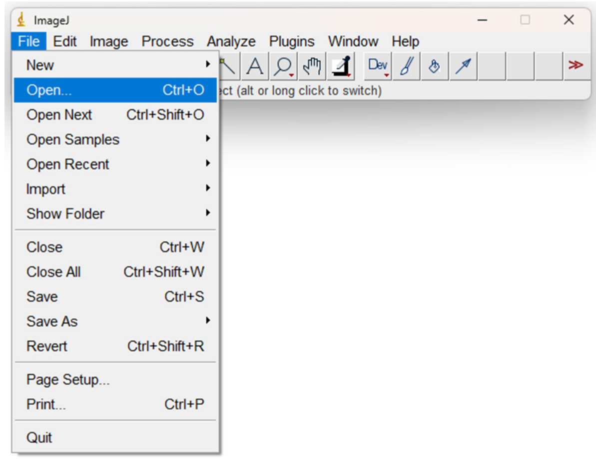
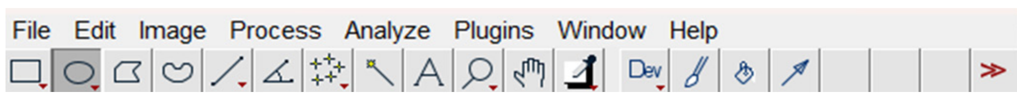
Data Analyses

Quantification of cell invasion using ImageJ software

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

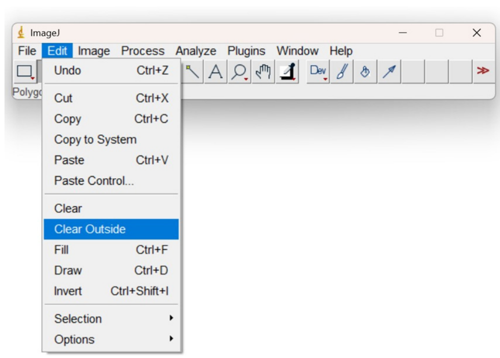


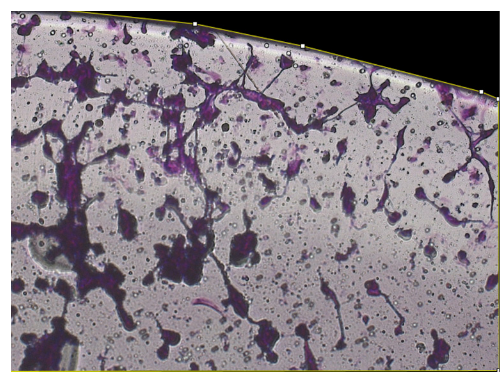
2. Select the regions of the image that will be analyzed by using the shape tools.
   <u>Note:</u> If no edges need to be excluded, proceed to step 4.



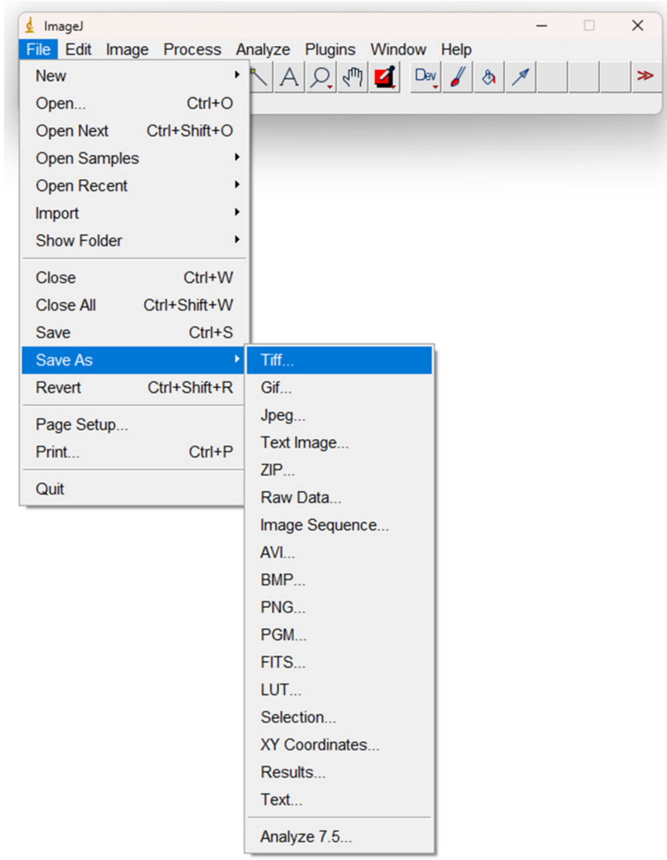
   Tip: Exclude edges to avoid the software recognizing them as cell colonies.

