

Generation of Spheroids

1. General Information

In recent years, three dimensional (3D) culture systems have gained increasing recognition as an effective tool for biological research. Cells cultured in 3D more closely mimic the physiological environment of living organisms compared to conventional monolayer culture systems. One widely used 3D culturing technique is the application of multicellular spheroids. Spheroids are microscale, spherical cell clusters which grow free of foreign material.

2. Principle

There are several methods available for the generation of spheroids which are used more or less frequently. All the methods available prevent cells from attaching to the culture ware substratum, thereby increasing interactions with neighboring cells and extracellular matrix. In this Application Note we present the liquid-overlay technique for spheroid generation. This method is one of the simplest approaches for spheroid formation and has several advantages. First, single spheroids can be generated and monitored allowing an easy evaluation of growth kinetics and spheroid characteristics. Second, a simple medium exchange allows longer cultivation times. Last, no special equipment is needed. In short, wells are coated with agarose before adding cell suspension. Besides providing a nonadhesive surface, the agarose-coated wells also increase cell-to-cell contact as cells collect on their concave bottoms.

3. Material

For this protocol the following material is necessary:

- 96-well plates, flat bottom (for example: Corning, 3370)
- 1.5% agarose (dissolved in PBS or water) (for example: Sigma-Aldrich Chemie GmbH, A9539)
- Trypsin or alternatives for cell detachment
- Cell culture medium

For this protocol the following equipment is necessary:

- Thermal incubator, microwave or autoclave to melt agarose
- Inverted microscope for determining growth kinetics
- Incubator

4. Procedure

Preparation of the 96-well plate:

1. Prepare 1.5% agarose.
2. Coat the inner wells with 50 μ l of 1.5% agarose. Agarose solidifies and cools down to room temperature in 20 minutes. A volume of 50 μ l agarose is appropriate to entirely cover the surface of the well and produce a concave surface.
3. Fill 200 μ l PBS in the outer wells of the 96-well plate to create an evaporation barrier.

Spheroid formation:

1. Prepare cell suspension, as usual.
2. Enumerate the cell number and dilute the cell suspension to the desired cell concentration.
3. Transfer 50 μ l of the cell suspension into each well. The total volume of agarose and cell suspension per well should not exceed 200 μ l.
4. Incubate plate in a humidified atmosphere with 5% CO₂ in air at 37 °C.
5. Culture medium should be changed every other day after an initial incubation time of four days. Minimize movement of the plate, particularly during spheroid initiation.
6. Spheroid formation and growth can be evaluated using phase-contrast microscopy and spheroid viability using fluorescein diacetate (FDA)/ propidium iodide (PI) staining as described in [Application Note 33](#) "Live/dead staining with FDA and PI".

