TECHNICAL PROTOCOLS

used for GrowDex cultures

revvity An authorized distributor

Contents:

1 Most frequently asked protocols

1.1 Handling and pipetting GrowDex hints and tips:

1. We recommend using smaller well plate formats, such as 96-well plate, for the tests. In smaller wells the well walls provide support to keep the hydrogel more stable.
2. Low-retention pipette tips should be used to avoid GrowDex sticking to the tip. Low-adhesion pipette tips, e.g. TipOne RPT 1250ul CAT NO S1161-1820.
3. Pipette tips can be cut for better flow in the beginning of mixing. When the mixing progresses, uncut tips should be used to increase mixing efficiency.
4. Using a wider bore pipette tip or one that has been cut can help with the initial mixing step.
5. Aspirating and dispensing GrowDex should be performed slowly to avoid air bubbles and to ensure an accurate volume.
6. A positive-displacement pipette is useful for pipetting viscous materials like undiluted GrowDex.
7. For an exact amount of undiluted GrowDex the product can also be weighed before dilution.
8. Electric dispensing pipettes and automated dispensing systems can be used for dispensing and mixing.
9. A multi-stepper pipette or automated dispensing system is recommended for repeat dispensing of GrowDex into the well-plates for high throughput applications
10. Dispense first the cell culture media in the vial and after that dispense GrowDex - this makes diluting GrowDex easier.
11. Swirling the pipette tip along the wall of the tube and then by pipetting up and down for a minimum of 90 seconds or continue until a homogenous solution is achieved by visual inspection. Increase the speed of pipetting towards the end of mixing and make sure the hydrogel flows smoothly through the pipette tip.
12. Please follow the link to the calculator tool where you can easily input the product you are using along with other information such as your desired final concentration, the volume of hydrogel needed from the syringe and the cell suspension volume. Calculator for quick start assay setup.
13. Videos for instructions:
    - I. GrowDex syringe in 3D cell culture
    - II. GrowDex Instruction of Use
    - III. GrowDex Quickstart Guide

1.2 Passaging cells in GrowDex:

Passaging cells/spheroids/organoids/tissues embedded within or cultured on top of GrowDex may be performed in 2 possible methods:

1. With the aid of GrowDase, please follow the instructions for use found HERE.
   - **a.** Briefly, using the calculation equation, work out the required volume of GrowDase needed to digest the known amount of GrowDex per wall.
   - **b.** The concentration of GrowDase can be increased in order to decrease the time to liberate the cells from GrowDex.
   - **c.** Following addition of the GrowDase to the well, incubate the cells and plate until cells/spheroids/organoids/tissues have been liberated.
2. By dilution and shear stress pipetting.
   - **a.** Make up a solution of GrowDex at the same concentration which is equal to the concentration of your culture system.
   - **b.** Carefully remove excess media from the culture vessel which has your cells/organoids within GrowDex.
   - **c.** With the use of the pre-prepared GrowDex (in step a), dilute the contents of the culture vessel by pipetting.
   - **d.** Ensure to pipette vigorously to disrupt and mix cells/organoids.
   - **e.** Re-dispense the newly diluted culture matrix with cells/organoids into a new culture vessel.
   - **f.** Remember to add new media to the cells above the layer of GrowDex with cells/organoids.

1.3 Using GrowDex in a dome or droplet culture:

The GrowDex hydrogels do not crosslink and therefore it is not recommended to do droplet or dome cultures as is traditionally performed with Matrigel. However, it is possible to create dome cultures with 1µl droplets of high concentrations of GrowDex (≥ 0.7%) and GrowDex-T (≥ 0.5%) when using the Nunc Immobilizer Amino Clear Well Plate. Please note that using these high adhesion plates may cause your cells to adhere and form a monolayer on the bottom of the well rather than growing in 3D suspension.

Nevertheless, we recommend using a well plate as the support from the well makes handling GrowDex and GrowDex-T easier. The dome and droplet cultures are usually used in order to reduce the volume of hydrogel or Matrigel used. Instead it is easily possible to use 384 or 96 well plates with the GrowDex and GrowDex-T instead. Alternatively, GrowDex can be used in a hanging drop format. Please have a look for further information in the following application note [1]:

- Hanging drop culture of mesenchymal stem cells within nanofibrillar cellulose [1]

1.4 Crosslinking GrowDex-T for cell culture applications:

Calcium chloride (CaCl2) can be used to ionically crosslink GrowDex-T. However, it is advised to consider the cell type which is being used, and the effects of high CaCl2 on cell viability and differentiation. Therefore, it is recommended to use concentrations between 0.05 - 0.1M CaCl2 for 5-10 mins followed by 2-3 wash steps with PBS (with Calcium and Magnesium). Depending on the application and intended model design crosslinking can be performed for cells embedded within GrowDex-T or a layer of GrowDex-T can be formed and crosslinked where cells can then be seeded on top.

- For cells embedded within GrowDex-T:
  - Embed cells within GrowDex-T at the desired concentration and seeding density
  - Add 0.05M CaCl2 to the cell culture for 10mins
  - Remove CaCl2 solution and wash the culture with PBS

- For crosslinking a GrowDex-T layer followed by cell seeding:
  - Place the desired volume of GrowDex-T into the appropriate culture vessel
  - Add 0.1M CaCl2 for 5mins
  - Remove CaCl2 solution and wash 3 times with PBS
  - Seed cells onto the crosslinked GrowDex-T as necessary

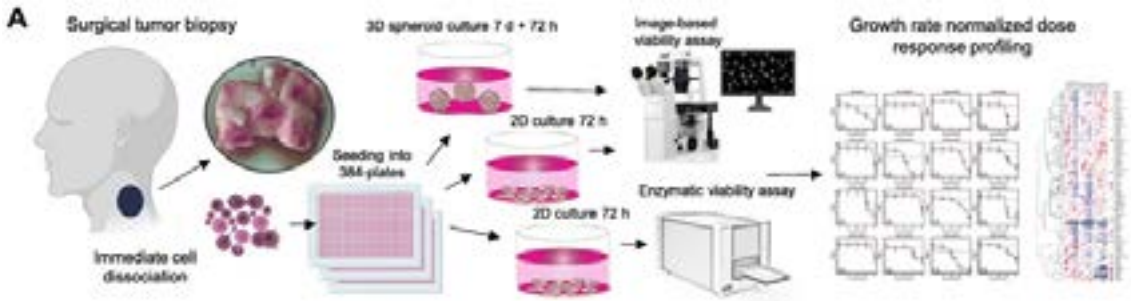
1.5 Organoid culture in GrowDex

Many customers have reported the successful culture of primary healthy and tumour tissue which has been excised, dissociated and sub-cultured in GrowDex. These ex vivo tissues include placental tissue, ovarian cancer tissue, kidney tumour tissue, and urachal carcinoma tissue.

