Chapter 20

Microfluidic-Based Generation of 3D Collagen Spheres to Investigate Multicellular Spheroid Invasion

Fabien Bertillot, Youmna Attieh, Morgan Delarue, Basile G. Gurchenkov, Stephanie Descroix, Danijela Matic Vignjevic, and Davide Ferraro

Abstract

During tumor progression, cancer cells acquire the ability to escape the primary tumor and invade adjacent tissues. They migrate through the stroma to reach blood or lymphatics vessels that will allow them to disseminate throughout the body and form metastasis at distant organs. To assay invasion capacity of cells in vitro, multicellular spheroids of cancer cells, mimicking primary tumor, are commonly embedded in collagen I extracellular matrix, which mimics the stroma. However, due to their higher density, spheroids tend to sink at the bottom of the collagen droplets, resulting in the spreading of the cells on two dimensions. We developed an innovative method based on droplet microfluidics to embed and control the position of multicellular spheroids inside spherical droplets of collagen. In this method cancer cells are exposed to a uniform three-dimensional (3D) collagen environment resulting in 3D cell invasion.

Key words Multicellular spheroids, 3D model, Cancer cells invasion, Droplet microfluidics, Extracellular matrix, Collagen

1 Introduction

The metastatic cascade is a multistep process that requires cancer cells to overcome many obstacles: cells need to breach the basement membrane they rest on, invade the surrounding stroma, find their way to the circulation that will allow them to travel throughout the body before they arrest in capillaries, extravasate, and colonize secondary organs [1]. Each step is crucial for cancer cells to move on to the next one. However, the study of cancer cell invasion and cell dynamics is a complex task, mostly because of the lack of proper model systems.

In 2003, Nature published an editorial article entitled "Goodbye, flat Biology?" that highlighted the necessity of switching from two-dimensional (2D) to 3D cell cultures, as it became evident that 3D models recapitulate the complexity of in vivo cell's behavior more faithfully. This article also predicted that it

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was "only a matter of time before 3-D techniques become standardized and cost-benefit ratios become irresistible in many areas of biology."

It is also becoming accepted that genetic and epigenetic modifications of cancer cells are not sufficient to drive metastasis formation. During their metastatic journey, cancer cells constantly interact with their microenvironment and modify it. In turn, microenvironment plays an active role in throughout the metastatic tumor progression, stimulating tumor growth, survival, and invasion capacity. However, "normal" microenvironment can also have the ability to revert cancer cells to a "normal" phenotype [2].

The tumor microenvironment consists of different cell types, such as endothelial cells, pericytes, immune cells, and fibroblasts; and the extracellular matrix (ECM), a 3D mesh of proteins that constitutes a scaffold for those cells [1]. Tumor-associated ECMs are mostly composed of collagen I fibers. Other matrix proteins such as fibronectin, laminin, or tenascin C are present in different amounts [3]. The ECM is generated and deposited by stromal cells, primarily fibroblasts.

Self-derived matrices generated by stimulating fibroblasts by hyaluronic acid to deposit their own matrix are commonly used as model of the tumoral ECM [4]. Fibroblasts are then removed and cancer cells are plated on those decellularized matrices. The major limitation of this model is that those matrices are rather thin, thus cells are usually not fully embedded [5].

Alternative model is based on artificial hydrogels. Synthetic scaffolds such as poly(ethylene glycol) (PEG) hydrogels allow embedding of the cells while polymerizing the matrix. Some hydrogels can reproduce the fibrillary structure of biological matrices and, most importantly, offer complete control over its mechanical properties such as stiffness and pore size [6]. However, the drawback of the artificial gels is that they lack signals necessary for cell survival, proliferation, and migration.

In order to overcome this issue, it is possible to use biological component of in vivo matrices, such as collagen I that can be polymerized in vitro. If the right polymerization conditions are used, these matrices resemble the matrices found in vivo [7, 8]. Even though their composition is simplified, they allow generation of truly 3D scaffolds.

