Spheroid Invasion Assay With the Xeno-free Functional VitroGel® Hydrogel Matrix

Application Note

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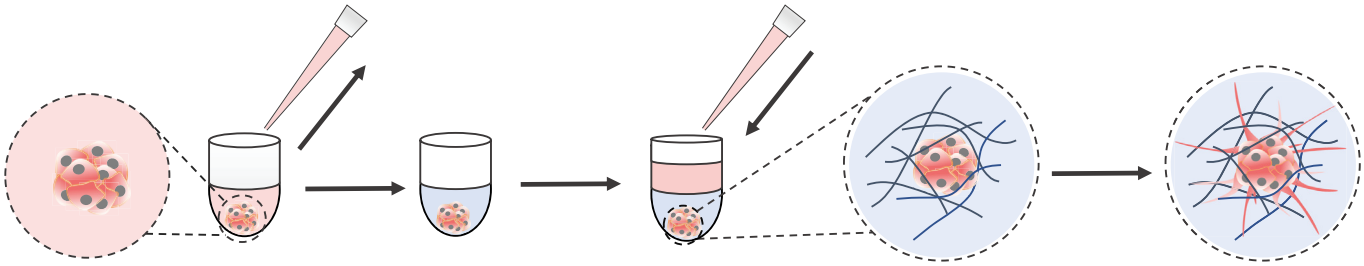


Introduction

Cell migration and invasion play important roles in various physiological processes such as gastrulation, embryonic morphogenesis, development of the nervous system, and immune cell trafficking. Cells need to be able to change, interact with their environment, and reach their proper spatial orientation in a physiological setting to ideally execute their functions. Similarly, cell invasion is also crucial in the regulation of many pathological processes such as inflammation, cancer metastasis, cardiovascular disease, and preeclampsia. Recently, the utility of a spheroid invasion assay has increasingly become appreciated[1,2]. In this assay, cells are grown in a three-dimensional culture, in which their biological properties better mimic what would occur in a live tissue environment. For cancer study, spheroid culture allows better preservation of the interactions between cells and/or between cells and the extracellular matrix (ECM)[3]. The development of reliable, versatile, and easy-to-use spheroid invasion assays is thus very desirable.

In cancer invasion, there are three basic steps involved: (1) attachment of tumor cells to the extracellular matrix (ECM); (2) biodegradation of the ECM via proteolysis; and (3) cell migration through the compromised ECM. Each of these steps is important for tumor invasion to be successful, which means that the ECM must have components to promote attachment and biodegradation. Current animal-based ECM contains components from non-human sources that introduce unpredictability to 3D cell invasion study. Our synthetic, xeno-free functional hydrogel – VitroGel® system was developed to rectify this shortcoming. The state-of-the-art hydrogel system can closely mimic the physiological and functional properties of the native ECM and give an outstanding balance of biological complexity and operating ease to establish robust 3D cell models.

Tumor spheroids exhibit several characteristic physiological traits such as increased cell survival, hypoxic core, strong cell-cell interactions, etc. In this study, we establish a spheroid invasion assay by using U-87 MG glioblastoma cell line to study the tumor mobilization in a different hydrogel matrix. The cancer cell spheroids were created using the ultra-low attachment U-shaped plate (**S-BIO PrimeSurface® 96U Plate** (Cat# MS-9096UZ) before the invasion study. The spheroid invasion was established by adding the cell spheroid directly on top of a layer of hydrogel or encapsulating inside of the hydrogel matrix. In traditional animal-based ECM, the complexity of the hydrogel compositions makes it extremely difficult to understand how the hydrogel properties can affect cell mobility. However, the xeno-free VitroGel system can be easily manipulated in hydrogel strength, binding ligand, degradability, and supplement compositions, making it an excellent system to give an in-depth understanding of the relationship between the microenvironment and cell behaviors. To demonstrate that, we select two VitroGel hydrogels as examples in this study to show the effects functional binding ligands have on the invasion process: 1) **VitroGel Hydrogel Matrix** (Cat# VHM01), a ready-to-use hydrogel of multi-functional ligands; and 2) **VitroGel 3D** (Cat# TWG001), a high concentration hydrogel without binding ligand modification. The same concept can be explored in other types of VitroGel systems for different hydrogel properties. The protocol described here provides a reproducible method to form cancer spheroids and to observe invasion through an ECM. Our results demonstrate the VitroGel Hydrogel Matrix contains crucial biochemical and mechanical properties allowing for the replication of *in vivo* physiological processes and show the hydrogel's potential to be used for high throughput screening pharmacological studies, culture of patient-derived culture models, and other types of translational research.