Culture of excised tumour organoid in GrowDex [2].

A subcutaneous metastasis tissue sample was collected for the study during palliative surgery. In conjunction with the surgical procedure, part of the dissected tumor tissue was placed in sterile RMPI-1640 medium (Gibco) for transport to the consulting pathologist for preparation and further delivery to the research laboratory (Fig. 1a). The live tissue was dissociated into a single cell suspension as described before [3]. Following the enzymatic dissociation, the resulting cell suspension was counted using a Cellometer Mini cell counter (Nexcelom). In total 6.5 × 10^6 cells with an average size of >13 µm was derived from ~2cm3 of the tumor tissue. The suspension was diluted to RPMI-1640 medium (Gibco) containing 5% FBS to achieve a suspension with 1000 cells per 45 µL of medium. 5 × 10^6 cells were used for the initial ex vivo drug screening and the rest were placed to cell culture in standard cell culture conditions (37 °C, 5% CO2). Following 4 days in culture, the cells presented a semi-adherent phenotype with cells growing both as loose aggregates and adhered to the plastic cell culture surface (Fig. 1b).

The 3D cell culture assays were performed using GrowDex® hydrogel (UPM, Helsinki, Finland) as the matrix supporting 3-dimensional cell growth. To minimize adherent cell growth in the bottom surface of the wells, the microwells were first coated with 1.2% pHEMA (poly (2-hydroxyethyl methacrylate; Polysciences). For the experiment the 1.5% hydrogel stock was mixed with complete cell culture medium (RPMI-1640 + 5% FBS) to achieve a 0.6% w/v hydrogel solution. 30 µL of cell suspension containing 2000 cells was mixed with 30 µL of the diluted hydrogel to achieve a 0.3% w/v hydrogel solution containing ~ 30 cells/µL. Required amount of hydrogel-cell solution was prepared by pipetting the hydrogel using a wide-mouth 10 mL pipetting tip into a 50 mL tube and gentle mixing with a vortex shaker. The hydrogel-cell solution was dispensed to 384-plate wells using a Multidrop plate dispenser (ThermoFisher Scientific). The total volume of sample added into a single 384-well was 60uL/well (2000 cells). Following dispensing, plates were centrifuged for 1 min at 100 g and left on incubation at + 37 °C, 5% CO2 for 7 days prior to addition of the drugs. At day 4, 20 µL of medium was carefully aspirated from the wells using a multichannel pipet and 20 µL of fresh complete cell culture medium was added to each well. On day 7, the same was repeated followed by addition of 6 µL of the 10× drug stocks per well and additional 72-h incubation. For analysis of cell growth, the cells were stained using Hoechst 33342 cell permeant live cell DNA dye (Invitrogen). 8 µL of 10× stock dilution was added per well and incubated for 45 min. Following Hoechst staining, the plates were centrifuged for 2 min at 200 g to settle the cell spheroids/aggregates to the bottom plane of the wells (Supplementary Figure 1). Imaging and image analysis were then performed using an Olympus scan^R high content imager and image analysis suite as described above for the 2D assays.



1.6 Using GrowDex for coating culture plates as a semi 2D monolayer culture:

You can use GrowDex to coat the surface of cell culture plates, however, please note the difference in the material properties. GrowDex is an inert hydrogel which does not contain adhesion proteins, so the result may be different to other coating molecules such as Laminin or Poly-D-Lysine. When using GrowDex as a surface coating, the culture can be referred to as a 2.5D as there is still space for the cells to move in the gel. It is recommended to make a very low concentration solution of GrowDex or GrowDex-T, such as 0.1-0.2%. Spread this evenly over the bottom of the well of a normal tissue culture plate, ensuring it covers the whole surface. Allow it to dry or dehydrate and it will form a dry 'cake layer' of NFC on the surface.

1.7 Making a surface low attachment using PolyHEMA for coating:

You can coat the well surface with polyHEMA to make the surface low attachment. This will form a hydrophobic and nonadherent polymer layer on the surface of the plastic which will not interfere with the cell viability, however it will ensure the cells do not bind to the plastic surface.
Make the polyHEMA to 0.4% in ethanol. Add to the well and allow to evaporate until completely dry.

- For a 96 well plate, use 40ul, ensuring the whole of the bottom of the well is covered.
- For a 24 well plate, use 100ul, ensuring the whole of the bottom of the well is covered.
  Alternatively, you can use 1% agarose on the bottom of the well.

2 Downstream analysis of cells

2.1 Protocol for DNA quantification from cells cultured in GrowDex [3]:

Genomic DNA was quantitated with Quant-iTTM PicoGreen dsDNA assay kit (Molecular probes, Invitrogen). At first, the cell cultures were washed with dPBS. Hydrogel cultures were washed and ruptured by pipetting up and down with dPBS followed by centrifugation (2500 G 5 min). After dPBS removal the cultures were lysed in RLT buffer (RNeasy Mini kit, Qiagen). Additionally hydrogel cultures were disrupted with TissueRuptor (Qiagen) to release the nucleotides from the gel and centrifuged (3000 G 5 min) to remove the hydrogel. Then, the RLT cell lysates were diluted 1:20 with autoclaved milliQ water, 5 ml of each diluted sample and standard were pipetted into DNA-free black 96-well plate (Greiner bio-one), and 100 ml of PicoGreen (diluted 1:100) was added to the wells. The final buffer concentration of 1:400 in the analysis is known to be compatible with the PicoGreen signal [31]. After 5min incubation at RT the emission of dsDNA samples at 520 nm was recorded with fluorometer (Varioskan Flash, Thermo). Readings from samples were compared to 1e500 ng/ml of control dsDNA provided with kit. Samples from three independent experiments of both low-density and high-density experiments were analyzed. Each lysate was analyzed as triplicate.