In order to model tumor invasion, cell biologists either use single cancer cells or cell aggregates (spheroids), aiming to mimic small tumors [8, 9]. Cell aggregates can be easily generated using nonadhesive substrates, such as agarose or by more sophisticated methods, for example in alginate capsules made using microfluidic strategies [10]. Spheroids could be then embedded in collagen I gels, mimicking a small tumor invading the ECM [9–11]. However, because spheroids are objects denser than a water phase solution, when deposited in a non-polymerized collagen, they have the tendency

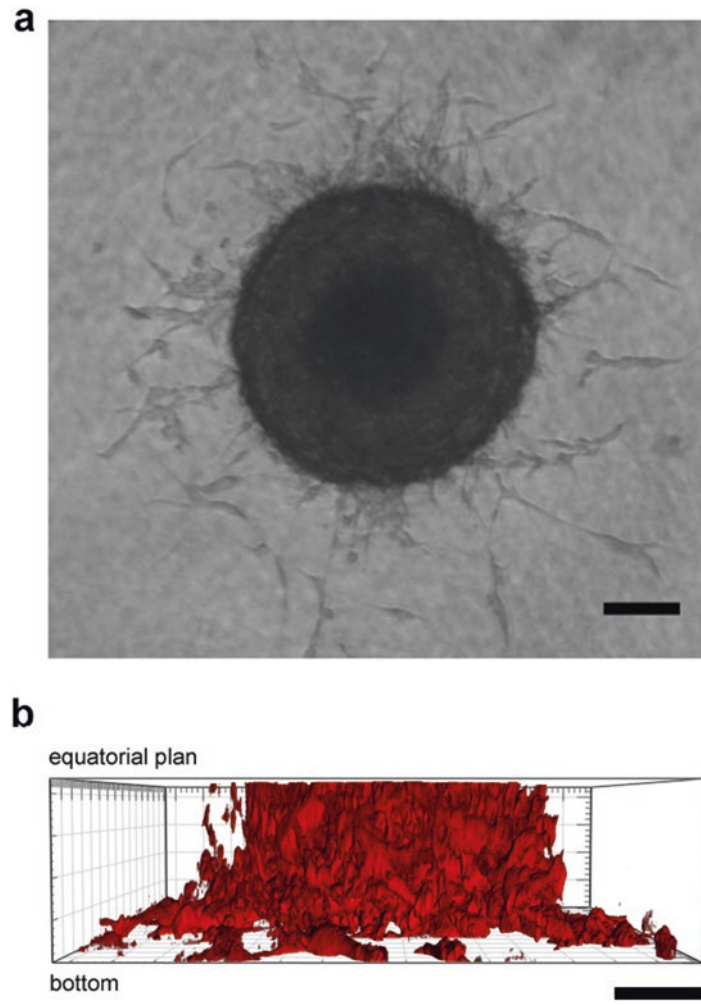

Fig. 1 2D spreading of a CT26 spheroid embedded in collagen droplet. Spheroid sank onto the glass surface of the dish and cells migrate in 2D. Scale bar, 50 μm. (**a**) Phase image of cancer cell invasion. The spheroid appears to invade in 3D but all the cells that have escaped are in the same focus plan, possibly, migrating on the glass surface. (**b**) Side view of a reconstructed 3D stack of a spheroid (LifeAct-Cherry, *red*) embedded in collagen (*not shown*). All cells that have escaped the spheroid migrated on the glass surface. The spheroid was imaged using an inverted two-photon microscope with a 40× oil objective

to sink onto the plastic dish while the collagen is polymerizing. Once on the hard and flat surfaces, cells tend to invade only in 2D (Fig. 1). To prevent spheroids' sinking, it is possible to polymerize collagen faster, for example by raising the collagen polymerization temperature. Nevertheless, when polymerized at higher temperatures, resulting gels are made of thin collagen fibers with small pores that do not resemble the structure of the ECM in vivo [7, 8]. Alternatively, collagen can be still polymerized at room temperature,