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

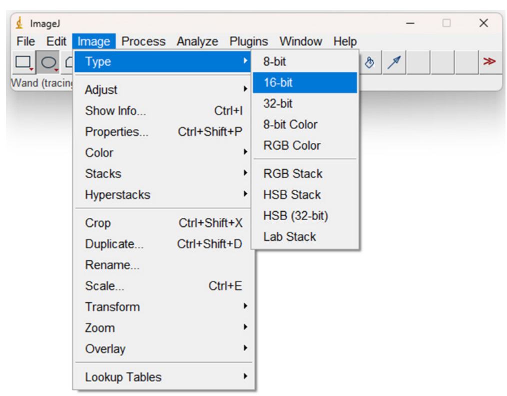


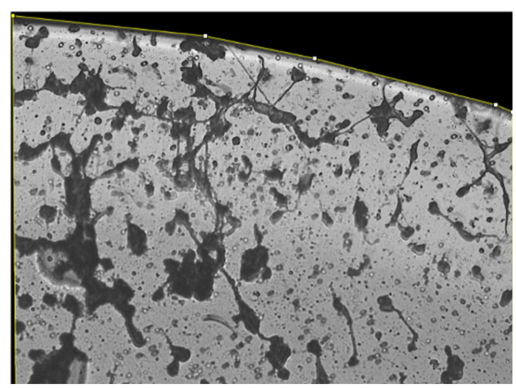


4. Save the image by going to *File* and then selecting *Save As*.



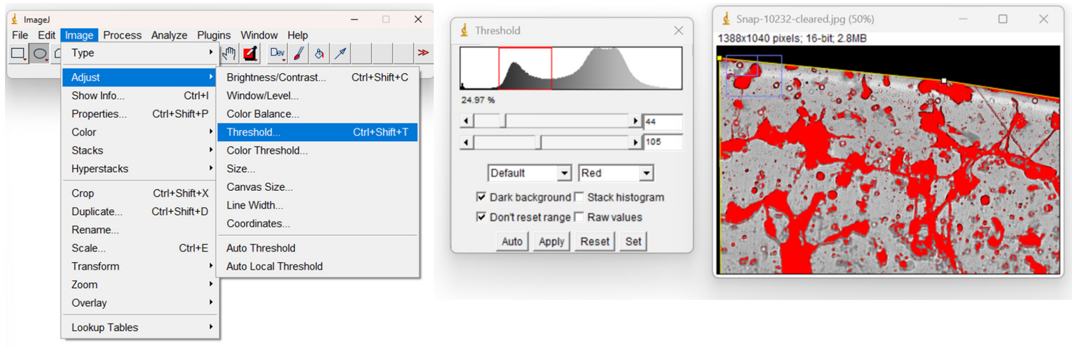
5. Change image format to grayscale by selecting *Image, Type*, and then clicking *16-bit*.