2.2 Cryopreservation and Cryosectioning of cell samples in GrowDex [4]:

Each 3D-cryo well insert was processed one at a time. Accordingly, tweezers were used to transfer the 3D-cryo well insert from its culture plate to liquid nitrogen. Within 20–30 s in liquid nitrogen, the inserts were frozen (Figure 2A). In another well plate, we stored the liquid nitrogen frozen 3D-cryo well insert in a −80 °C freezer overnight or further processed them for cutting right away. The further processing involved embedding the 3D-cryo well insert into optimal cutting temperature (OCT) (cryostat tissue embedding chemical) (1708801018; Algol), a liquid used to freeze cryosectioning samples. This OCT embedding step resulted in having the 3Dcryo well insert inside an OCT frozen cylinder. To perform this OCT embedding step we created a mold (well insert mold [W.I. mold]) built from a 15mL conical tube cut at the 12 mL mark (Figure 2C). The embedding was performed as follows: first we filled the empty W.I. mold to 90% with 37% OCT and submerged it in liquid nitrogen (OCT should not contact the liquid nitrogen) (Figure 2B). Then, once we noticed a thin circular freezing ring of the OCT we removed the W.I. mold from the liquid nitrogen. At this point, we glided the −20 °C or −80 °C preserved 3D-cryo well insert into the cold OCT liquid of the W.I mold (Figure 2C). The W.I mold containing the 3Dcryo well insert was placed again into liquid nitrogen

for 1–2 min (Figure 2D) until the OCT changed to white and bubble emission decreased. At that point, we removed the W.I. mold and its contents from the liquid nitrogen. When frost was seen accumulating on the outer plastic of the W.I. mold, the lid could be removed and an OCT cylinder containing the 3D-cryo well insert could be ejected with slight pressure (Figure 2E). After ejection, the cylinder on its own without the W.I. mold was cooled again in liquid nitrogen until bubble emission had decreased. OCT cylinders were incubated overnight in a −80 °C freezer or transferred to the cryotome for sectioning (Figure 2F,G) or to −20 °C for long-term storage. Sectioning was performed on a cryotome (2800E Frigocut; Jung) with the blockhead near −25 °C and the chamber near −30 °C. On histology glass slides (SuperfrostPlus; Thermo Fisher Scientific) we mounted 20 µm sections (Figure 2H). Over the cutting period, the sections mounted on glass slides were air dried and afterward stored at −20 °C. This whole procedure is shown in Video S1 (Supporting Information).

3 Dyes and Stains

3.1 Calcofluor White Staining of GrowDex-T [5]:

Nanofibrillar Cellulose and Calcofluor White Staining. The aNFC hydrogel (GrowDex®-T) and the cellulase enzyme (GrowDase™) were kindly provided by UPM Biomedicals (Helsinki, Finland). 0.2% w/v aNFC was prepared by diluting 1.0% stock solution in PBS (Sigma-Aldrich, Irvine, United Kingdom), and the hydrogel (100 µl) was transferred into wells of a low-adhesion 96-well plate using low-retention pipette tips (Sarstedt Ltd., Leicester, United Kingdom) followed by the incubation with Calcofluor white (25 µl, 0.01% w/v, Sigma-Aldrich) for 5 min at room temperature (RT). Calcofluor-stained aNFC was visualised using a DAPI filter on an inverted fluorescence microscope (VertA1, Carl Zeiss Ltd., Cambridge, United Kingdom).

3.2 CellTracker™ Green Fluorescent Dye Staining [1]:

Lyophilised CellTracker™ Green CMFDA Dye was resuspended in DMSO to a stock concertation of 10mM according to manufacturer's instructions. The stock (10mM) was diluted 1:1000 to a working concentration of 10µM in warm complete medium. Before adding the dye to the cells, old supernatant was removed (e.g. 80µl), ensuring to not disturb the cells embedded in GrowDex-T. The media with dye (e.g. 80µl) was then added to the cells and incubated for 45 mins.
Following incubation, the cells were fixed with 4% PFA for imaging purposes.
Alternatively, the media with dye can be removed and the cells washed gently with complete media (2 - 3 washes with 5 min incubations per wash). Following washing, it should be possible to observe the cells for longer as detailed in the manufacturer's instructions.

Alternative Cell Tracker Dyes for live of fixed imaging include:

- Cell Tracker™ Green BIODIPY™ Dye
- CellTracker™ Red CMTPX Dye

3.3 Crystal violet staining of cells in GrowDex-T [5]:

Crystal Violet Staining. For crystal violet staining, ADSCs were cultured in 0.2% aNFC (1 × 105 cells/ml) within TC cell culture inserts (24-well format, Ø3.0 µM, 83.3932.300, Sarstedt Ltd.) for 72 h. Following cell culture and PFA fixation as detailed above, ADSCs were stained with crystal violet (0.05% v/v) for 2 h at 4°C. After incubation with the crystal violet dye, samples were washed 5 times with PBS. Images were collected using brightfield microscopy on an inverted microscope (EVOS XL, AMG, WA, USA).

3.4 Phalloidin Fluorescent Dye Staining [1]:

Phalloidin-Atto 555 (Sigma-Aldrich) was used to label the actin filaments of fixed ADSCs (1 × 105 cells/ml) cultured in 0.2% aNFC within x-well cover glass cell culture chambers for 72 h.

Following cell culture and PFA fixation as detailed in previous sections, ADSCs were gently washed with PBS for 5 min. The PBS was replaced with PBS/0.1% Triton X-100 (100 µl) per well for 30 min at RT. The PBS/0.1% Triton X-100 was removed carefully, and the scaffold with cells was washed once with PBS for 5 min.

Staining solution (100 µl) was added to the cells and incubated overnight at 4°C.