and sinking of the spheroids could be prevented by flipping the plastic dish continuously upside/down until collagen fibers are formed and position of the spheroid locked in the center of the collagen matrix. Although robust, this method requires an extensive amount of work and time. In order to overcome these limitations, we have developed an innovative strategy based on droplet microfluidics to embed cancer cells spheroids in the center of the collagen spherical droplets. In particular, droplet microfluidics mainly represents the possibility of generating stable and regular emulsion of two or more fluidic phases. Additionally, due to its large spreading during the last 10 years, droplet microfluidics is considered one of the most promising candidates for the generation of innovative and useful tools for new generation of biological experiments [12]. The capability of encapsulating cells in droplets has been already used for spheroids production [10, 13]; however, there are no examples concerning their encapsulation in collagen matrix especially with the final aim to control their position within the collagen matrix. Therefore, we have established a microfluidic platform that generates trains of non-polymerized collagen droplets containing spheroids (one spheroid per collagen droplet) flowing in a spiral capillary (Fig. 2). During collagen polymerization, which is performed at room temperature, spheroids are continuously moving inside the collagen droplet miming the flipping gesture of the plastic dish described above. Additionally, we worked with a confined droplet (droplet diameter is larger than the capillary size) [14] allowing the control of the final shape of the collagen droplet. Therefore, adjusting the droplet volume, at the end of the spiral, spheres of polymerized collagen containing spheroids in its center can be easily collected in conventional culture medium.

The validation of our approach has been performed with two different cell lines (CT26 and NIH 3T3), monitoring the spheroids position in the collagen droplet, their proliferation and invasion in the 3D matrix (Fig. 3).

2 Materials

2.1 Cell Culture

1. CT26-LifeAct-GFP cells: CT26 cells (ATCC CRL-2638) stably transfected with LifeAct-GFP (courtesy of S. Geraldo, Institut Curie).
2. NIH 3T3-GFP cells: NIH 3T3 cells (Cell Biolabs) stably transfected to express cytoplasmic green fluorescent protein (GFP).
3. Cell culture medium: 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin in Dulbecco's Modified Eagle's Medium (DMEM) with GlutaMAX.
4. 0.05% Trypsin/EDTA.

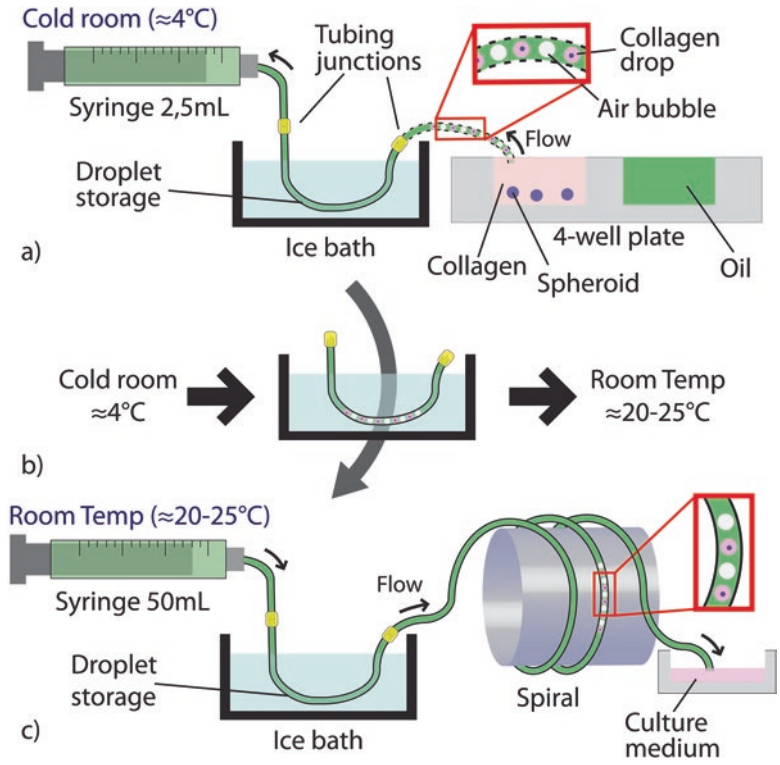

Fig. 2 Scheme of the microfluidic experimental setup for collagen droplet generation and polymerization. The *continuous black line* represents the larger PTFE capillary, *dashed black line* is the smaller capillary, and the *yellow connections* are silicon capillaries used as junctions. (**a**) Droplets are generated by alternating sucking of the oil solution (*green*), collagen containing spheroids (*light red* and *violet*, respectively) and the air, with the aspiration step performed by the syringe pump. The train of droplets is stored in the portion of capillary between *yellow connections* and it is immersed in the ice bath. (**b**) This part is detached from the rest of the setup, transferred to the room temperature and connected between the second syringe and the spiral (**c**). The train of collagen/spheroids droplets is pushed into the spiral. Collagen is polymerized during the flow and the collagen droplets containing spheroids positioned centrally are collected in culture medium