Materials and Methods

Cell Culture

U-87 MG cells were maintained in Alpha Modified Eagle Medium (αMEM) with 10% Fetal Bovine Serum (FBS). The cells were passaged when the cultures reached 80% confluence. The cells were harvested using trypsin and suspended in complete cell culture medium with 10% FBS at a concentration of 5 X 10^5 cell/ml for 3D cell spheroid formation.

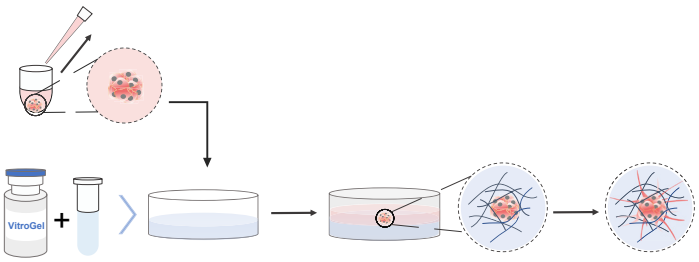
Overnight Spheroid Formation using U-Shaped Plate

1. 50 µl of the cell suspension was added carefully into each well of a 96 well ultra-low attachment U-shaped plate (**S-BIO PrimeSurface® 96U Plate,** Cat# MS-9096UZ).
2. The plate was incubated at 37°C, 5% CO_2 overnight to obtain uniform glioblastoma spheroids for the invasion assay.
3. The spheroids were carefully harvested before incorporating into the VitroGel hydrogels in two different invasion methods: 3D encapsulating invasion and 2D hydrogel coating invasion.

Hydrogel Preparation

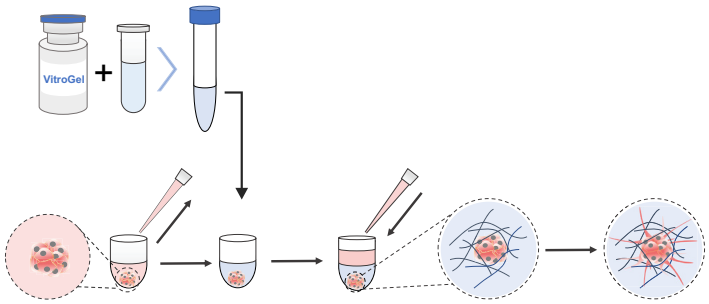
The ready-to-use **VitroGel Hydrogel Matrix** (Cat# VHM01) and the high concentration **VitroGel 3D** (Cat# TWG001) were selected in this experiment. VitroGel Hydrogel Matrix can be directly mixed with cell culture medium for hydrogel formation. The VitroGel 3D was prepared at a 1:3 dilution with VitroGel Dilution Solution TYPE 1 (v/v) and 4:1 mixing (v/v) with the cell culture medium (αMEM + 50% FBS, please check VitroGel 3D user handbook for details).

2D Hydrogel Coating Invasion
(Using VitroGel Hydrogel Matrix as an example)