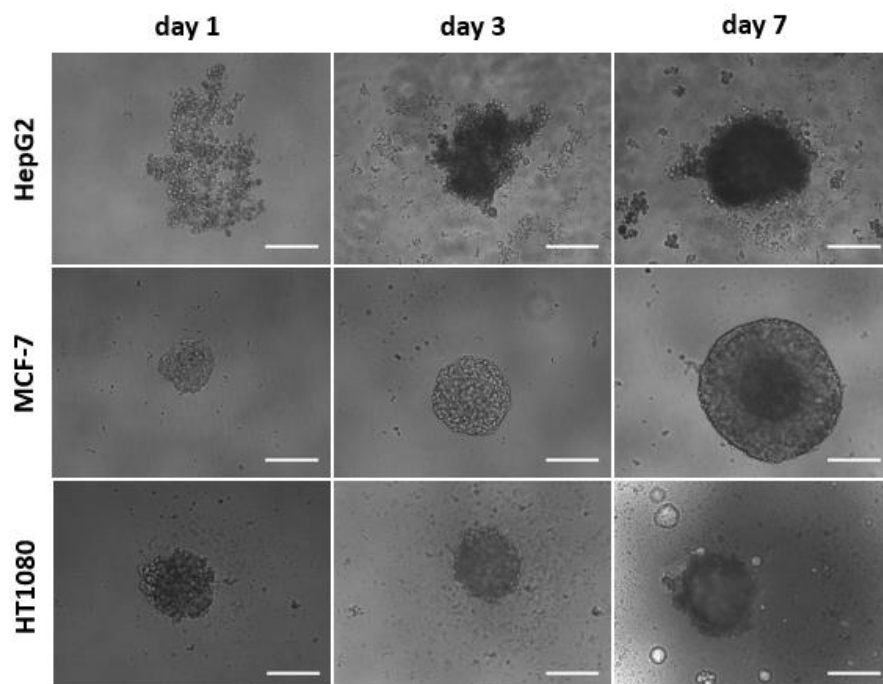


Figure 1 Spheroid formation and growth of different cancer cell lines. 500 cells/well were used as seeding concentration. (Scale bar: 200 μ m)

5. Notes:

- Size and thus characteristics of spheroids can be controlled by adapting seeding concentration or incubation time (Figure 1).
- Spheroid formation, growth kinetics and spheroid characteristics are cell type dependent (Figure 2).
- Serum quality and type critically affect spheroid formation and growth.
- Irregular or insufficient agarose coating can result in the formation of several irregular spheroids per well. Do not allow agarose to cool down below 60 °C to minimize the risk that agarose solidifies during dispensation.
- Formation of irregular, noncircular spheroids can be attributed to small particles in medium and/or serum. Monitor media and media supplements and filter supplemented media through sterile filter if required.