5. Cell culture dishes or flasks (T25).
6. Inverted light microscope.

2.2 Multicellular Cancer Cell Spheroids

1. Ultrapure agarose (Life Technologies).
2. 96-well flat-bottom plates.
3. 0.4% Trypan blue.

2.3 Collagen Mix

1. Rat tail collagen type I (Corning, 354236).
2. 10× phosphate buffered saline (PBS): Combine 2 g KCl, 2.4 g KH_2PO_4, 80 g NaCl, and 11.45 g Na_2HPO_4 and add H_2O to 1 L. Autoclave.

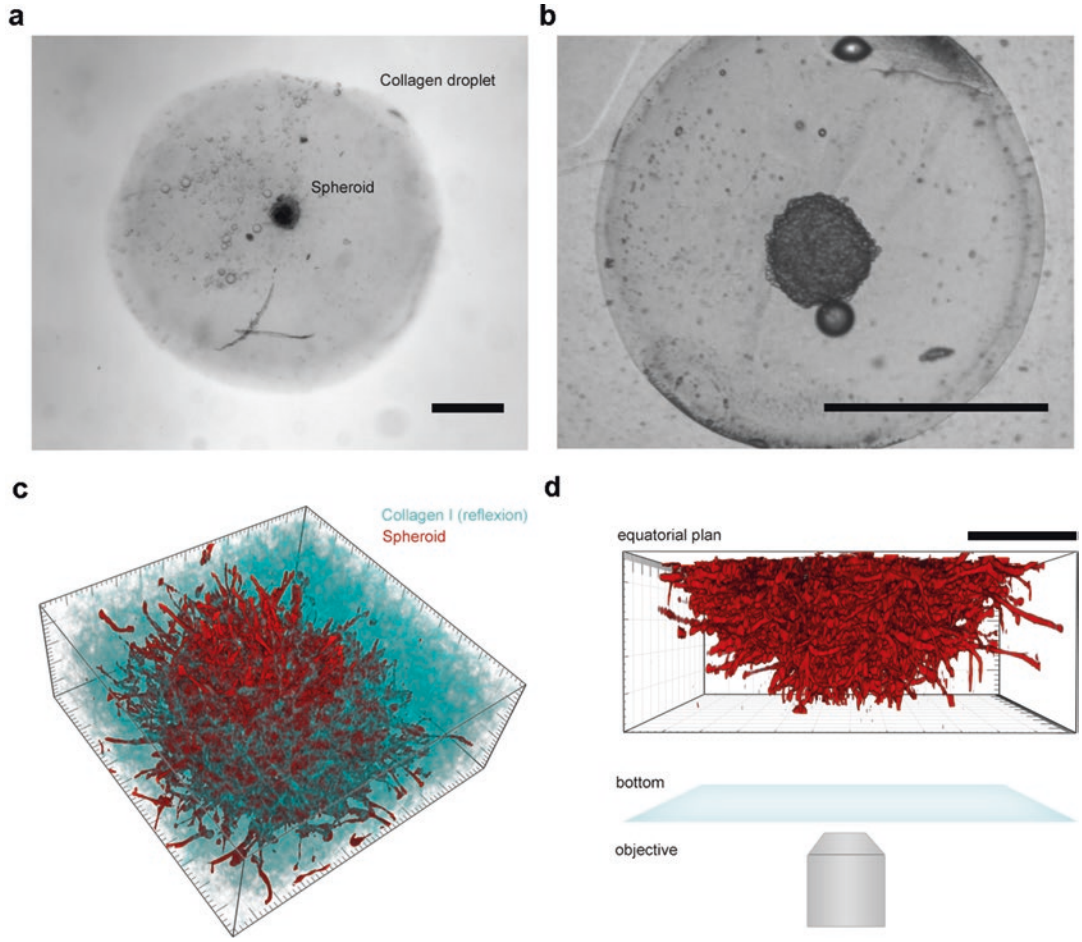

Fig. 3 CT26 and NIH 3T3 spheroids embedded in collagen droplets, before and after invasion. Scale bar, 500 mm. (**a**) 2 mm droplet with a spheroid of NIH 3T3 cells. (**b**) 800 μm droplet with a spheroid of CT26. (**c**) 3D reconstruction of a spheroid (LifeAct-Cherry CT26 cells, *red*) invading collagen (reflection, *cyan*) droplet in 3D. The spheroid was imaged using an inverted two-photon microscope with a 40× oil objective. Only one half of the spheroid has been imaged. (**d**) Side view of the invading spheroid. Spheroid is not in contact with the glass bottom of the dish and all cells invade in 3D

3. 1 N NaOH, sterile.
4. DMEM.

