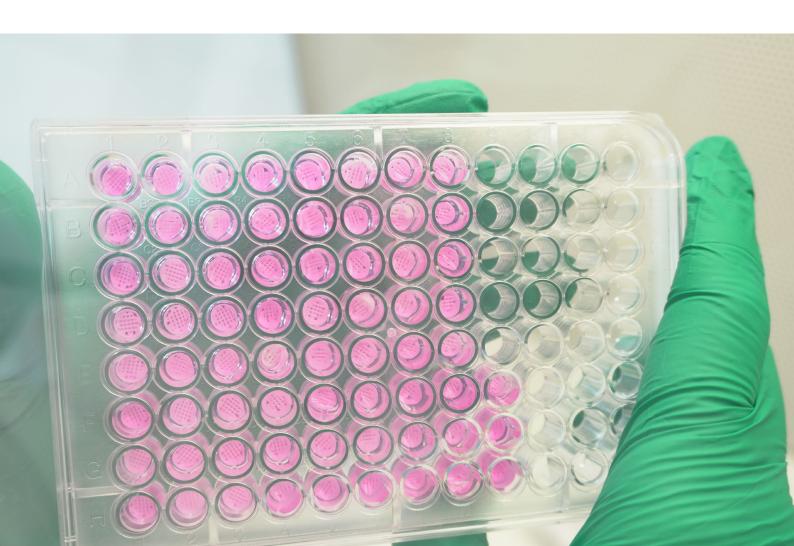




# Scaffolds-based 3D cell culturing Research Resource Handbook

- April 2023





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## **ABOUT OSSIFORM**

Ossiform is dedicated to improving the lives of people with bone defects. We provide patient specific, biomimetic, and naturally resorbable bone implants to reduce complications, augment functional and aesthetic outcomes and obtain faster recovery.

We are continuously working on redefining bone replacements through progressive research and close collaboration with physicians and industry partners. Indeed, our patient-centric approach and commitment to innovation, quality, and progress drive our efforts to provide best-in-class implant solutions that facilitate the natural forming of new bone.



Our P3D Scaffolds establish more relevant tissue- and disease models that capture the complex reciprocity between cells, as well as promoting bone research by providing researchers with relevant bone-mimicking cell cultures.



Our bones provide essential support to our bodily posture, as well as reducing inertness. A wide range of bone-related diseases affect individuals worldwide, which Ossiform aspires to decrease, by advancing the development of personalized treatments through furnishing porous-like scaffolds based upon 3D printed ß-tricalcium phosphate (ß-TCP).



Oslogade 1, 5000 Odense C, Denmark



## REVITALIZING CELL CULTURE SYSTEMS

## Piloting the future of cell culturing systems and biomimetic materials

P3D Scaffolds are unique, xeno-free bioceramic scaffolds designed to mimic the architecture and complexity of the natural tissue to enable personalized tissue- and disease models, which capture the complex reciprocity between various cell types. The scaffolds are compatible for a wide range of platforms such as cell- and bacteria studies, in vivo studies, tissue engineering, disease modeling, and drug screening.

## Advance your research with customizable P3D Scaffolds

The P3D Scaffolds are provided with several customizable features including design, pore size and height of the scaffolds, which allows the customer to fully tailor the scaffolds to suit their research.

Find more information about the P3D Scaffolds at Ossiform: P3D Scaffolds.

## Introducing scaffold-based 3D cell culture systems

Cell culture systems, applied in disease modeling, tissue engineering, and drug screening, have markedly contributed to reducing laboratory animal use and ensured progressive research, pharmaceutical discovery, and the development of evolutionary medicine (Habanjar et al., 2021; Fischbach et al., 2009). The two-dimensional (2D) in vitro cell culture system constitutes a traditional application on uniform support for cell growth in a monolayer (Kramer et al., 2013; Pampaloni et al., 2007). Although the 2D cell culture system has been a revolutionary milestone for research since the early 1900s (Becker & Blanchard 2007; Ferreira et al., 2018), this type of culturing presents copious limitations, such as failing to effectively mimic physiological conditions and the natural microenvironment, such as cell-matrix interactions. Indeed, the cell-to-cell communication with the surrounding extracellular matrix (ECM), which is predominantly absent in 2D, regulates cell growth, proliferation, and overall function (Amer et al., 2015; Bray et al., 2015).