Staining solutions were prepared as follows:

- DAPI with phalloidin; DAPI working solution (0.5 µg/ml) from a stock (1 mg/ml) by diluting it 1:2000 in sterile PBS; phalloidin working solution (0.2nM) from a stock (10nM) by diluting the stock with the DAPI working solution;
- Calcofluor with phalloidin; and phalloidin working solution (0.2 nM) was prepared from a stock (10 nM) by diluting it 1:50 with PBS. Calcofluor dye was diluted 1:33 with the phalloidin working solution.

Following overnight incubation, the staining solutions were gently removed, and the cells were washed once with PBS. Following the final wash, PBS was replaced with 0.02% PBS-sodium azide and the samples were imaged using the Nikon A1R inverted confocal microscope.

3.5 PKH67 Staining and Fluorescence Imaging [1]:

Living cells within the aNFC (0.1%) matrix were visualised using the PKH67 Green Fluorescent Cell Linker Kit for General Cell Membrane Labelling (Sigma-Aldrich).

AdMSCs (5 × 10^5) were labelled according to the manufacturer's instructions. Briefly, and embedded within a 0.1% aNFC hydrogel. For the detection of the labelled cells, the CQ1 (Yokogawa Electric Corp., Japan) high-content confocal imaging system was used.

4 Fixing cells with PFA

4.1 Fixing cells in GrowDex with PFA [6]:

Structural polarity of the HepaRG cultures was analyzed by fixing the cell cultures in 4% paraformaldehyde for 15 min at 4°C. After washing with PBS, the cells were permeabilized with 0.1% Triton X-100 for 15 min and subsequently incubated overnight with a blocking buffer (10% goat serum and 0.2% BSA in PBS) at 4°C. Then, the cultures were incubated with a primary antibody, anti-MDR1 (1:100, Sigma P7965) or anti-MRP2 (1:300, Abcam ab3373) (diluted in 5% goat serum in 1 x PBS buffer) overnight at 4°C in a humidified chamber. After washing unbound primary antibody, samples were incubated with goat anti-mouse or goat anti-rabbit antibody conjugated with Alexa Fluor 488 (Invitrogen, diluted 1:200 in 5% goat serum in PBS buffer) for 4 h at RT in a humidified chamber. The cultures were washed with 1 x PBS (four times for an hour) and 1 µg/ml Hoechst 33258 (SigmaeAldrich, bisbenzimide) together with Alexa Fluor 594-labelled phalloidin (Invitrogen, diluted 1:100 or 1:50 in 1 x PBS buffer) was placed and held for overnight to visualize the nuclei and cellular distribution of the filamentous actin (F-actin) cytoskeleton, respectively.

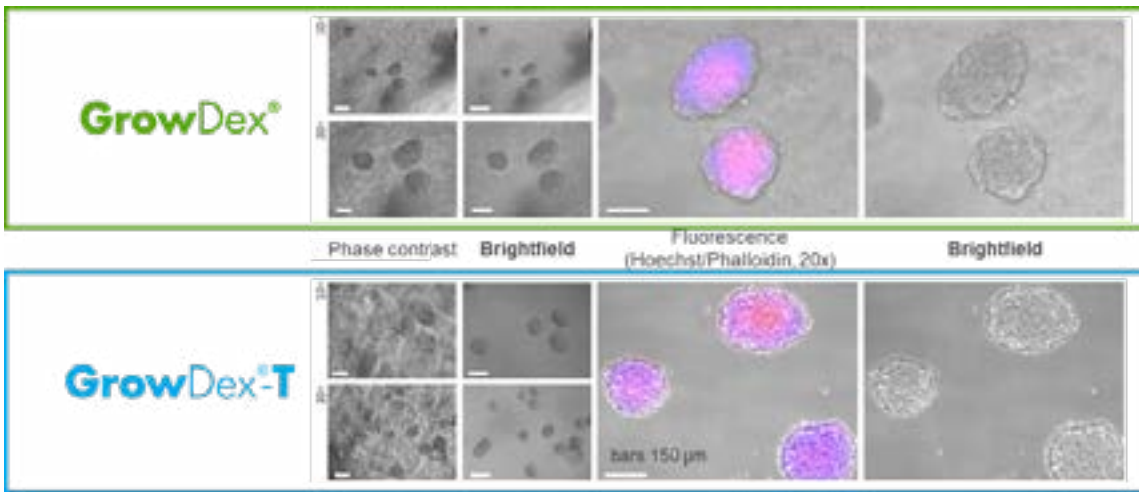
4.2 Fixing cells in GrowDex-T with PFA [5]:

ADSCs were cultured in 0.2% aNFC (1 × 10^5 cells/ml) within x-well cover glass cell culture chambers for 72 h. Following culture, media was removed and cells were gently washed with PBS, followed by fixation using 4% PFA for 20 min at RT. Subsequently, PFA was removed and cells were washed twice with PBS for 5 min.

5 Imaging

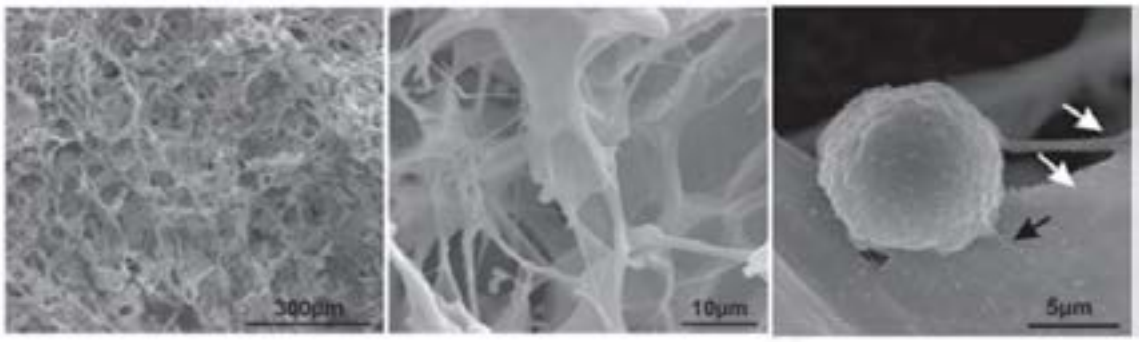
5.1 Bright Field vs Phase Contrast

It is recommended to use **bright field microscopy**, without any phase contrast rings, when imaging or visualising cells, spheroids or organoids cultured in GrowDex and GrowDex-T. The visual differences using different magnifications can be seen in the images below.