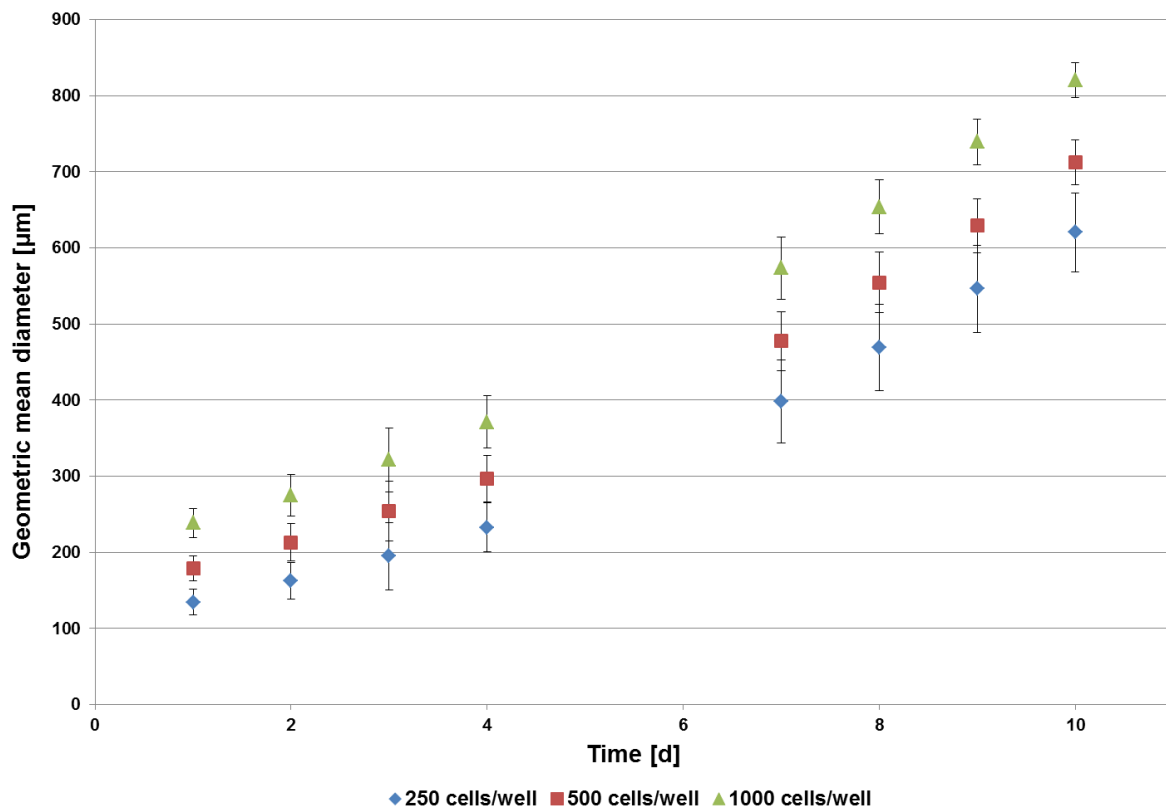


Figure 2 Spheroid growth of MCF-7 spheroids is dependent on the seeding concentration and the cultivation time.

Application Note 32

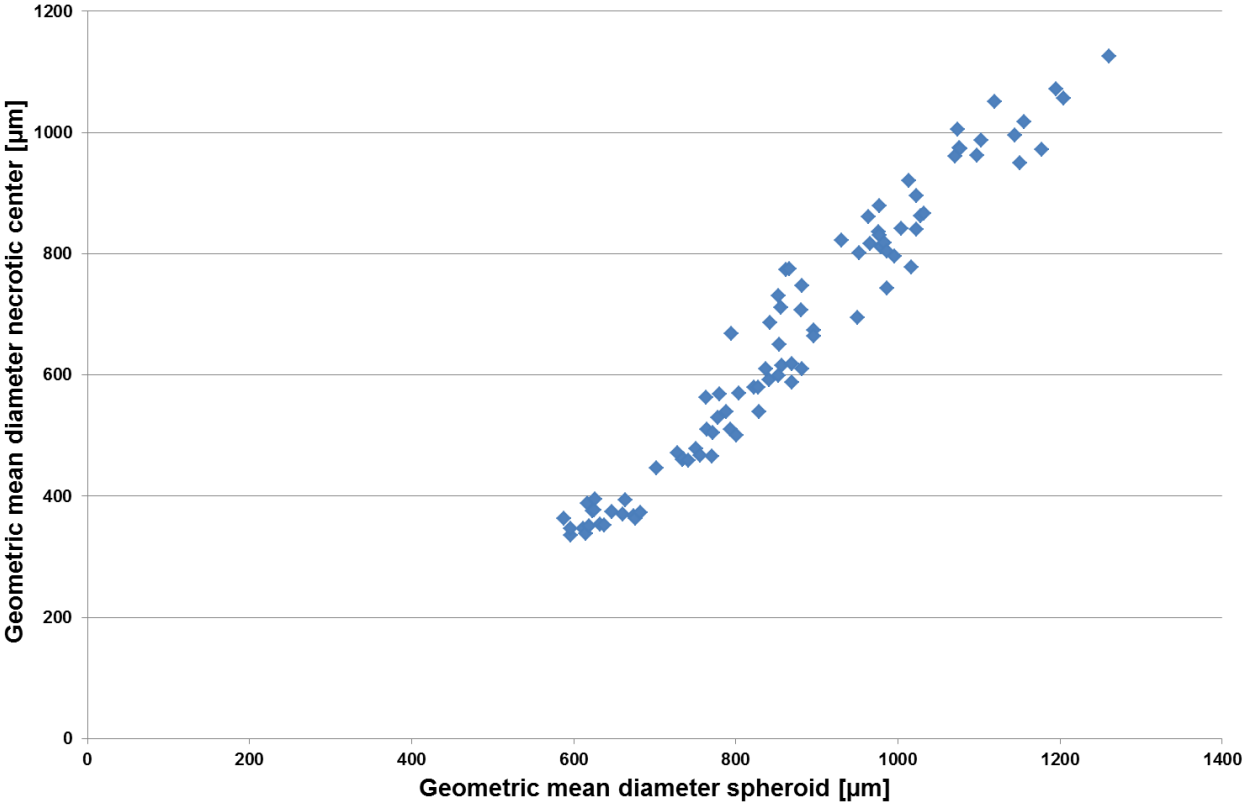


Figure 3 Correlation between MCF-7 spheroid size and the size of the necrotic center.