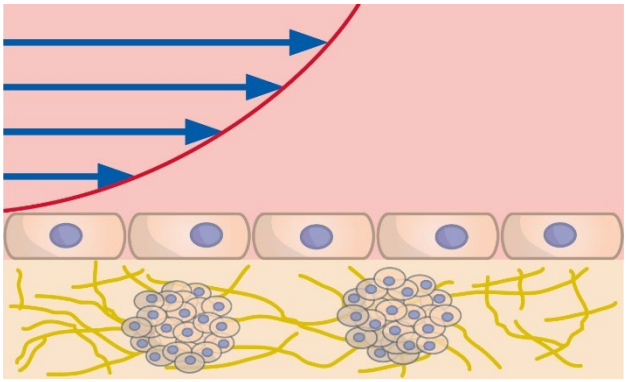
Co-culture of Tumor Spheroids and HUVEC Under Flow Using the µ-Slide III 3D Perfusion

As tumors are complex 3D structures, 3D tumor spheroid models became a relevant model system for mimicking physiologically relevant tumor microenvironments such as tumor angiogenesis or metastasis. This protocol describes the co-culture of tumor spheroids embedded in a Collagen Type I gel and endothelial cells (human umbilical vein cells, HUVECs) using the µ-Slide III 3D Perfusion. This model also allows for the imitation of physiological conditions by applying flow.



ibidi offers various solutions for spheroid and organoid cultivation with perfusion:

- µ-Slide III 3D Perfusion
- µ-Slide Spheroid Perfusion
- µ-Slide I Luer 3D
- µ-Slide With Multi-Cell µ-Pattern ibiTreat
- ibidi Collagen Type I
- ibidi Pump Systems and Accessories

Related Documents

- Application Note 13: Endothelial Cells Under Perfusion (PDF)
- Application Note 16 Immunofluorescence Staining Using the µ-Slide 8 Well high (PDF)
- Application Note 26: Collagen I Gel for 3D cell culture (PDF)
- Application Guide 34: Cell Culture Under Flow (PDF)

Related Software

- ibidi Collagen Calculator

Keywords:

3D culture models, Tumor microenvironment, 3D Co-culture; Endothelial cells, Tumor spheroids, Tumor angiogenesis

1 Material

Note: This protocol is optimized for Hep-G2 tumor spheroids and HUVEC cells; please adapt the reagents and buffers, when using other spheroids or endothelial cells

1.1 Reagents and Buffers

- Human umbilical vein endothelial cells (HUVEC, 12203, Promocell)
- Tumor-Spheroids (e.g., Hep-G2 Leibniz-Institut DSMZ, ACC 180)
- Endothelial Cell Growth Medium (Promocell, C-22010)
- Endothelial Cell Growth Medium 2 (Promocell, C-22011)
- PBS (14190144, Gibco)
- Accutase (A1110501, Gibco)
- Collagen Type I, Rat Tail, (ibidi, 50201) or Bovine (ibidi, 50301), non-pepsinized, 5 mg/ml diluted to 4 mg/ml in 17.5 mM or 0.1 M acetic acid
- 17.5 mM and 0.1 M acetic acid
- 10x DMEM (Sigma, D2429)
- 1x DMEM (Sigma, D5796)
- Media supplements (e.g., L-glutamine, depending on your cell type)
- NaOH in ultrapure H_2O, 1 M
- $NaHCO_3$ 7.5% (Sigma, S8761)
- Sterile, ultrapure water

1.2 Equipment

- µ-Slide III 3D Perfusion (80376)
- ibidi Pump System (10902) including a Perfusion Set (10968)
- Standard cell culture equipment (sterile working bench, cell detachment kit, culture flasks, pipets, tips, etc.
- Inverted microscope
- Ice and cooling rack

Important Note: To avoid air bubbles, the degassing of the Perfusion Set and the medium is critical. Place the following parts inside the incubator **one day before starting** the experiment. Sterility is maintained as long as the packaging is not opened.

- Perfusion Set(s) (within the packaging)
- Cell culture medium for cell seeding (add the volume needed to a small vessel, and loosen the cap slightly)

This procedure is necessary because of the temperature dependency of gas solubility in water and plastic. At higher temperatures, water and plastic can absorb less gas than at lower temperatures.