5.2 Protocol for Scanning Electron Microscopy (SEM) of GrowDex and cells in GrowDex [7]:

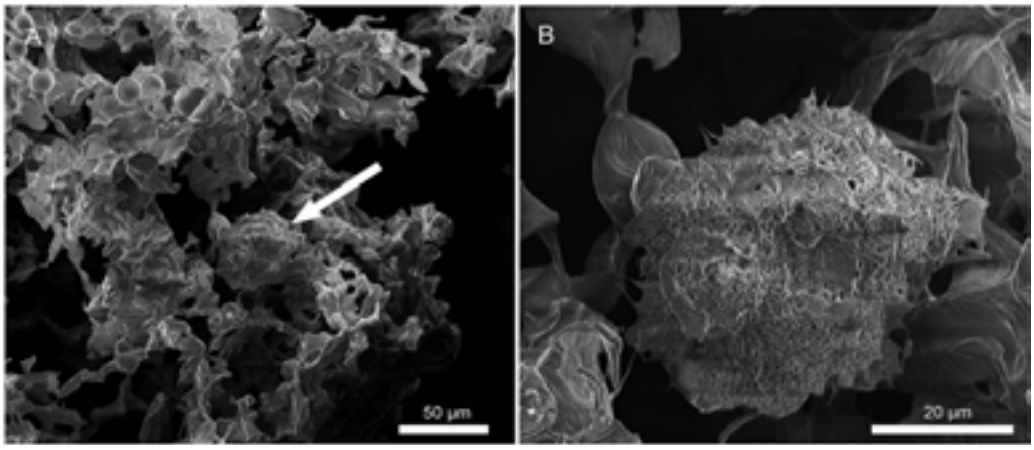
Cell within 0.2% hydrogel or 0.5% NFC hydrogel alone were transferred into a 24 mm cell culture insert membrane (Corning, St. David's Park, UK; pore size 0.4 µm), incubated for 24 h at 37 °C and fixed with 2% paraformaldehyde and 2.5% glutaraldehyde for 1 h, washed three times with phosphate-buffered saline (PBS) for 30 min followed by dehydration in ethanol series then transferred on top of a stub. The samples were plunged into liquid nitrogen slash, and then freeze dried for 1.5 h followed by gold coating (25 nm). Analysis was performed using a scanning electron microscope (FEI Quanta FEG 600 SEM, Thermo Fisher, Paisley, UK). Fiber size was extracted from seven SEM images suing the ImageJ Fiji software. The histogram was created with GraphPad Prism software (GraphPad, La Jolla, California).



5.3 Protocol for Scanning Electron Microscopy (SEM) of GrowDex and cells in GrowDex [8]:

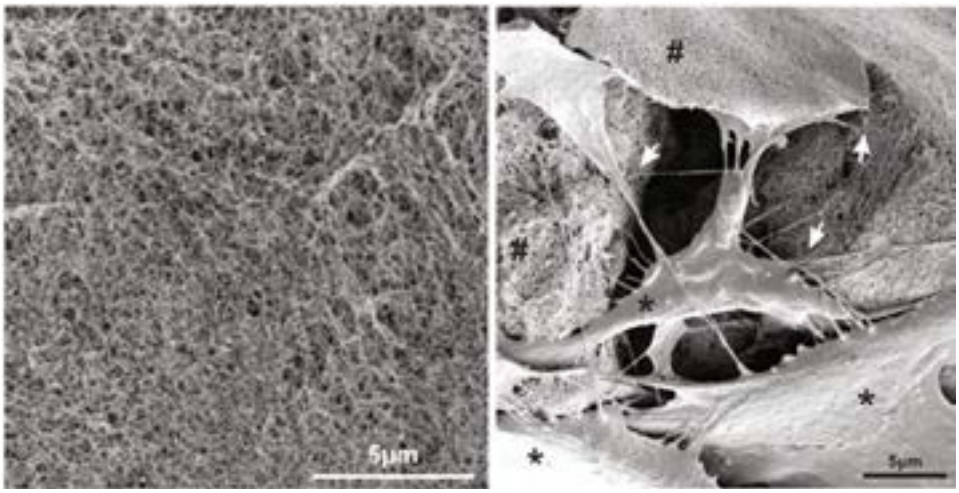
Please refer to the publication for full details of their freeze-drying techniques prior to SEM imaging.

HepG2 cell spheroids were imaged with a scanning electron microscope (SEM) to study the effects of freeze-drying on the morphology and the structure of the cell spheroids. In another setting, freeze-dried HepG2 cell spheroid samples were rehydrated and freeze-dried again to evaluate the effects of rehydration on the integrity of cell membrane. The SEM samples were stored in a desiccator at 4 °C until imaged. The samples were not coated nor fixed for the SEM imagining which was performed with FEI Quanta 250 Field Emission Gun SEM using 2.0 kV and 2.5–3.0 spot in high vacuum.



5.4 Scanning Electron Microscopy (SEM) of GrowDex-T [5]:

aNFC at 0.5%, with cells (1 × 106 cells/ml) or without cells, was fixed with 2% formaldehyde and 2% glutaraldehyde in 0.05 M cacodylate buffer for at least 24 h before being rinsed. Hydrogels were washed twice with deionised water (DIW) to remove the fixative and placed on 10mm ∅ melinex coverslips (mesh-side down); excess water was removed. The samples were then plunge-frozen in liquid-nitrogen-cooled ethane. Samples were freeze-dried overnight in an Emitech K775X liquid nitrogen-cooled freeze dryer (Quorum Technologies). Melinex coverslips were mounted on aluminium SEM stubs using silver DAG (TAAB); small amounts of silver-DAG around the bottom rim of the mesh/sample were used to secure the sample on the coverslip and ensure conductivity. Then, samples were coated with 35nm gold and 15nm iridium using an EMITECH K575X Sputter Coater (Quorum Technologies). Samples were viewed in a FEI Verios 460 scanning electron microscope at an accelerating voltage of 2 keV and a probe current of 50 pA. Images were acquired in a secondary electron mode using either an Everhart-Thornley Detector (ETD) or a Through-Lens Detector (TLD) in an immersion mode.