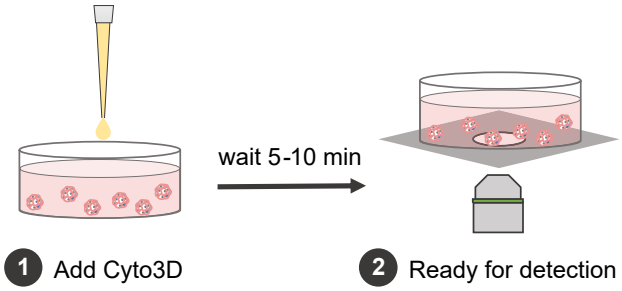
1. The hydrogel solution was brought to room temperature or warmed up to 37°C.
2. The hydrogel solution was mixed with the cell culture medium (αMEM + 30% FBS) at 2:1 (v/v) ratio in a microcentrifuge tube. (The mixing ratio was adjusted to 4:1 (v/v) when using VitroGel 3D.)
3. 50 µl of the hydrogel-cell culture mixture was added to each well in a new flat-bottom 96 well plate.
4. Incubation of 15–20 minutes at room temperature was allowed for smooth gel formation and stabilization. Do not disturb the hydrogel by tilting or shaking the well plate during the formation process.
5. The 50 µl of the medium and the formed spheroid from the U-Shaped plate was carefully pipetted out. Care was taken not to disrupt the structure of the formed spheroid.
6. The spheroid and medium were added to the top of the formed hydrogel in the flat-bottom plate. Care was taken not to introduce bubbles into the wells.
7. The plate was immediately incubated at 37°C to complete the polymerization process.
8. The cover medium was changed every 2–3 days by pipetting half of the cover medium out and replacing it with 50 µl of fresh medium. Care was taken not to disturb the hydrogel.

3D Encapsulation Invasion
(Using VitroGel Hydrogel Matrix as an example)



1. The hydrogel solution was brought to room temperature or warmed up to 37°C.
2. About 45 µl of the medium was carefully pipetted out of each well of the U-shaped plate. Care was taken not to disturb the structure of the formed spheroid nor to pipette it out.
3. The hydrogel solution was mixed with the cell culture medium (αMEM + 30% FBS) at 2:1 (v/v) ratio in a microcentrifuge tube. (The mixing ratio was adjusted to 4:1 (v/v) when using VitroGel 3D.)
4. 50 µl of the hydrogel mixture was immediately and carefully added to the U-shaped plate well with the spheroid.
5. The mixture was pipetted twice to make sure the spheroid was correctly suspended in the hydrogel mixture. Care was taken not to pipette too hard to introduce bubbles nor to break apart the spheroid.
6. The hydrogel was allowed to form for about 20 minutes at room temperature. The hydrogel was not disturbed by tilting or shaking the well plate during the formation process.
7. The hydrogel was carefully covered with 50 µl cell culture medium (αMEM + 10% FBS) and incubated immediately at 37°C to complete the polymerization process.
8. The cover medium was changed every 2-3 days by pipetting half of the cover medium out and replacing it with 50 µl of fresh medium. Care was taken not to disturb the hydrogel.

Measuring Cell Viability Using the Cyto3D Live-Dead Assay Kit



1. The **Cyto3D Live-Dead Assay Kit** (Cat# BM01) was brought to room temperature.
2. The Cyto3D reagent was added to the cover medium of the selected well (2 µl of Cyto3D reagent to every 100 µl total volume in a well).
3. The plate was incubated at 37°C for 5-10 minutes, after which the cells were ready for imaging to detect viability (following the product protocol for fluorescence microscope imaging details).

Calculating Cell Surface Area

The invasion process was tracked by imaging the cells over 7 days. The imaging was performed using an auto-microscope system, ImageXpress (Molecular Devices), and the image analysis was performed on MetaExpress High Content Image Acquisition and analysis software. We measured the cell area occupied by the growing U-87 MG spheroid and the metastatic invadopodia (the outgrowth of epithelial structures) produced through the invasion process.

Results

The U-87 MG cell spheroids were harvested from the ultra-low attached U-shaped plate and placed on top of the hydrogel matrix (2D hydrogel coating invasion). Growth of the cell spheroids on both VitroGel 3D and VitroGel Hydrogel Matrix can be seen over a 7-day course (Figure 1). The cells on VitroGel 3D maintained the spheroid morphology with an expansion in size from day 1 to day 7 (Figure 1a; 1b).