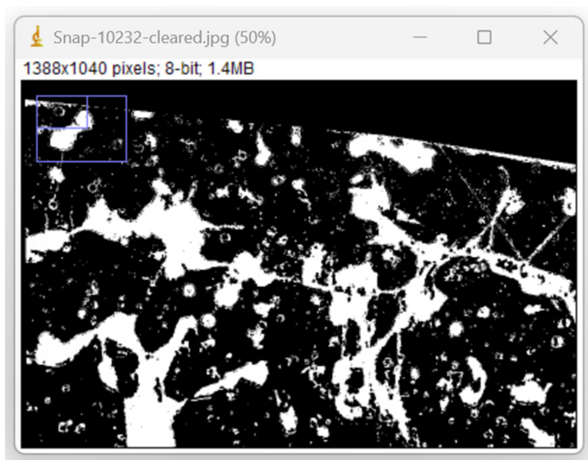




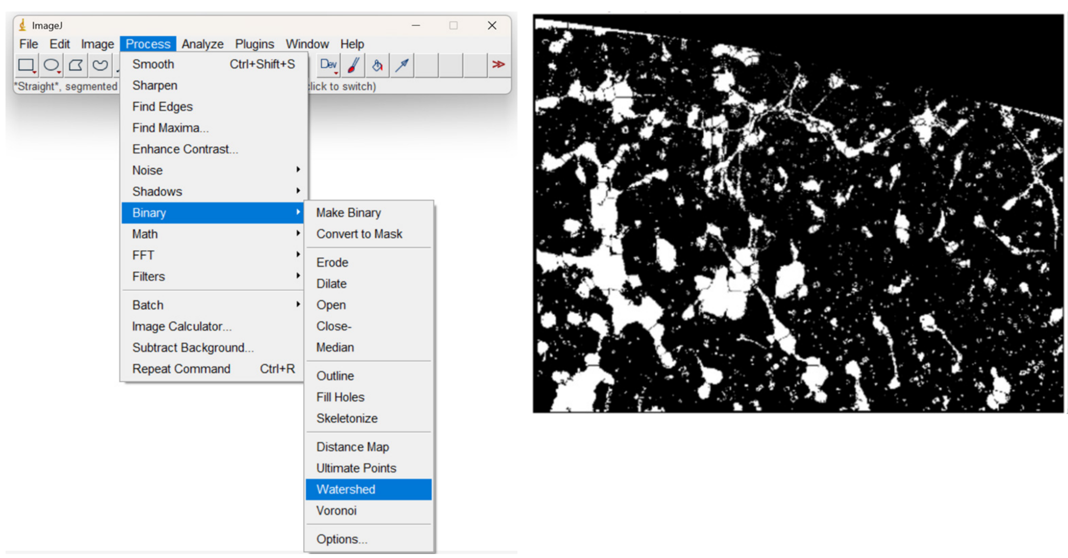
6. Select image icon, followed by adjust, threshold, and apply to select the cell colonies. The threshold can be adjusted to accurately select the cell colonies.

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

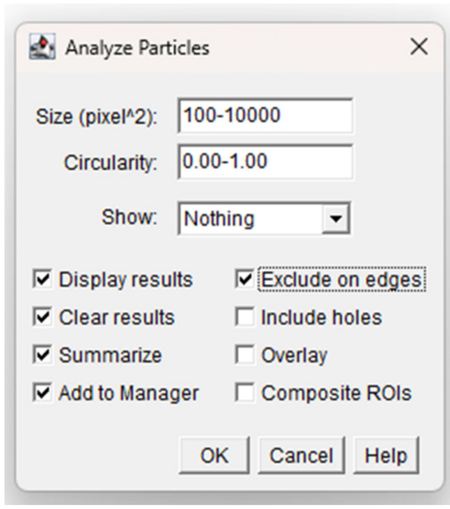




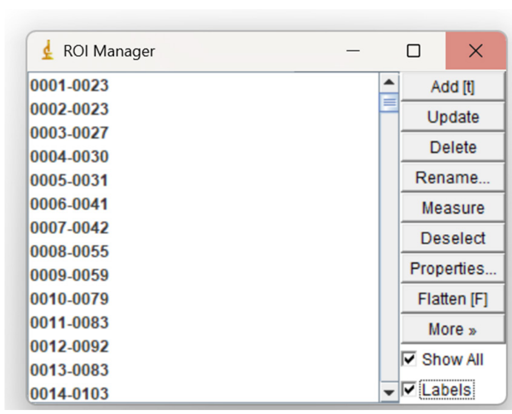
7. Divide clumped cell colonies into discrete ones by selecting *Process, Binary,* and *Watershed*.

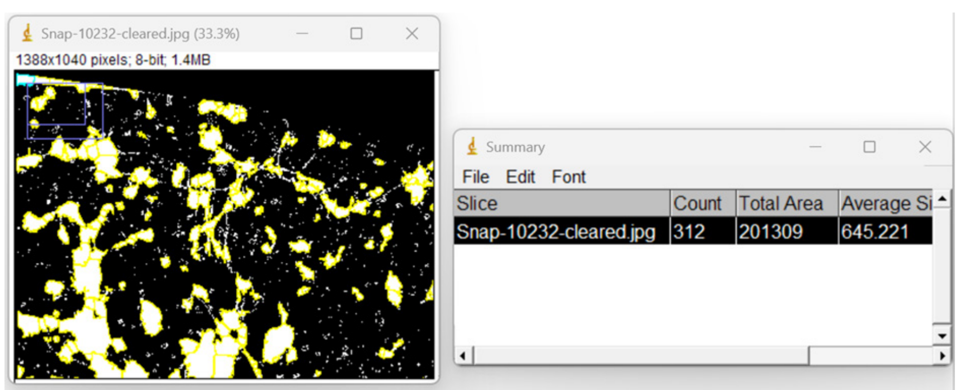


8. Press the *Analyze* icon and choose *Analyze* particles to calculate how many cell colonies are on the image. Specify the following parameters:
   - Size pixel^=100-10000. This range can be customized to exclude the membrane pores from being counted as cell colonies.
   - Circularity=do not alter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