6 Immunofluorescent staining of cells

6.1 Immunofluorescence staining of cells in GrowDex [6]:

Structural polarity of the HepaRG cultures was analyzed by fixing the cell cultures in 4% paraformaldehyde for 15 min at 4°C. After washing with PBS, the cells were permeabilized with 0.1% Triton X-100 for 15 min and subsequently incubated overnight with a blocking buffer (10% goat serum and 0.2% BSA in PBS) at 4°C. Then, the cultures were incubated with a primary antibody, anti-MDR1 (1:100, Sigma P7965) or anti-MRP2 (1:300, Abcam ab3373) (diluted in 5% goat serum in 1 x PBS buffer) overnight at 4°C in a humidified chamber. After washing unbound primary antibody, samples were incubated with goat anti-mouse or goat anti-rabbit antibody conjugated with Alexa Fluor 488 (Invitrogen, diluted 1:200 in 5% goat serum in PBS buffer) for 4 h at RT in a humidified chamber. The cultures were washed with 1 x PBS (four times for an hour) and 1 µg/ml Hoechst 33258 (SigmaeAldrich, bisbenzimide) together with Alexa Fluor 594-labelled phalloidin (Invitrogen, diluted 1:100 or 1:50 in 1 x PBS buffer) was placed and held for overnight to visualize the nuclei and cellular distribution of the filamentous actin (F-actin) cytoskeleton, respectively.

6.2 Immunofluorescence Staining of ADMSCs in GrowDex-T [1]:

ADSCs were cultured in 0.2% aNFC (1 × 10^5 cells/ml) within x-well cover glass cell culture chambers for 72 h. Following culture, media were removed and cells were gently washed with PBS, followed by fixation using 4% PFA for 20 min at RT. Subsequently, PFA was removed and cells were washed twice with PBS for 5 min. Unspecific antibody binding was blocked by incubation with 0.02% PBS-Tween with 5% goat serum for 30 min. Primary antibody against actin (1 : 250, rabbit polyclonal, Sigma-Aldrich), nestin (1 : 250 mouse monoclonal, clone #196908, R&D Systems), osteocalcin (1 : 100, mouse monoclonal, clone G-5, Santa Cruz Biotechnology Inc.), and osteopontin (1 : 100, mouse monoclonal, clone AKm2A1, Santa Cruz Biotechnology Inc.) were added in 0.02% PBS-Tween with 5% goat serum and incubated overnight at 4°C in agitation.

Cells were then washed twice with PBS and incubated in PBS overnight at 4°C in agitation. PBS was replaced with the secondary antibody (1 : 300) in 0.02% PBS-Tween with 5% goat serum and incubated overnight at 4°C in agitation. Cells were then washed twice with PBS and incubated in PBS overnight at 4°C in agitation. Cells were counterstained with DAPI (1 : 2000, Sigma-Aldrich) in PBS overnight at 4°C in agitation and washed twice with PBS. Following the final wash, PBS was replaced with 0.02% PBS-sodium azide and the samples were imaged using the Nikon A1R inverted confocal microscope.

7 *In vivo* application of NFC

7.1 Drug compound release from GrowDex *in vivo* [9, 10]:

The full step by step protocol of how GrowDex was used in vivo for drug compound delivery can be found in the application note and publication:

- Study of radio-labeled model drug compound (123I-NaI) release from GrowDex® in vivo [10]
- Laurén, P., Y.-R. Lou, M. Raki, A. Urtti, K. Bergström and M. Yliperttula (2014). "Technetium-99m-labeled nanofibrillar cellulose hydrogel for in vivo drug release." European Journal of Pharmaceutical Sciences 65: 79-88.

Hydrogel implants were injected subcutaneously in the pelvic region, and the mice were observed non-invasively over a 24 h period with the use of a SPECT/CT system.

8 Mixing NFC with extracellular matrix molecules

8.1 Synthesis of collagen-nanocellulose hydrogels [11]:

Nine parts of type I collagen solution from bovine skin (3 mg/mL, Sigma-Aldrich C4243) was mixed to one part of 10X filtered PBS. A volume of 10 µL of 1M NaOH was added to adjust the pH to 7, measured by pH paper strips. Collagen and nanocellulose hydrogels were combined to achieve the final concentration of 0.2 wt.% and 0.1-0.3 wt.%, respectively. The hydrogels were sterilized under ultraviolet radiation exposure during 20 minutes prior to use.