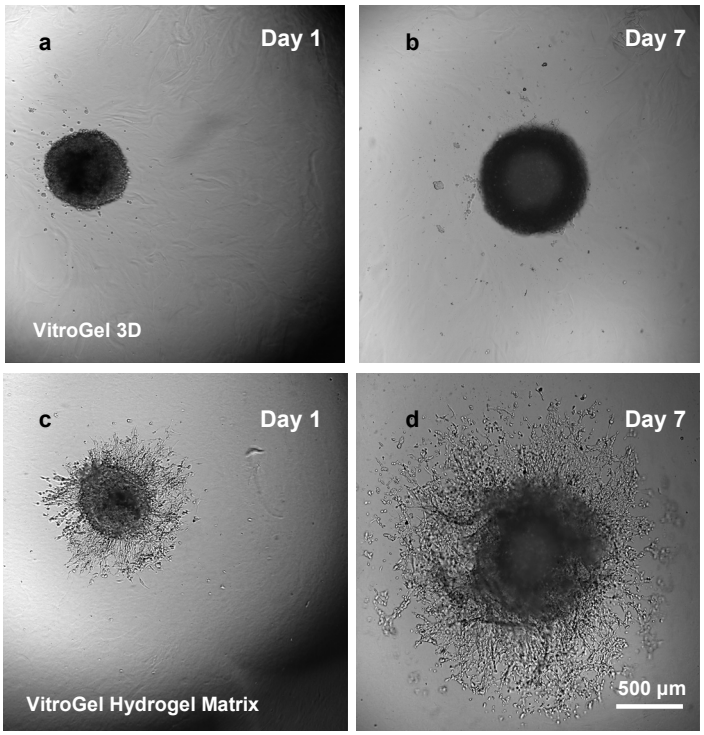


Figure 1. Growth of cell spheroids over time in the VitroGel 3D (a and b) and the VitroGel Hydrogel Matrix (c and d).

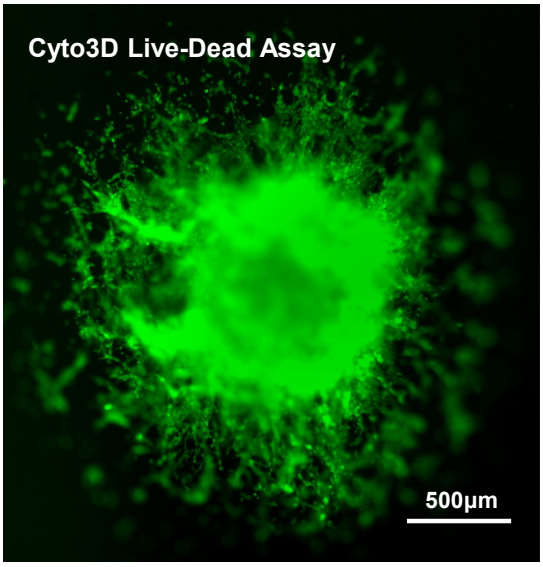


Figure 2. Live-dead assay on day 7 cells cultured with VitroGel Hydrogel Matrix.

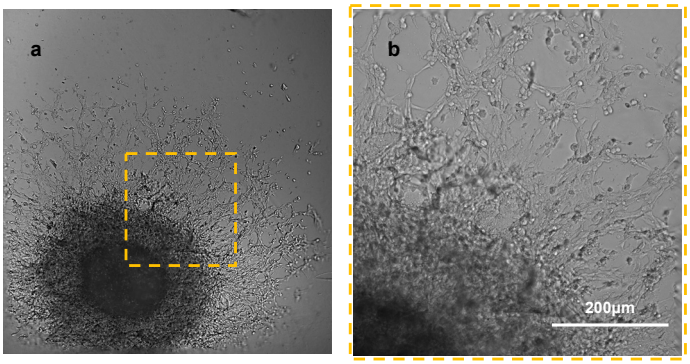


Figure 3. Day 7 of culture with VitroGel Hydrogel Matix. Panel b is the enlargement of the area in panel a.