2.4 Microfluidic Setup

1. Syringe Pump neMESYS (Cetoni GmbHS).
2. Syringes of 2.5 mL and 50 mL of maximum capacity.
3. Capillaries (not necessarily sterilized, *see* **Note 1**):
   (a) About 6 m of PTFE capillary inner/outer diameter of 2 mm/4 mm.
   (b) About 50 cm of PTFE capillary inner/outer diameter of 1.5 mm/2 mm.

(c) About 10 cm of silicon capillary inner/outer diameter of 3 mm/4 mm.

4. Oil solution: 2% (w/w) 1H,1H,2H,2H–perfluorodecan-1-ol (Fluorochem) in FC-40 oil (3 M).
5. 4-well plate.
6. Plastic rectangular bar (about 4 × 2 × 30 cm).
7. 24-well cell culture plate.

3 Method

3.1 Cell Culture

1. Maintain the NIH 3T3-GFP and CT26-LifeAct-mCherry cells in cell culture medium at 37 °C in 5% CO_2 humidified air.
2. Passage cells at a ratio of 1:10 every 3 days to maintain their exponential growth.
3. To prepare single-cell suspension for cells for spheroid preparation, trypsinize a subconfluent culture of cells for 5 min at 37 °C (in cell culture incubator).
4. Stop trypsinization by adding fresh cell culture medium. Pipette the cell suspension repeatedly to create a single-cell suspension.
5. Pellet the cells by centrifugation at 500 × *g* for 3 min.
6. Resuspend the cells in fresh cell culture medium, count the cells, and adjust cell number per mL as needed.

3.2 Multicellular Spheroid Preparation

1. Prepare a fresh 1% agarose solution in deionized H_2O and boil it.
2. Immediately after boiling, add 50 μL of the hot agarose solution to each well of a 96-well plate in a laminar hood.
3. Let the agarose in plate cool down at room temperature for 30 min and by eyes check the gelation of agarose.
4. Prepare a single-cell suspension of 5000 cells/mL (*see* Subheading 3.1 and **Note 2**).
5. Add 200 μL of cell suspension to each well of a 96-well plate; one spheroid per well is generated.
6. Incubate the plate for 24 h (NIH 3T3) or 72 h (CT26) at 37 °C in 5% CO_2 humidified air (*see* **Note 3**).

3.3 Collagen Mix Preparation

Collagen polymerization mix is prepared including multicellular spheroids. The final concentration of the collagen mix is 2 mg/mL. From the initial stock collagen concentration *ci* and for a final volume of 1 mL of solution, calculate the volume of stock collagen necessary.

1. Transfer spheroids from the 96-well to a centrifuge tube.
2. Let spheroids sediment to the bottom of the centrifuge tube. Check by eyes.
3. Aspirate the medium without aspirating spheroids and resuspend in 315 µL of DMEM (*see* **Note 4**). Incubate in ice bath for 5 min.
4. In a 2 mL eppendorf tube, add 100 µL (1/10 of the final volume) of 10× PBS stocked at 4 °C.
5. Add 12 µL of 1 N NaOH stocked at 4 °C.
6. Add 315 µL of the suspension of spheroids.
7. Add 573 µL of collagen I. Gently mix the solution, avoiding formation of air bubbles.
8. Check the pH of the final solution with a pH paper indicator to ensure a pH of 7.5. Incubate the solution into an ice bath until its use.