9 Transwell Inserts

9.1 Transwell inserts have been used in the following application notes and publications:

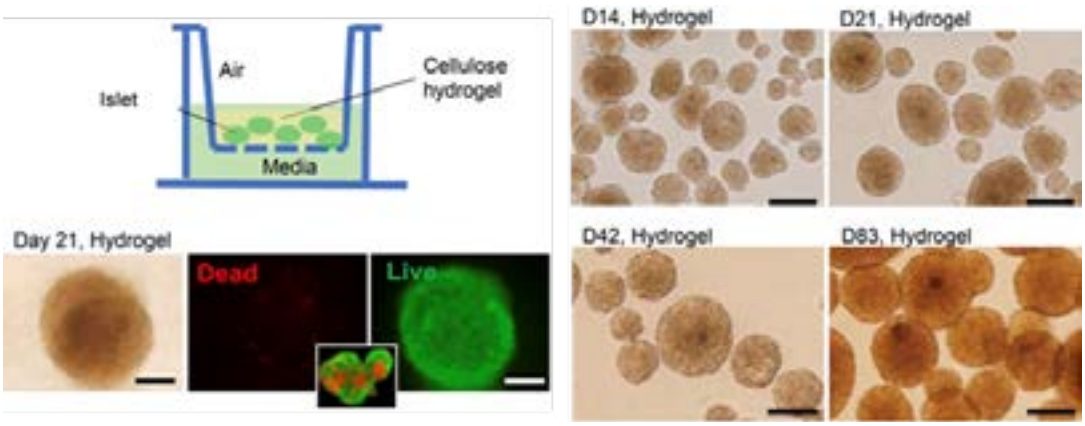
- Cells seeded in GrowDex or GrowDex-T in the inner chamber of a transwell insert:
  - Azoidis, I., J. Metcalfe, J. Reynolds, S. Keeton, S. S. Hakki, J. Sheard and D. Widera (2017). "Three-dimensional cell culture of human mesenchymal stem cells in nanofibrillar cellulose hydrogels." MRS Communications 7(3): 458-465.
  - App note: 24 - Osteogenic and adipogenic differentiation of human mesenchymal stem cells
  - Sheard, J. J., M. Bicer, Y. Meng, A. Frigo, R. M. Aguilar, T. M. Vallance, D. Iandolo and D. Widera (2019). "Optically Transparent Anionic Nanofibrillar Cellulose Is Cytocompatible with Human Adipose Tissue-Derived Stem Cells and Allows Simple Imaging in 3D." Stem Cells International 2019: 12.
  - App note:10 - Mesenchymal Stem Cell Differentiation in GrowDex-T
  - Chen, Y.-J., T. Yamazoe, K. F. Leavens, F. L. Cardenas-Diaz, A. Georgescu, D. Huh, P. Gadue and B. Z. Stanger (2019). "iPreP is a three-dimensional nanofibrillar cellulose hydrogel platform for long-term ex vivo preservation of human islets." JCI Insight 4(21).
- Cells seeded on the underside of a transwell insert coated with GrowDex:
  - Zaderer, V., M. Hermann, C. Lass-Flörl, W. Posch and D. Wilflingseder (2019). "Turning the World Upside-Down in Cellulose for Improved Culturing and Imaging of Respiratory Challenges within a Human 3D Model." Cells 8(10): 1292.

9.2 Culturing cells in a transwell insert with GrowDex or GrowDex-T [5, 7]:

For adipogenic and osteogenic differentiation, ADSCs (5 × 10^5 cells/ml) in normal cultivation medium were embedded in 0.2% aNFC as described above and placed into TC cell culture inserts (24-well format, Ø3.0 µM, 83.3932.300, Sarstedt Ltd.). Medium was changed after 24 h to StemPro Adipocyte or Osteocyte Basal Medium supplemented with StemPro Adipogenesis or Osteogenesis Supplement (Life Technologies) according to the supplier's instructions. For the controls, medium was changed to fresh standard cultivation medium. Control and differentiation media were replaced every 2-3 days and maintained at 37°C for 21 days. Following the differentiation period, media were removed from the wells and TC inserts. Cells were washed with PBS and subsequently fixed with 4% PFA for 30 min.

9.3 Culturing pancreatic islet cells in a transwell insert with GrowDex [12]:

On the day of arrival, human islets were handpicked into the tubes (50 islets per tube) and assigned to different groups to investigate the effect of medium, Transwell filter, and 3D NFC hydrogel culture. CMRL medium was CMRL 1066 (Corning) supplemented with 10% heat-inactivated FBS, 1× GlutaMAX (Life Technologies), 100 U/mL penicillin, and 100 µg/mL streptomycin. PIM(R) (Prodo Laboratories Inc.) medium was supplemented with 5% human AB serum and provided by the Penn Center for Islet Transplantation. CIT medium was prepared from CMRL 1066, CIT Modification (Corning) supplemented with 0.5% human albumin, 10 U/mL heparin, and 0.1 µg/mL IGF-1 (21). For standard culture, 50 islets were distributed into the well of a 96-well plate (Corning) and cultured in CMRL 1066, PIM, or CIT medium. Transwell Permeable Inserts, 6.5 mm with 8-µm pores (Corning), were used in both Transwell culture and 3D NFC hydrogel culture. To set up the Transwell culture, 50 islets were suspended and loaded into the insert with CMRL, PIM, or CIT medium. For 3D NFC hydrogel culture, 50 human islets were mixed with 0.75% (w/v) NFC hydrogel (GrowDex; UPM Biomedicals) and then dispensed into the insert of Transwell. CIT medium was added to the top of hydrogel and the lower compartment. The culture media were replenished every 3–4 days.



9.4 Culturing cells under a transwell insert with GrowDex [13, 14]:

The full step by step protocol of how GrowDex was used to coat the underside of the transwell insert prior to cell seeding can be found in the application note and publication:

- Turning the World Upside-Down for Improved Culturing and Imaging of Respiratory Cells Within a Human 3D Model [14]
- Zaderer, V., M. Hermann, C. Lass-Flörl, W. Posch and D. Wilflingseder (2019). "Turning the World Upside-Down in Cellulose for Improved Culturing and Imaging of Respiratory Challenges within a Human 3D Model." Cells 8(10): 1292.

10 Viability analysis

10.1 alamarBlue™ viability analysis of cells in GrowDex [15]:

Cell viability was also assessed by Alamar Blue assay (Invitrogen). 10% of dye solution was prepared in complete medium and incubated with cells for 4 h at 37°C. Supernatant was then transferred to 96 well plate and absorbance at 570 nm and 600 nm was recorded to calculate reduction in dye (reduction in dye corresponds to cell viability).

10.2 CTG, CTG-3D and RTG vibility kits:

The application and protocols for CTG, CTG -3D and RTG viability analysis of cells in GrowDex or in GrowDex-T are extensively discussed in the following application notes:

- Robust and accurate 3D cell viability assays combing Promega viability kits and GrowDex [16]
- Robust and accurate 3D cell viability assays combing Promega viability kits and GrowDex-T [17]

10.3 Live/Dead staining on ADMSC in GrowDex-T [5]:

Live and Dead Staining and Confocal Laser Scanning Microscopy. ADSCs were cultured in 0.2% aNFC (1 × 10^6 cells/ml) for 24 h within x-well cover glass cell culture chambers (Sarstedt Ltd.) and stained using LIVE/DEAD Viability/Cytotoxicity Kit according to the manufacturer's instructions (Thermo Fisher Scientific), fixed in 4% PFA for 20 min and counterstained with DAPI (Sigma-Aldrich). Confocal images were acquired using the Nikon A1R inverted confocal microscope with the Nikon Plan Apo VC 20x DIC N2 optic lens, running NIS-Elements AR. DAPI was visualised at an excitation/emission of 405/450 nm, calcein at 494/517 nm, and ethidium homodimer-1 at 528/617nm with the Chroma 405/488/561/647 quad mirror. A z-stack depth of 200 µm (z-plane) was created for an area of 554 × 550 µm, and a 3D reconstruction was generated for all the channels using the NIS-Elements AR software (v4.0).