2 Preparation of the 3D Gel Containing Spheroids

Perform all the following steps under sterile conditions.

Important Note: Previously generated spheroids are required for the next steps of this co-culture experiment. Some ibidi solutions for generating spheroids are the µ-Slide Spheroid Perfusion (ibidi, 80350) described in Application Note 63, or Bioinert ULA Labware (ibidi 81150, 80800 and 80420).

1. Precoat the culture wells with 5 µg/cm^2 Collagen Type I (bovine or rat tail, depending on the required gel). Apply 18 µl of a 0.07 µg/µl Collagen Type I solution and incubate for 1 hour at room temperature. After incubation time, wash gently with PBS.

   Note: This step is crucial for preventing the detachment of the 3D gels. Therefore, it is important to dilute the Collagen I with H_2O or PBS to a final concentration of 0.07 µg/µl.
2. Retrieve previously generated spheroids (e.g., using the µ-Slide Spheroid Perfusion according to Instructions µ-Slide Spheroid Perfusion) and collect them in a tube under the flow hood at room temperature.
3. If supplements are required in the gel matrix, add them to the 1x cell culture medium and put it on ice in the flow hood (Application Note 26).
4. Place all further ingredients and a sterile tube, with sufficient capacity for the total gel volume, on ice in the flow hood. Unpack the slide and place it in the flow hood as well.

Important Note for Pipetting the Collagen Gel

Always use precooled pipet tips (4°C) for pipetting the gel.

For the preparation of the Collagen I gel matrix, reverse pipetting is recommended for all steps due to the high viscosity. Press the pipette to the second pressure point and fill the complete pipette tip with gel. Dispense the gel only until the first pressure point is reached. This leaves a residual amount of gel in the pipette tip to be discarded, but the volume is much more accurate. Alternatively, you can use pipettes designed for high viscosity solutions. Among others, we recommend Eppendorf Visco Tips or Gilson Microman E.

Note: even at 4°C, the gel mixture can be used for a maximum of 5 minutes before partial gelation occurs.

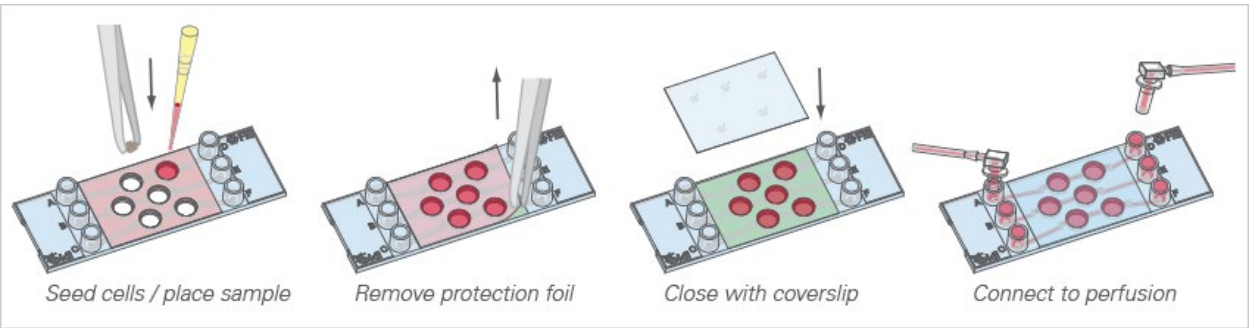
5. Pipet all ingredients except the collagen and the cell suspension in the order listed in Tables 1 and 2 to the tube, keeping it on ice. Mix by pipetting up and down and put back on ice.

   Note: The following steps refer to Table 1 and 2. Use the ibidi Collagen Calculator for variations of the protocols below, i.e. if different concentrations are needed of the collagen stock solution, the NaOH or the collagen gel.
6. Make sure the Collagen Type I, Rat Tail is diluted to 4.0 mg/ml in 17.5 mM acetic acid and the Bovine is diluted to 4.0 mg/ml in 0.1 M acetic acid. Check the Certificate of Analysis (CoA) for the lot-specific collagen concentration, described in more detail in Application Note 26: Preparation of Collagen I Gels.

   Note: Before diluting the collagen, it must be thoroughly mixed by pipetting up and down several times to create a homogeneous solution.