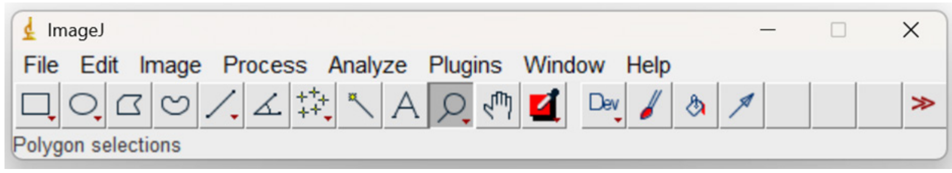


9. Unclick the labels option on the ROI manager to view discrete cell colonies instead of visualizing the cell count. The total colony count can be obtained from the summary tab.

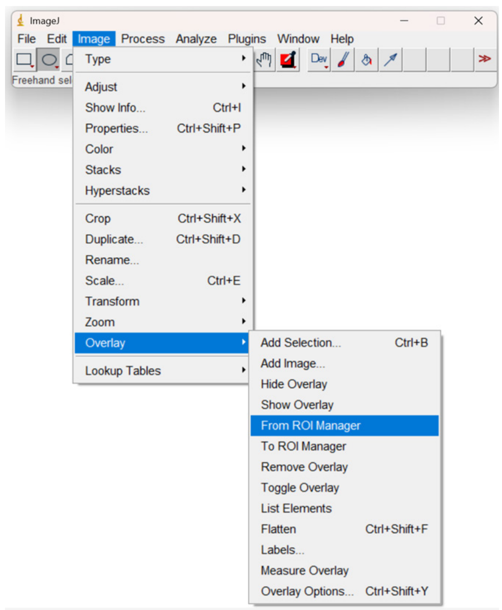


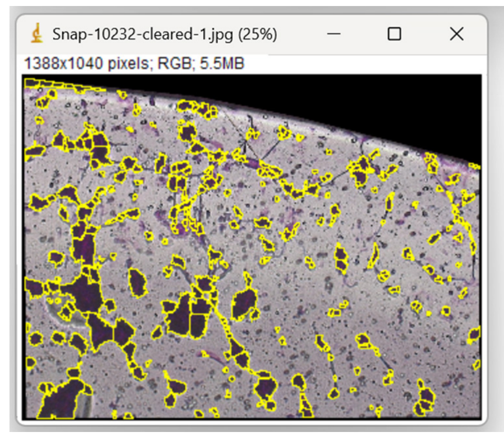


10. Use the magnifying glass to verify if ImageJ counted membrane pores or regions within the edges as cell colonies.



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

Study the effect of different hydrogel mechanical strengths on cell mobility

Materials

- **Cell Invasion Assay Kit: VitroGel® RGD Cell Invasion Assay (High-concentration, Catalog #: IV-HC003-1P)**
- **Insert: VitroGel® RGD hydrogel at 1:1, 1:3, and 1:5 dilution ratios**
- Outer well: MEM basal medium (without serum) or MEM with 20% FBS
- Cells: U87-MG cells (3x 10^4 cells per insert)
- Cell incubation time: 24 hrs.

To evaluate whether the hydrogel mechanical strength impact cell invasion, we diluted VitroGel® RGD hydrogel solution with VitroGel® Dilution solution at 1:1, 1:3, and 1:5 v/v ratios and then mixed with MEM basal medium at 4:1 ratio according to Table 1 of our standard protocol (Figure 1A). The hydrogel mixture (100 µL) was added homogeneously to each insert and allowed to solidify for 20 minutes at room temperature. U87-MG cells (3x 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). Then, the cells were incubated for 24 hours at 37°C followed by an examination of cell invasion using crystal violet staining (Figures 1 B-C).