10.4 PrestoBlue viability analysis of cells in NFC [18]:

PrestoBlue was mixed 1:10 with the cell culture medium needed for the used cells. Depending on the size of the wells, this mixture was added to the printed structures so that they were covered completely. Afterwards, the samples were incubated for 2 hours. Besides the cell seeded samples, several sample blank dummy samples were needed as well to account for impurities from solvents & chemicals used with the same dilation and volume of PrestoBlue that should be incubated alongside the printed samples. After the 2 hours of incubation, 100 µl of each well was transferred in duplicate into a 96 plate for further inspection. Considerations of the PrestoBlue solution was read with a Plate Reader (H1 Synergy, biotech, Germany) to obtain relative fluorescence values that were collating with the metabolic activity of the cells. Excitation wavelength of the Plate Reader was set to 560 nm and the emission wavelength was set to 590 nm. The readout was set to bottom-read and the gain was set to 50. For normalization, the mean of all sample blanks was then subtracted from the mean of each tested sample. When observing the same samples on different time points everything should be repeated in the same way. So, the same type of well-played should be used, the incubation time should be the same and the amount of sample blank should be the same as well.

10.5 XTT viability analysis of cells in GrowDex [7]:

Cell Viability Assay (XTT). Cell viability assays were performed for cells in both 2D and 3D cultures using the Cell Proliferation Kit II (Sigma-Aldrich) according to the manufacturer's instructions. Absorbance of the XTT metabolite was measured at an excitation wavelength of 490nm and a reference wavelength of 650nm on a SpectraMax iD3 plate reader (Molecular Devices, Wokingham, United Kingdom). Measurements were taken after 2 hours of incubation. Values were corrected for the reference values (650 nm), as well as the appropriate aNFC controls.

11 References:

1. 1.Sheard, J., Hanging drop culture of mesenchymal stem cells within nanofibrillar cellulose, in UPM Biomedicals Application Note. UPM-Kymmene Oyj, Helsinki, Finland.
2. Mäkelä, R., et al., Ex vivo modelling of drug efficacy in a rare metastatic urachal carcinoma. BMC Cancer, 2020. **20**(1): p. 590.
3. Malinen, M.M., et al., Differentiation of liver progenitor cell line to functional organotypic cultures in 3D nanofibrillar cellulose and hyaluronan-gelatin hydrogels. Biomaterials, 2014. **35**(19): p. 5110-5121.
4. Charbonneau, A.M., et al., 3D Culture Histology Cryosectioned Well Insert Technology Preserves the Structural Relationship between Cells and Biomaterials for Time-Lapse Analysis of 3D Cultures. Biotechnology Journal, 2019. **0**(0): p. 1900105.
5. Sheard, J.J., et al., Optically Transparent Anionic Nanofibrillar Cellulose Is Cytocompatible with Human Adipose Tissue-Derived Stem Cells and Allows Simple Imaging in 3D. Stem Cells International, 2019. **2019**: p. 12.
6. Malinen, M., Development of organotypic liver cell cultures in three-dimensional biomaterial hydrogels, in Faculty of Pharmacy. 2014, University of Helsinki.
7. Azoidis, I., et al., Three-dimensional cell culture of human mesenchymal stem cells in nanofibrillar cellulose hydrogels. MRS Communications, 2017. **7**(3): p. 458-465.
8. Auvinen, V.V., et al., Effects of nanofibrillated cellulose hydrogels on adipose tissue extract and hepatocellular carcinoma cell spheroids in freeze-drying. Cryobiology, 2019. **91**: p. 137-145.
9. Laurén, P., et al., Technetium-99m-labeled nanofibrillar cellulose hydrogel for in vivo drug release. European Journal of Pharmaceutical Sciences, 2014. **65**: p. 79-88.
10. Laurén, P., Study of radio-labelled model drug compound (123I-NaI) release from GrowDex® in vivo, in UPM Biomedicals Application Note. UPM-Kymmene Oyj, Helsinki, Finland.
11. Curvello, R., et al., A thermo-responsive collagen-nanocellulose hydrogel for the growth of intestinal organoids. Mater Sci Eng C Mater Biol Appl, 2021. **124**: p. 112051.
12. Chen, Y.-J., et al., iPreP is a three-dimensional nanofibrillar cellulose hydrogel platform for long-term ex vivo preservation of human islets. JCI Insight, 2019. **4**(21).
13. Zaderer, V., et al., Turning the World Upside-Down in Cellulose for Improved Culturing and Imaging of Respiratory Challenges within a Human 3D Model. Cells, 2019. **8**(10): p. 1292.
14. Zaderer, V., et al., Turning the World Upside-Down for Improved Culturing and Imaging of Respiratory Cells Within a Human 3D Model, in UPM Biomedicals Application Note. UPM-Kymmene Oyj, Helsinki, Finland.
15. TejalV. Pant and R. Jain, Development of a 3D model for toxicity studies using lung adenocarcinoma cell line A549, in UPM Biomedicals Application Note. UPM-Kymmene Oyj, Helsinki, Finland.
16. Sheard, J., Robust and accurate 3D cell viability assays combing Promega viability kits and GrowDex®, in UPM Biomedicals Application Note. UPM-Kymmene Oyj, Helsinki, Finland.
17. Sheard, J., Robust and accurate 3D cell viability assays combing Promega viability kits and GrowDex®-T, in UPM Biomedicals Application Note. UPM-Kymmene Oyj, Helsinki, Finland.
18. Theiner, S., Extrusion 3D Bioprinting of nanocellulose based bioinks, in Faculty of Mechanical Engineering and Business Administration. 2021, Vienna University of Technology.

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