7. Add the collagen to the mixture prepared in step 5. Mix well by pipetting, while always keeping the tube on ice.
8. Add the prepared spheroid suspension to the mixture. Try to collect as many spheroids as possible in a 50 µl volume. Then, mix the sample with a brief vortexing.
9. The mixture is now ready to be pipetted into the µ-Slide III 3D Perfusion. Keep the slide on ice during pipetting.

   Tip: To avoid scratches originating from the ice, put the µ-Slide in a petri dish and put the petri dish with the slide on ice.
10. Remove the protection foil on the upper side of the slide and fill each well with 30 µl liquid gel. Avoid air bubbles.



Step-by-step graphical protocol using the µ-Slide III 3D Perfusion.

11. Next, place the coverslip on the sticky part of the slide. Make sure the adhesive area is tightly sealed by pressing to tighten the connection.
12. Cover the Luer adapters with the supplied caps to maintain sterility and put the cell culture vessel with the gel into a cell culture incubator (37°C, 5% CO_2) for 45 minutes for gelation.
13. After gelation, collagen fibrils will be visible using phase contrast microscopy with a 10x objective lens.

Table 1: Pipetting scheme for making gels using Collagen I, Rat Tail with DMEM. Volumes in µl. All ingredients are listed in the order of pipetting.

DMEM Final Collagen Type I, Rat Tail Concentration 2 mg/ml)	
10x DMEM	20
NaOH 1M	2.6
H_2O	17.4
$NaHCO_3$ 7.5%	10
1x DMEM (optionally with supplements)	50
Collagen I 4 mg/ml	150
Cell suspension in 1x Medium	50
Total volume	300

Table 2: Pipetting scheme for making gels using Collagen I, Bovine with DMEM. Volumes in µl. All ingredients are listed in the order of pipetting.

DMEM Final Collagen Type I, Bovine Concentration 2 mg/ml)	
10x DMEM	20
NaOH 1M	15
H_2O	5
$NaHCO_3$ 7.5%	10
1x DMEM (optionally with supplements)	50
Collagen I 4 mg/ml	150
Cell suspension in 1x Medium	50
Total volume	300

3 Cell Seeding of the Endothelial Cells

Perform all the following steps under sterile conditions.

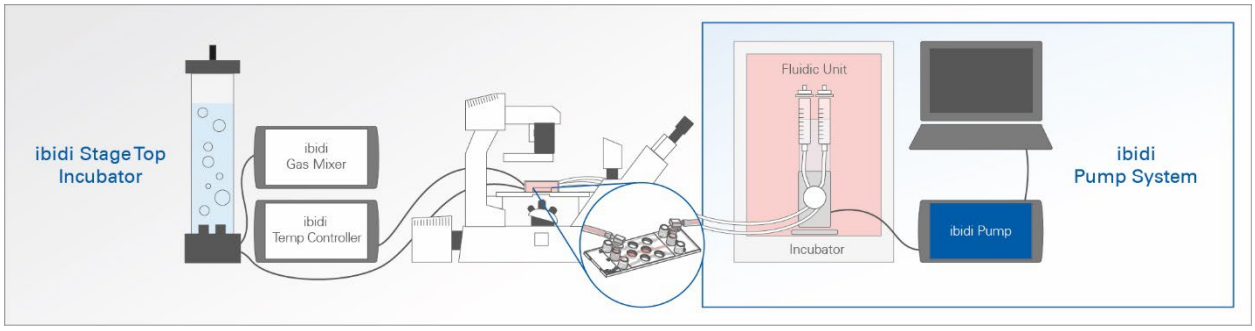
1. Before starting perfusion with the ibidi Pump System, prewarm the Perfusion Set and the Endothelial Growth Medium (ECGM and ECGM 2) inside a 37°C incubator one day before the experiment.
2. Treat the HUVECs with Accutase for 1–2 min for detachment.
3. Harvest the cell suspension, then centrifuge and dilute it in a low amount of culture medium (ECGM) for counting.
4. Count the cells and adjust them to the final concentration of 2 × 10^6 cells/ml in culture medium.
5. Apply ca. 70 µl cell suspension into each channel. Remove the leftover cell suspension from the Luer adapters and repeat the seeding step for more cell homogeneity.
6. Remove the leftover cell suspension from the Luer adapters with a standard pipette tip and cover the slide with the supplied lid to maintain sterility.
7. Place the covered slide and a wet tissue into a Petri dish. Then, add the dish into a cell culture incubator (37°C, 5% CO_2).
8. In the case of static cultivation, the medium must be changed every 2–3 days.