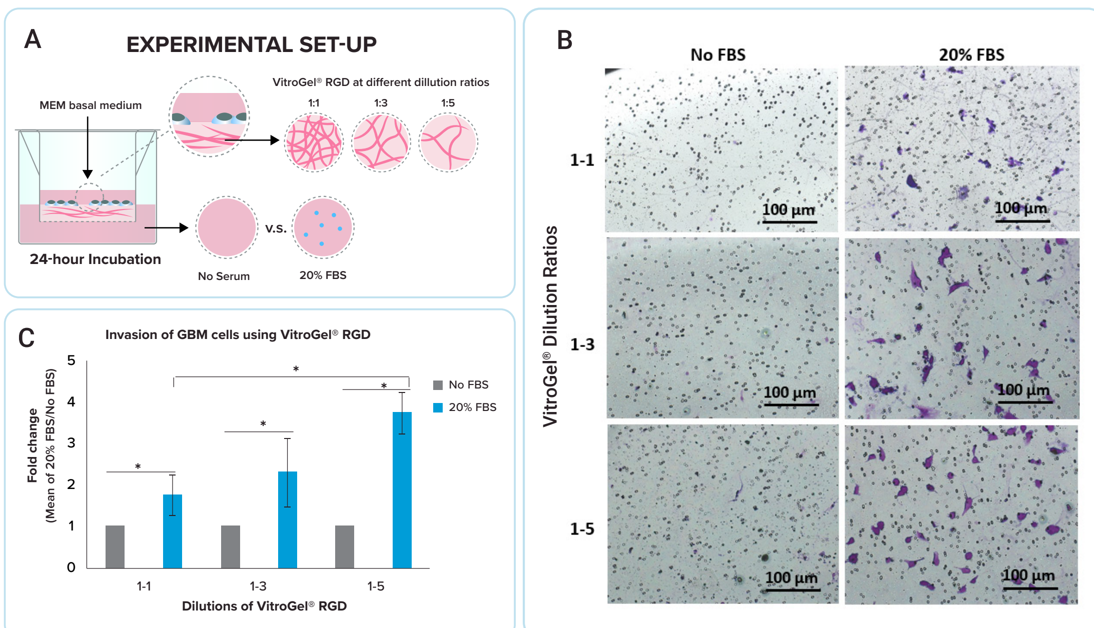


Figure 1. Invasion of U87-MG glioblastoma (GBM) cells using VitroGel® RGD high-concentration hydrogel.

A. Schematic representation of experimental setup. **B**. Microscopy images showing U87-MG glioblastoma cell invasion through VitroGel® RGD. The hydrogel was diluted with VitroGel® Dilution Solution in a 1:1, 1:3, and 1:5 ratio. Images were obtained with a Zeiss microscope at a 10X magnification. **C.** Fold change of cell invasion in the FBS group relative to the No FBS group for each dilution of VitroGel® RGD. The No FBS group was normalized to 1. The asterisk denotes $p < 0.05$.

CASE STUDY 2

Study the effect of hydrogel functional ligands and degradability on cell mobility

Materials

- **Cell Invasion Assay Kit: VitroGel® 3D, RGD, & MMP Cell Invasion Assay (High-concentration, Catalog #: IV-HC001-1P, IV-HC003-1P, IV-HC010-1P)**
- **Insert: VitroGel® 3D, MMP, or RGD hydrogel at 1:3 dilution ratio**
- 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: 48 hrs.

VitroGel® High-Concentration hydrogels are customized with various functional ligands for cell-based applications. To examine how bio-functional ligands within hydrogel matrices modulate cell invasion, we used VitroGel® 3D, an unmodified hydrogel; VitroGel® RGD, a hydrogel modified with fibronectin functional ligand that support cell adhesion; and VitroGel® MMP which is modified with matrix metalloproteinases sensitive functional ligand that enhance matrix degradability to study cell mobility (Figure 2A).

To perform the cell invasion assay, the VitroGel® hydrogel solutions were diluted with VitroGel® Dilution Solution at a 1:3 ratio. Then, the diluted hydrogel was mixed with MEM basal culture medium at a 4:1 ratio. The hydrogel mixture (100 µL) was added to each insert and allowed to solidify for 20 minutes at room temperature. After this, U87-MG cells were prepared in MEM basal medium and incorporated on top of the hydrogels (3 x 10^4 cells per insert). The outer wells were replenished with MEM basal medium or MEM medium supplemented with 20% FBS (500 µL per well). The cultures were incubated for 48 hours at 37°C. Following incubation, crystal violet staining was performed to evaluate cell invasion (Figures 2 B-C).