To overcome these limitations, Ossiform has developed P3D Scaffolds which function as novel *in vitro* and *in vivo* 3D cell culture systems. With them, Ossiform aims to refine research by properly mimicking the natural physiological properties and conditions of the cellular environment, temporal and spatial contexts (Figure 1A), as well as improving the development of novel treatments at the preclinical stage. Thereby, our P3D Scaffolds (Figure 1B) successfully provide a pragmatic physiological microenvironment with a potential role in cancer drug discovery, thus eliminating the lack of preclinical models relevant to 2D cultures (Chopra et al., 1997; Lei & Schaffer, 2013; Soares et al., 2012; Horning et al., 2008).



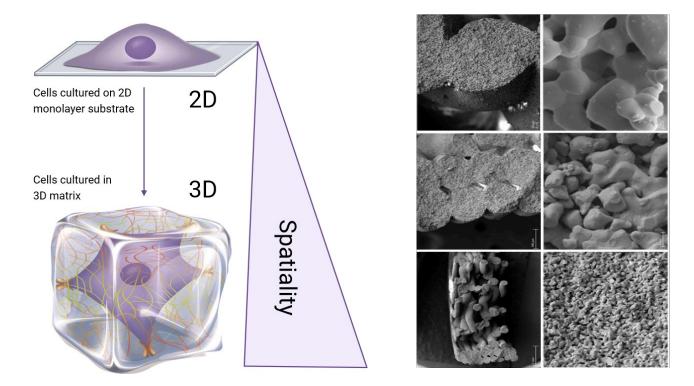


Figure 1: **3D cell culture systems. (Right)** Schematic demonstrating cells cultured in 2D versus 3D. 3D cell culture systems drastically increase spatiality. Based on the schematic by Huang et al. (2017). **(Left)** Scanning electron microscopy (SEM) images of the microporous P3D Scaffolds at different magnifications.

## Optimize cell culturing with the disruptive technology

Ossiform is founded on a widely applicable proprietary technology revolving around three-dimensional (3D) printing of natural biocompatible ceramic structures for generating P3D scaffolds and patient specific bone implants (PSI). The technology is based on a bio-ink composed of powder particles suspended in a solid but highly meltable fatty acid matrix (Figure 2A–C), which allows for a novel additive manufacturing (AM) process in which virtual objects are directly constructed from a computer-aided design (CAD) file.



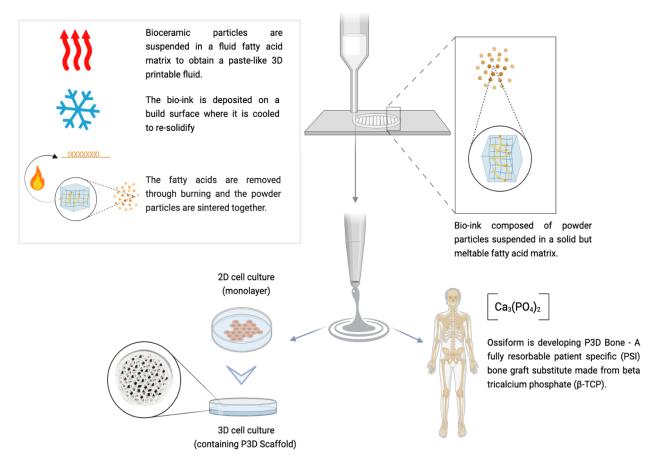


Figure 2: Bioprinting to construct 3D cell cultures and natural bone implants. Ossiform's bio-ink 3D printing setup and subsequent mechanism and applications.

## Elevate your research with P3D Scaffolds

3D cellular models yield a more effective and reliable research model for investigating the cellular environment as they maintain cell-to-cell and cell-to-matrix interaction. The P3D Scaffolds are a highly applicable platform for 3D cell culturing of bone harboring or bone related cell types. The cell culture system is compatible with the most common laboratory techniques and does not require significant alterations of preestablished protocols and workflows.