4 Connecting to the ibidi Pump System for Perfusion

Important Note: Endothelial Growth Medium 2 contain growth factors like VEGF and EGF inducing the migration and sprouting of the cells.

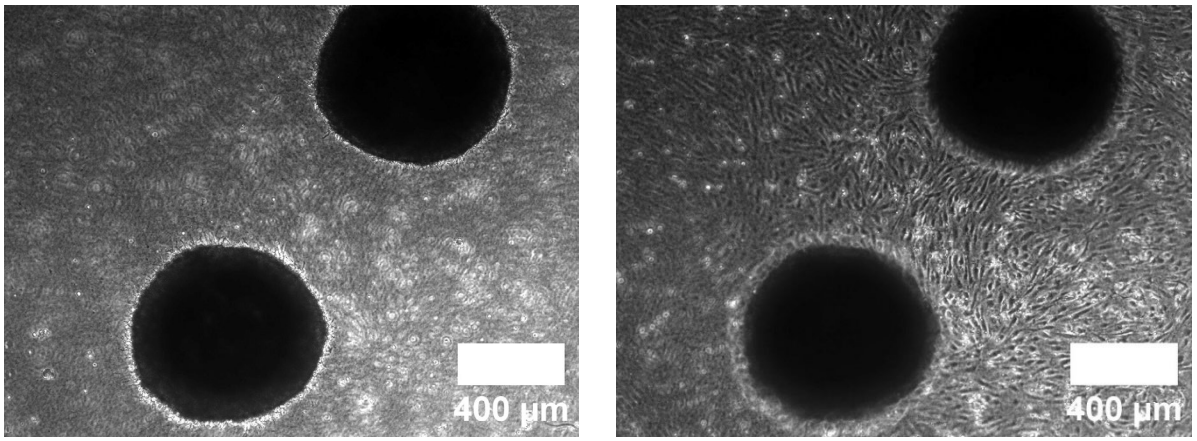
1. During cell incubation, prepare the ibidi Pump System as described in the ibidi Pump System Instructions. Fill the ECGM2 medium into the reservoirs.
2. After incubating for 2 hours, connect the slide to the perfusion system filled with the ECGM 2. (AN 13: Endothelial Cells Under Perfusion, AN 34: Cell Culture Under Flow)
3. If a time-lapse series is needed, place the slide into the stage top incubator (e.g., ibidi Stage Top Incubator) on the microscope and start the time-lapse measurement. If only

single frames are needed, prepare the microscope settings in advance to keep the imaging period as short as possible. To maintain ideal cell conditions, put the slide back into the incubator when it is not being imaged. For an endpoint analysis, please fix the cells at the end of the experiment and continue with your staining (see 5) or downstream protocol.

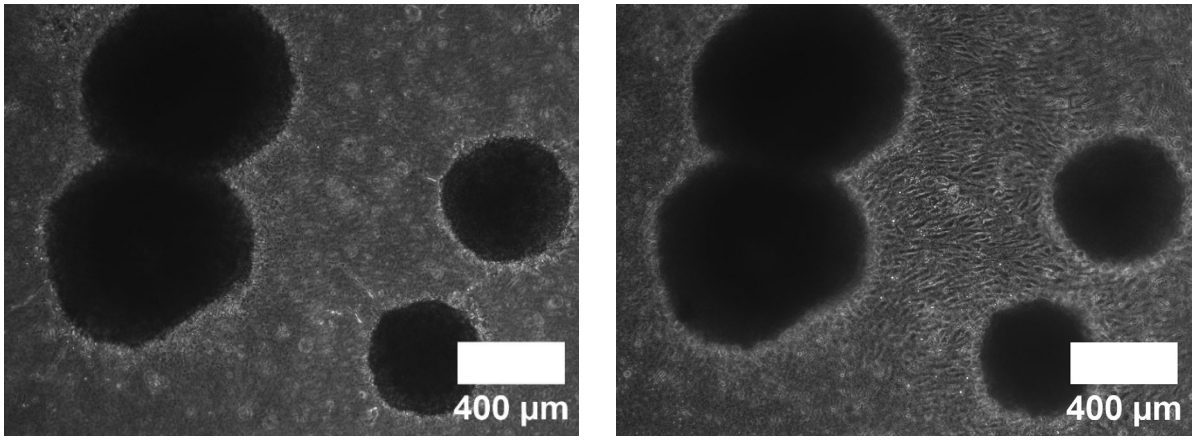


Experimental setup of the timelapse measurement under perfusion

5 Example of Live-Cell Phase Contrast Imaging



Phase contrast images 5 days of co-culture under perfusion showing the spheroids in focus (left) and the HUVEC cells in focus (right) (Collagen Type I rat tail gel)