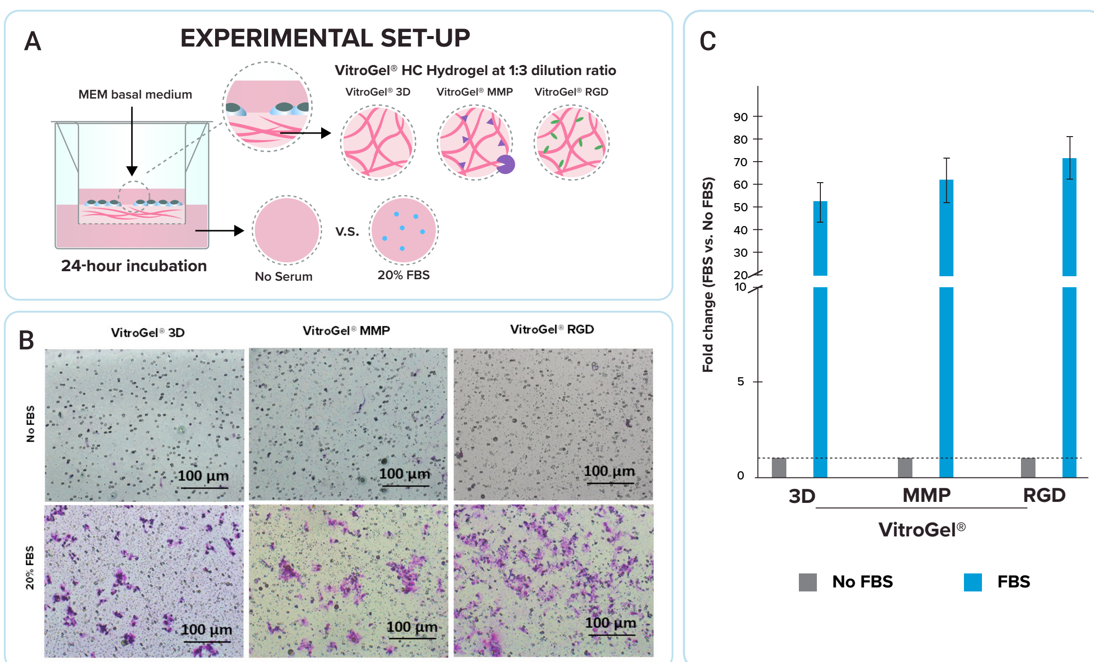


Figure 2. Bio-functional ligands inside hydrogel influence U87-MG glioblastoma cell invasion.

A. Schematic representation of experimental setup. Hydrogels were diluted with VitroGel® Dilution Solution in a 1:3 ratio, placed in the insert, and allowed to solidify for 20 mins. **B.** Light microscopy images showing U87-MG glioblastoma cell invasion through VitroGel® High-Concentration hydrogels with different bio-functional ligands. Images were obtained with a Zeiss microscope at a 10X magnification. **C.** Fold change of cell invasion in the FBS group relative to the No FBS group for each hydrogel.

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CASE STUDY 3

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

Materials

- **Cell Invasion Assay Kit: VitroGel® 3D and VitroGel RGD® Cell Invasion Assay (High-concentration, Catalog #: IV-HC001-1P, IV-HC003-1P)**
- **Insert: VitroGel® 3D and VitroGel® RGD hydrogels at 1:3 dilution ratio with TGF-β1 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: 48 hrs.

To examine U87-MG glioblastoma cell invasion towards a TGF-β1 gradient inside the hydrogel matrix, we first performed a 1:3 dilution of VitroGel® 3D and VitroGel® RGD with VitroGel® dilution solution, respectively. Subsequently, the hydrogel mixture was combined with MEM basal medium or MEM medium supplemented with TGF-β1 (30 ng/mL) in a 4:1 ratio to achieve a final concentration of 6 ng/mL of TGF- β1 inside the hydrogel matrix. The hydrogel mixture was then added to the inserts (100 µL per 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. Serum-free medium or medium supplemented with 20% FBS was placed in the outer wells (500 µL per well). The cells were incubated for 48 hours at 37°C. After that, cell invasion was assessed by performing crystal violet staining.