3.4 Collagen Droplets Generation

1. Set the microfluidic setup for the droplet generation as represented in Fig. 2a in a cold room to avoid premature collagen polymerization. The setup is composed by the 2.5 mL syringe connected to the large PTFE capillary (represented by a continuous black line in Fig. 2) that will be used as droplets storage, which is connected to the small PTFE capillary (dashed black line) that needs for the droplets generation. The connection between the two capillaries is assured by the silicon capillary (yellow parts in Fig. 2) (*see* **Note 5**). Additionally, the storage part is immersed in an ice bath to prevent the collagen polymerization during the transfer to the second part of the experiment outside the cold room. After the setup installation, the droplet generation is achieved by pipetting as follows.
2. Prefill the whole fluidic system with the oil solution (green solution in Fig. 2).
3. Fill one of the wells of the 4-well plate with the oil solution and another one with the mix of non-polymerized collagen with the spheroids. Droplet generation is observed by a conventional inverted microscope.
4. Set a negative flow rate of 1.5 µL/s and a volume of 5 µL.
5. Pipette repetitive sequences of "oil solution - collagen - oil solution - air bubbles-…" as shown in Fig. 2a to generate droplet of collagen containing spheroids separated with air bubbles. During the pipetting of the collagen droplet, take care of placing the capillary close to a selected spheroid (*see* **Note 6**). The air is used as a spacer to avoid any contact between the droplets during flow (*see* **Note 7**) [14].
6. After the generation of a desired number of droplets (typically we make 30–40), store the droplets in the portion of the tube kept in the ice bath by sucking.

7. Detach this portion of tube from the rest of the setup in correspondence of the junctions (yellow parts in Fig. 2) and transfer it to room temperature for the second part of the protocol, as shown in Fig. 2b.

3.5 Collagen Droplets Polymerization

The second part of the experimental setup (Fig. 2c) is composed by a second syringe pump (the same pump could be used) mounted with the 50 mL syringe filled with the oil solution. About 6 m of the large PTFE capillary is rolled up on a plastic rectangular bar forming a spiral of about 4 cm of high, 2 cm of width and 30 cm of length. This spiral assures that the droplets are continuously moved up and down in order to center the spheroids during the collagen polymerization.

1. After the completion of the protocol described in Subheading 3.4, connect the capillary part immersed in the ice bath, in between the syringe and the spiral as shown in Fig. 2c.
2. Set the syringe at 18 μL/s; this flow allows keeping the droplets in the spiral for about 17 min. Once the collagen is completely polymerized, the position of spheroids is fixed.
3. At the end of the process collect droplets in a 24-well plate filled with cell culture medium (*see* **Note 8**).
4. Wash three times with the medium to remove the oil (*see* **Note 9**).
5. Incubate samples at 37 °C in 5% CO_2 humidified air.
6. Observe cell invasion after 24–72 h by light microscopy.

4 Notes

1. The capillaries used for droplet generation and transportation do not need to be sterilized because, due to the presence of the oil solution, collagen droplets are never in contact with the capillary surface. As a matter of fact, there is always a small layer of oil between the water phase droplet and the capillary. For the same reason, the same capillaries can be reused without any particular precaution.
2. The initial spheroid size will mostly depend on the number of cells N and the size of your cell line. To estimate roughly the diameter D of spheroids use $D = N^{1/3}d$ with d the mean diameter of your cell type. For example, for NIH 3T3 cell line we calculated roughly that 1000 cells/well are needed to form a spheroid of 200 μm.
3. Aggregation time of the cells highly depends on the cell type. For the NIH 3T3 cells aggregation occurs within 10 h while for the CT26 it occurs within 3 days.
4. Sticking of spheroids to the pipette tip can occur occasionally. Coating the tip with 0.1% bovine serum albumin in PBS avoids sticking.

5. All the capillary connections must be done by putting in close contact two extremities of the tubes. The silicon capillary is used as a joint between the two parts.
6. While aspirating the collagen make sure not to transfer the capillary too early, even if the syringe software indicates that the sucking step is completed. A delay of 1 s must be adopted before transferring the capillary between the collagen and oil wells. This is due to the presence of the air that acts as a dampener in the fluidic system. This effect increases with the increasing number of the air bubbles in the capillary.
7. Spacing the droplets with air is fundamental because during the second part of the protocol, the collagen is polymerizing during the flow and therefore the viscosity of the droplet phase changes. As a consequence, the droplets flow at different speeds in the capillary and touch each other.
8. We recommend limiting the number of collected droplets in the same well to prevent sticking and fusion of the droplets.
9. Complete filtration of oil is not possible. Small leftovers are still present on the surface.

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