Phase contrast images 5 days of co-culture under perfusion showing the spheroids in focus (left) and the HUVEC cells in focus (right) (Collagen Type I bovine gel)

6 Staining Protocol

6.1 Material

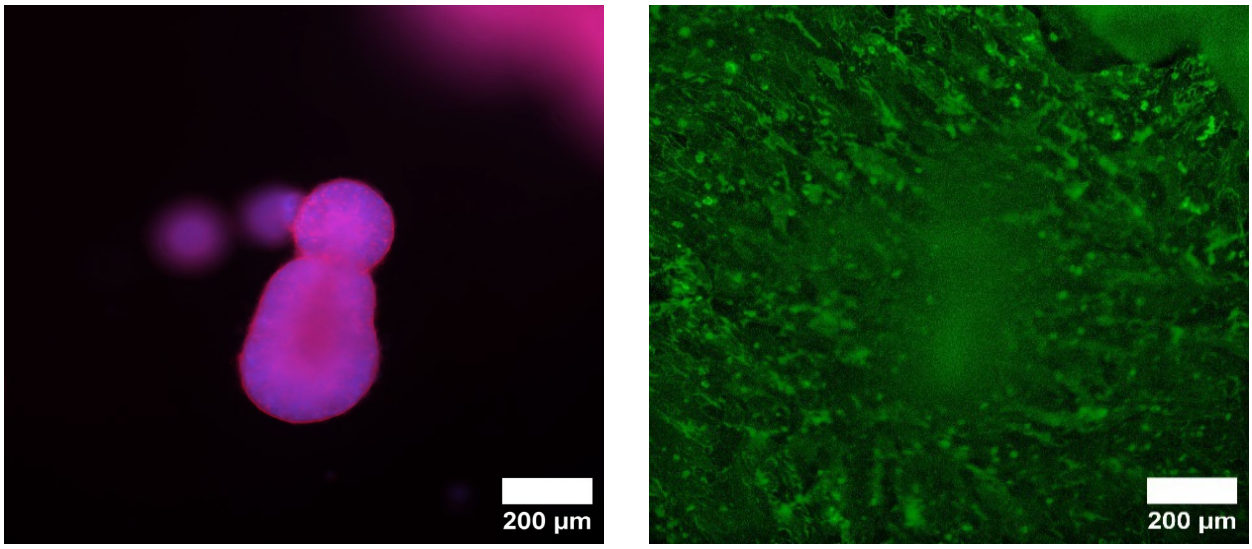
- PBS (14190144, Gibco)
- Formalin, 10%, ready to use (HT5011, Sigma Aldrich)
- Alexa Fluor 488 labelled Anti-CD31 Antibody, MA5-18135 Invitrogen).
- Phalloidin-iFluor 647 Reagent (ab176758, Abcam)
- 4′,6-diamidino-2-phenyl-indole (DAPI) (D9542 Sigma Aldrich)
- Triton-X-100 (A16046, Thermo Fisher Scientific)
- Perforation Buffer (0.5% Triton X-100 in PBS)
- Blocking Buffer (1% BSA + 0.2% Triton X-100 in PBS)
- Antibody Dilution Buffer (1% BSA + 0.05% Triton X-100 in PBS)
- Bovine Serum Albumin (BSA) (A1470-10G, Sigma Aldrich)