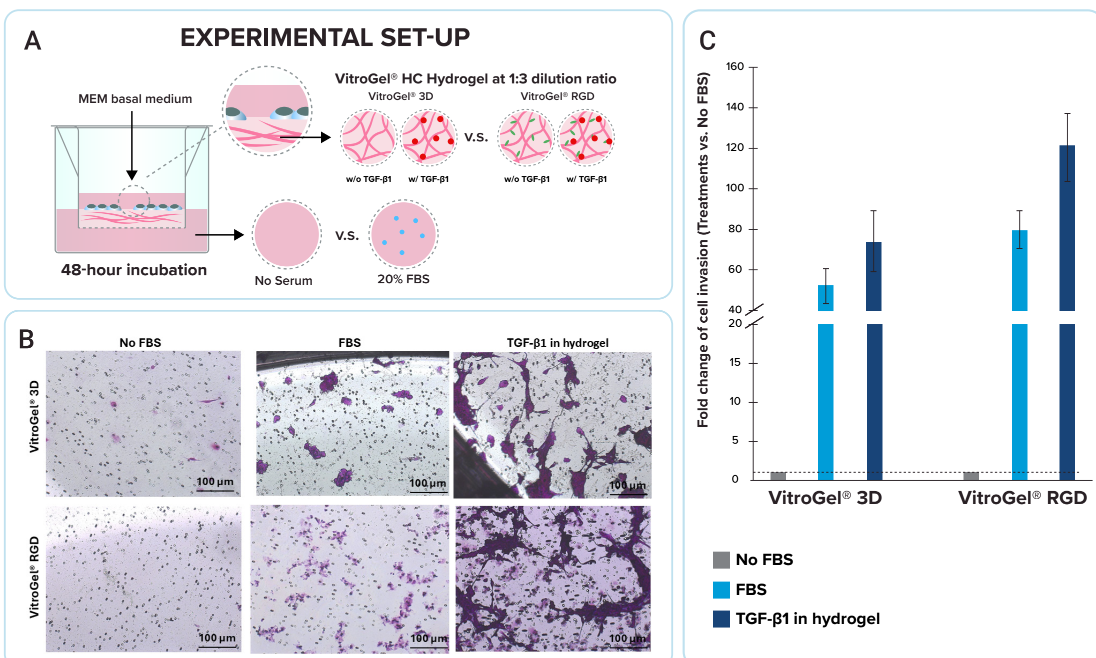


Figure 5. TGF-β1 inside VitroGel® 3D and VitroGel® RGD facilitates U87-MG glioblastoma cell invasion.

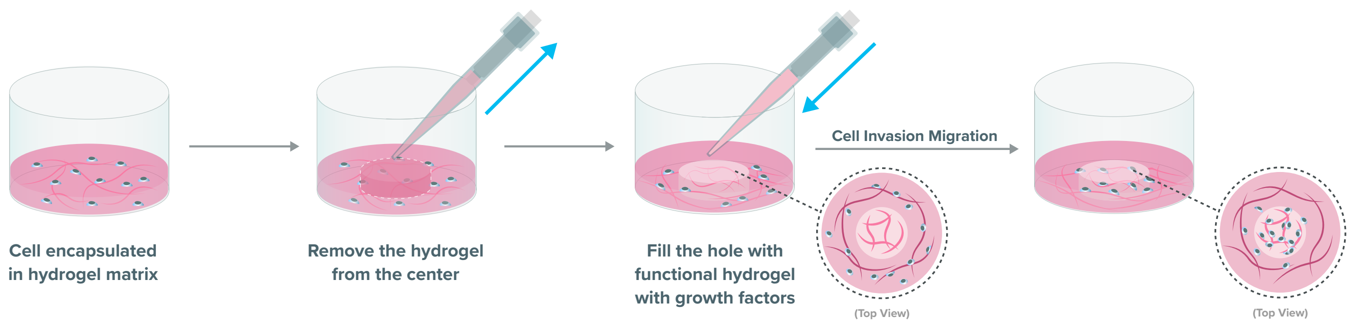
A. Visual representation of experimental setup. Cultures were incubated for 48 hours. **B.** Microscopy images demonstrating U87-MG glioblastoma cell invasion through VitroGel® 3D and RGD. Each hydrogel was diluted with VitroGel® Dilution solution in a 1:3 ratio and then combined with MEM basal medium or MEM with TGF-β1 (30 ng/mL) in a 4:1 ratio. Images were obtained with a Zeiss microscope at a 10X magnification. **C.** Fold change of cell invasion in the TGF-β1 in hydrogel and FBS groups relative to the No FBS group for each hydrogel. The No FBS group was normalized to 1.