However, the spheroid did not develop epithelial extensions characteristic of U-87 MG cells that would show the invasion of the cells through the hydrogel matrix. In contrast, the 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 (Figure 1c; 1d). The live-dead assay of day-7 cells on VitroGel Hydrogel Matrix (Figure 2) revealed significant cell viability when this functional hydrogel was used for 3D spheroid invasion assay. Comparing the images of spheroids in VitroGel 3D and VitroGel Hydrogel matrix, it is important to consider the pathophysiology of cell-matrix interaction within a 3D microenvironment.

The multifunctionality of the hydrogel-initiated cell-cell and cell-matrix interactions facilitates invasion within the hydrogel matrix. With the VitroGel Hydrogel Matrix, the morphology of the spheroid by day 1 is already seen to produce epithelial structures around the spheroid as the cells begin to engage with their microenvironment (Figure 1c). By day 7, we observed the clear cellular network structure around the cell spheroid, which indicated a strong invasion process during the culture period (Figure 3). This spheroid invasion assay replicates the metastasis progression, making it an excellent model for understanding the intricacies of the pathology and progression of the disease.

To qualify the differences of spheroid invasion between two hydrogels, we used the MetaExpress High Content Image Acquisition and analysis software to compare the cell surface area of the spheroid in VitroGel 3D and VitroGel Hydrogel Matrix on days 1 and 7. The blue masked images in Figures 4a and 4b indicate the surface area of the spheroid in day 1 that was used as an indicator of the invasion capacity of the glioblastoma cell line in the 3D system. The results show that VitroGel 3D produced about a 2-fold increase in surface area of the spheroid over 7 days, while VitroGel Hydrogel Matrix produced more than a 10-fold increase in surface area on account of the invasion properties of the spheroid in the system (Figure 4c).

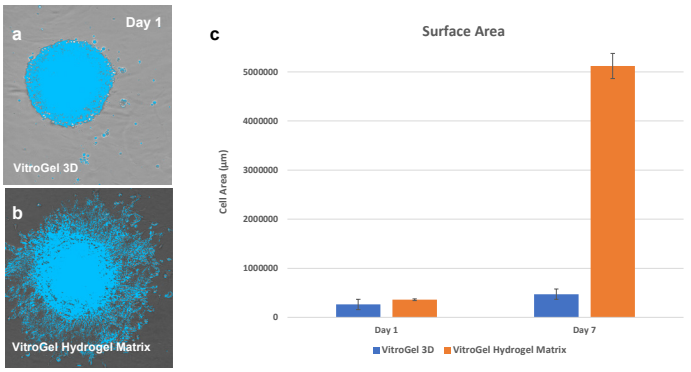


Figure 4. Surface area comparisons of culture growth in the two matrices.

Compared to the spheroid invasion by using the 2D hydrogel coating method, one could also directly add the hydrogel into the ultralow attached U-shape plate to encapsulate the U-87 MG cell spheroid in hydrogel matrix for 3D invasion. Similar to the results from the hydrogel coating method, the cell spheroids show great differences in the development of the invading structures depending on which hydrogel matrix was used (Figure 5). In VitroGel 3D, the cells maintain the spheroid structure without significant changing in the sizes or showing the cellular networking structure around the spheroid. In contrast, the spheroid grown in VitroGel Hydrogel Matrix paralleled the results from the hydrogel coating method. By day 1 the process of invasion in VitroGel Hydrogel Matrix had started, and by day 7 the malignant nature of the cells was visibly by the size and thickness of the spheroid, the surface area of the structure, and the development of cell-cell and cell-matrix interactions within the hydrogel.