P3D Scaffolds provide your research with a clinically relevant cell culture system of biomimetic materials and customized structures, which allows you to develop predictive research models of human physiology and pathology.



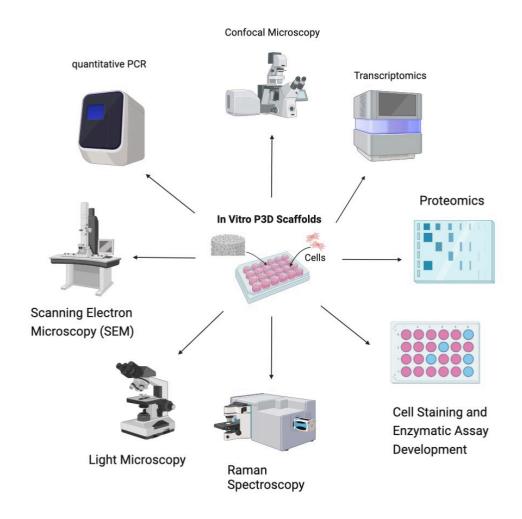


Figure 3: Areas of research compatible with P3D Scaffolds.

The P3D Scaffolds are, amongst other attributes, directly translatable from *in vitro* to *in vivo* preclinical research, which has enabled them to be incorporated in a broad spectrum of techniques, ranging from microscopy-based to protein-quantifying methods.

#### Create predictive models and obtain reliable results

The P3D Scaffolds have been employed by numerous methods for analyzing the biological activity occurring within the 3D printed structures (Figure 3). Ossiform has successfully applied various methods, which are listed on the website: Ossiform: Scientific Publications. 3D cell culture systems have demonstrated high yield in copious research areas, including tissue engineering, disease modeling, and drug discovery. By allowing cells to develop and migrate within a three —dimensional space, which is observed inside the human microenvironment, the P3D Scaffolds have been applied in predictive research models of human biology in several in vitro and in vivo pre-clinical studies.

The P3D Scaffolds have recently been highlighted in a 2022 research study, exploring the tissue development of subcutaneous calcium phosphate scaffolds seeded with hMSCs in mice by employing fibreoptic Raman spectroscopy. The researchers concluded that the methodology can be



further developed and tailored for several small animal subcutaneous implant models to study for instance ectopic bone formation, tissue regeneration, and scaffold resorption dynamics. Find the entire publication <a href="here">here</a>.

## Easily translate your research from in vitro to in vivo

#### P3D Scaffolds increase effectiveness

The P3D Scaffolds provide a more accurate duplicate of the native 3D structures and material within human bone than most other commercially available solutions. The scaffolds induce growth and adhesion of a 3D cell culture that proximately resembles cells in their native environment. Moreover, using P3D Scaffolds as a 3D cell culturing system enables the comprehension of complex biology in a physiologically relevant context, in which drug perfusion is fluctuating and where bacteria, cancer cells, and other pathogens may hide in pores. Thereby, the P3D Scaffolds can potentially increase research effectiveness and efficiency, due to several benefits, including more accurate, relevant, and reliable data derived from the research, which markedly facilitates the interpretation of research models by the researcher. Furthermore, P3D Scaffolds may be used both in vitro and in vivo. In using scaffolds originating from the same material and porosity across research methods affixes optimal conditions to achieve useful and relevant output.

#### Potential areas of investigation

Numerous cells and pharmaceuticals can be combined with the P3D Scaffolds to investigate the interaction. Potential usages of the P3D Scaffolds include, but are not limited to:

- Bone destruction in osteoporosis/arthritis can be replicated by seeding osteoclasts and macrophages onto the scaffolds.
- Osteomyelitis and the subsequent destruction of bone can be mimicked by adding bacteria
  and immune cells to the P3D Scaffolds. This enables you to investigate the mechanisms
  behind the bacteria's destruction of the vigorous calcified bone as well as how the bacteria
  can evade the immune system and antimicrobial pharmaceuticals.
- The development of bone tumors and spreading of cancerous cells through the outer calcified bone matrix can be investigated by seeding cancerous cells to the P3D Scaffolds.
- hMSCs or osteoblasts can be added onto the scaffolds to study how new bone develops in the bone grafts. Find more information on how to culture hMSCs in bioceramic 3D cell culture systems at <u>Ossiform: Protocols</u>.