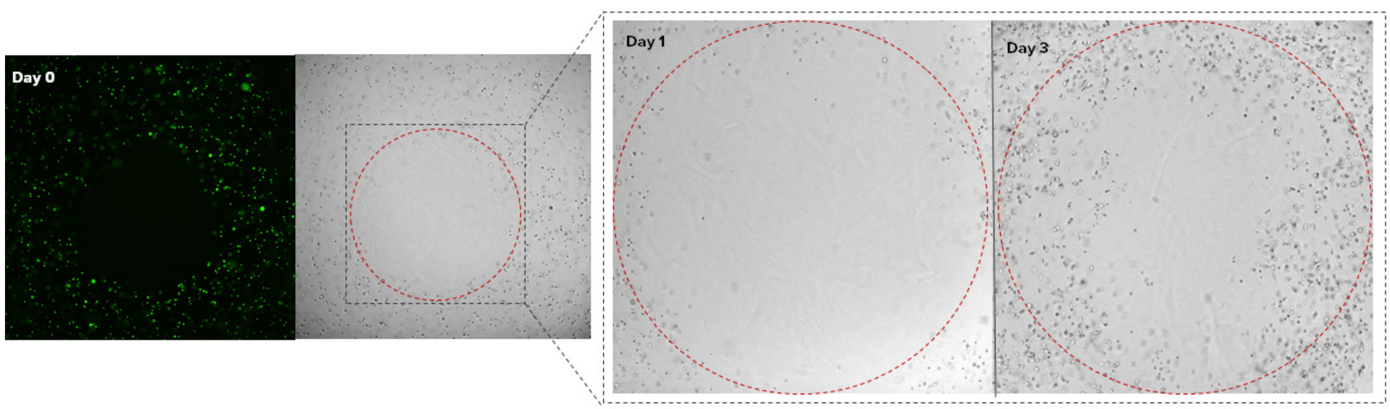
Other ways to use VitroGel® hydrogel for invasion assay

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 images 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/".

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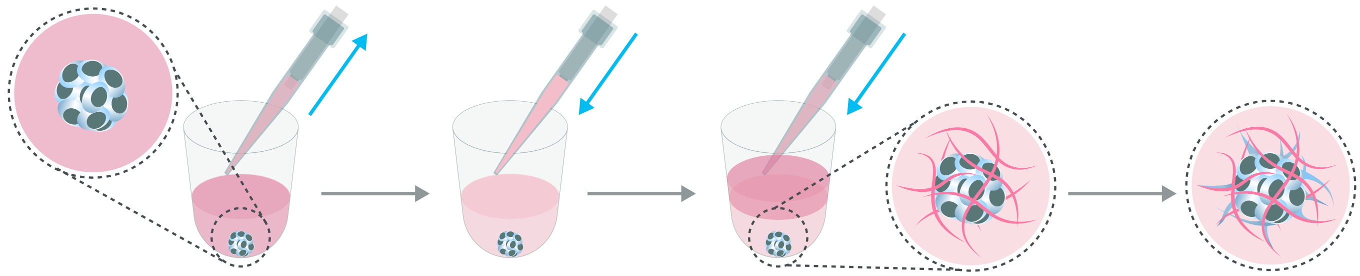
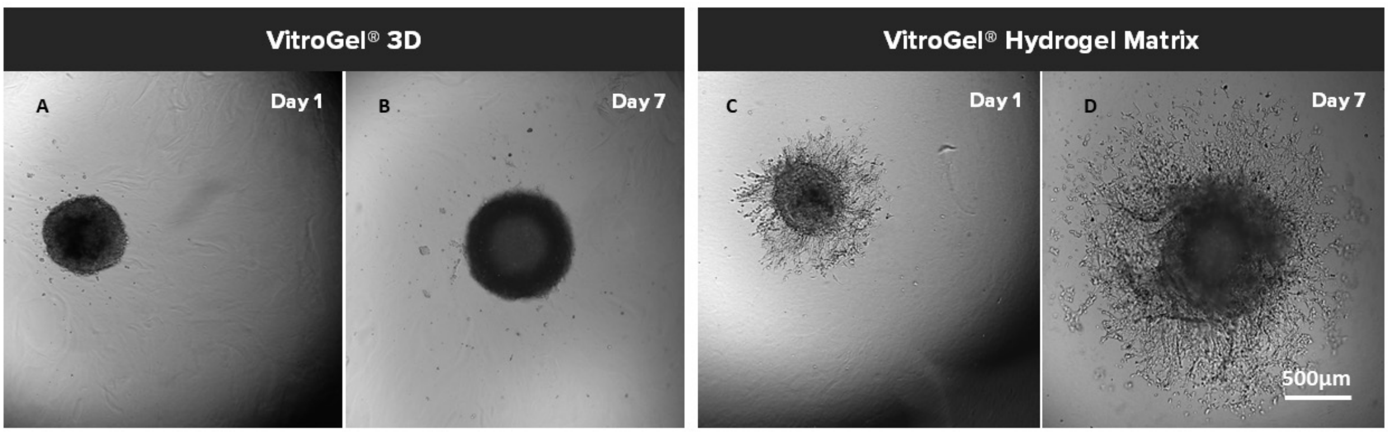
3D Spheroid Invasion Assay

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