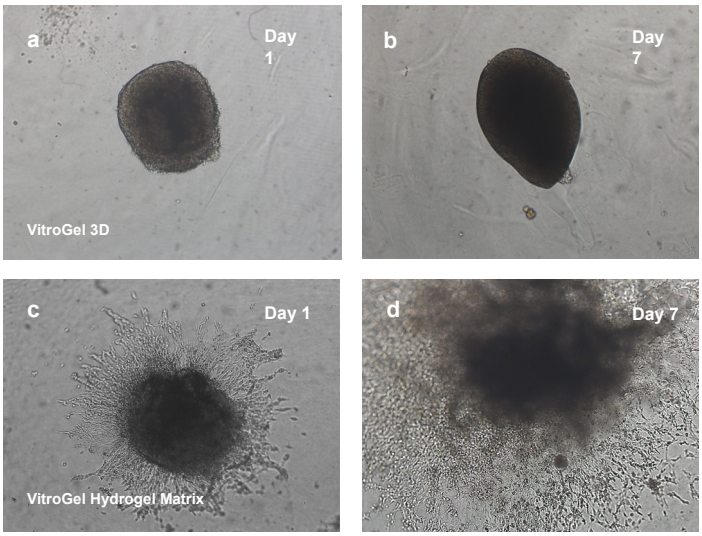


Figure 5. Images from direct attachment to U-shaped plate during the assay.

Discussion

This study has shown that the VitroGel hydrogel system can produce a comprehensive and physiologically related cancer invasion model for exploratory and pharmacological studies. The system allowed the spheroids to exhibit physiological characteristics such as epithelial structures, strong cell-cell bonds, increased cell survival, and movement of structures through the matrix. VitroGel Hydrogel Matrix supplies the spheroids with multiple binding ligands to facilitate the formation of strong and complex cell-cell and cell-matrix interactions while maintaining the viability of the cell. These interactions lead to the development of plasticity in the cells that result in chemoresistance in tumors. The organization of the cells and the interactions maintained result in the specialization of different populations of cells within the tumor. These populations end up initiating stem cell pathways that lead to tumor heterogeneity and the longevity of the disease. Using a system like VitroGel Hydrogel Matrix, this process can be understood to develop novel therapeutic interventions to combat the disease.

In addition, the support the hydrogel provides to the tumor microenvironment is also robust enough to support other cell types in the case of a co-culture between different systems. The VitroGel hydrogel system should have broad applicability in myriad cell models, not only in different cancer mimics but also in a wide variety of eukaryotic tissue environments, such as angiogenesis by endothelial cells. It will be of great interest to explore further the physical and physiological properties of the hydrogel matrix. The proliferation of 3D cell culture, both in academic and clinical milieus, opens up new frontiers for theoretical and practical advances[4]. Studies like these help bridge the gap between basic research and clinical research, which eventually will impact patient health. It will enable researchers to study disease in physiologically accurate contexts and to study the interaction of disease models and different biological systems in a living organism. The results will clearly be crucial in the innovation of medical and health practices.

References

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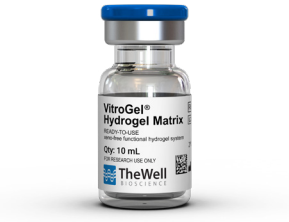
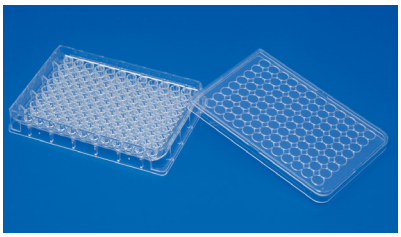
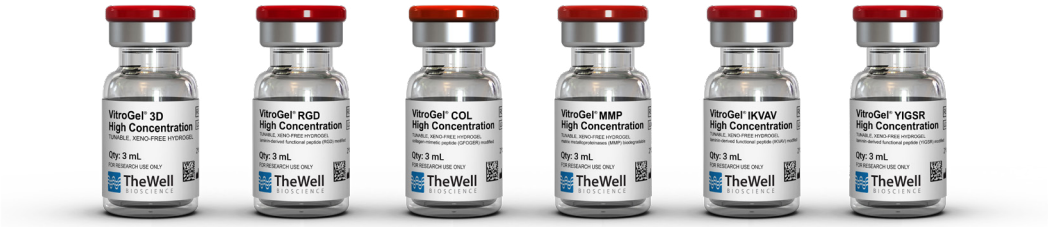


VitroGel® Hydrogel Matrix
(Cat# VHM01)

Cyto3D™ Live-Dead Assay
(Cat# BM01)



PrimeSurface® 96U Plate
(SBIO Cat# MS-9096UZ)



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