6.2 Method

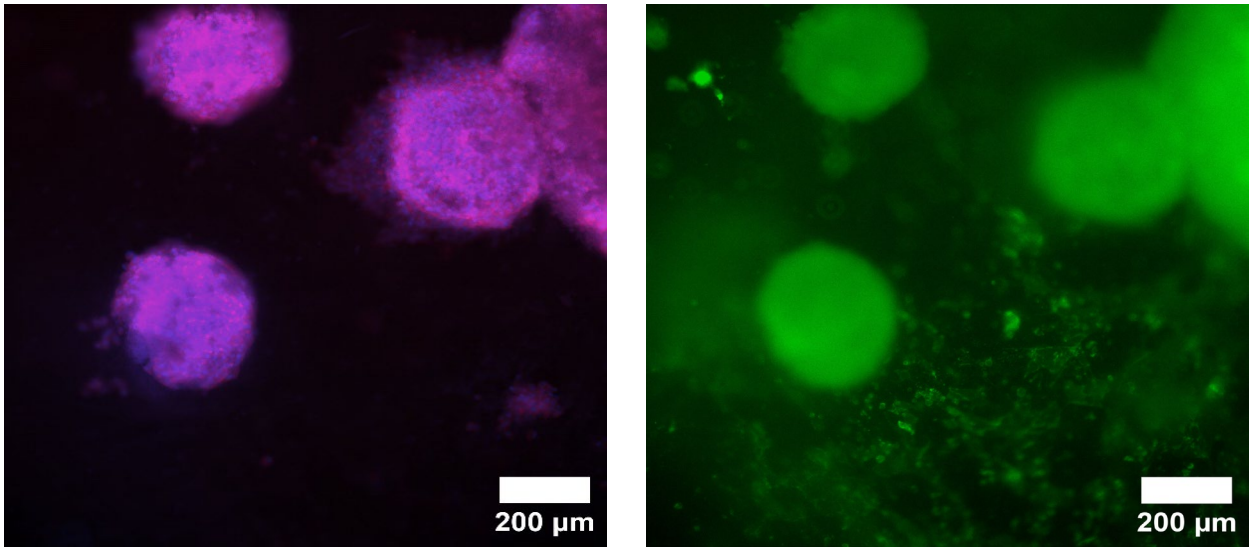
1. Prepare enough Perforation Buffer and Blocking Buffer for your experiment.
2. Disconnect the slide from the pump.
3. Remove the cell culture medium from the Luer port.
4. Gently wash the cells twice with 100 µl PBS by filling PBS in one reservoir until you see the fluid coming out at the other Luer port.
5. Fix the cells by exchanging the PBS with Formalin (10%). First, remove the PBS from the Luer port, then add 100 µl Formalin to a Luer port and remove it from the opposite Luer port. Then, add again 100 µl Formalin and incubate for 15 minutes.
6. Remove the formalin and wash the cells four times with 100 µl PBS.
7. Incubate the cells in 100 µl Perforation Buffer (exchange the PBS with the Perforation Buffer, similar to step 5 – Formalin exchange) for 10 minutes.
8. Remove the Perforation Buffer and wash the cells twice with 100 µl PBS.
9. Block with 100 µl Blocking Buffer (exchange the PBS with the Blocking Buffer like done with Formalin) for 30 minutes.
10. During the Blocking Buffer step, prepare enough Antibody Dilution Buffer to dilute the primary and secondary antibodies.
11. Dilute the labeled Antibody (or the primary antibody) in Antibody Dilution Buffer (1:100 dilution)
12. Exchange the Blocking Buffer with 100 µl of the primary antibody solution and incubate the cells overnight at 4°C
13. From this point on, the samples should be kept in the dark whenever possible.
14. Wash three times with 100 µl Blocking Buffer.
15. Dilute (the secondary antibody and) the phalloidin and DAPI in the same Antibody Dilution Buffer (Phalloidin 647 Conjugate 1:1000 dilution, DAPI 10 µg/ml).
16. Exchange the Blocking Buffer with 100 µl of the secondary staining solution and incubate overnight in the dark.

17. Wash three times with 100 µl Blocking Buffer.
18. Exchange the Blocking Buffer with PBS (100 µl per channel)
19. Store at 4°C in the dark until imaging. Optimally, proceed immediately with imaging since extended storage periods could reduce image quality.

7 Example images of the stained co-culture



Staining of the spheroids and HUVEC cells after 3 days in co-culture under perfusion; Nuclei: DAPI (blue), Actin Filaments: Phalloidin-iFluor 647 (red), CD31 (specific for the HUVEC): Alexa Fluor 488 labeled anti-CD31 Antibody (green), (Collagen Type I rat tail gel).



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