#### P3D Scaffolds in tested cell culture studies

A variety of cells can be grown on the 3D printed microstructures. Ossiform has successfully grown the following cells on the P3D Scaffolds:

- Stem cells, including hMSCs, for investigating bone development.
- Cancer cells, including oral carcinoma and lung cancer cells, used to investigate cancer invasion and metastasis in bones, a renowned site of cancer metastasis, and the treatment of bone tumors using therapeutic solutions.
- Pathogenic bacteria, including staphylococcus aureus, used to investigate bone-related diseases such as osteomyelitis, surgical site infections and implant biofilms, and the prevention and treatment of these conditions using pharmaceutical agents.
- Environmental microorganisms, including bacteria and fungi strains, used to investigate the
  interaction between environmental microorganisms and inorganic 3D structures that mimic
  environmental structures, such as soil, rock, sediment, and building materials.
- Resorption assays with human osteoclasts for studying osteoclast activation and re-sorption in various cell culturing conditions.

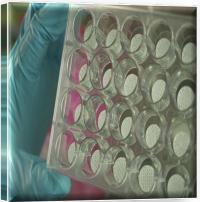
**Cancer & Oncology** 

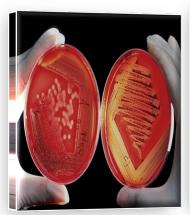
**Drug Screening Systems** 

Bacteria & Biofilm formation

**Bone-related diseases** 











# **SCIENTIFIC PROTOCOLS**

## Preparation of P3D Scaffolds

This protocol is designed for Ø12mm P3D Scaffolds for 24 —well plates. The protocol also applies to the other available sizes of the P3D Scaffolds. Volumes may require adjustments to accommodate for other well plate sizes.

Note: The material and structure of the P3D Scaffolds are not compromised during sterilization or disinfection, however, disengaged particles may occur.



- 1. Unpack the low-adherence 24 -well plate.
- 2. Place one P3D Scaffold in each well of a low attachment 24 -well plate.
- 3. **Optional:** Wash the P3D Scaffolds in 500  $\mu$ L PBS x3 for one minute in order to remove any redundant particles.
- 4. Add 500 µL culture media to each well and leave them to soak.

**Note:** The scaffolds must be completely submerged in the medium.

5. Incubate the plate in a humidified incubator for at least 30 min, 37°C and 5% CO<sub>2</sub>.

## Safety Data of P3D Scaffolds

Find all safety information about the P3D Scaffolds at P3D Scaffolds Safety Data Sheets.

## Handling

Avoid direct contact with eyes, skin, and clothes.

#### Storage

Keep the product tightly sealed.

#### Disposal

Disposal of the scaffolds as local guidelines dictate.

## Combine your P3D Scaffolds with the High Quality Ultra Low Attachment Plates

• Ultra Low Cell Adhesion.



- Comprised of a covalently bound hydrogel surface coating that minimizes cell attachment, protein absorption, and cellular activation.
- Available in 6 well, 24 well or 96 well plates
- Single wrapped sterile ready to use.
- Ideal for culturing stem cells and regenerative cell therapy.

Find more information about our recommended 3D cell culture systems at <u>High Quality Ultra Low Attachment Plates</u>. The plates are only available in Sweden, Norway, Denmark, Finland, and Italy.



## Seeding cells on P3D Scaffolds

#### Materials

- P3D Scaffolds (supplied sterile)
- Pipette
- Cell culture incubator
- Cell culture flask
- Cryopreserved hMSCs
- Hemocytometer

- Growth medium (e.g., Human Mesenchymal Stem Cell Growth Medium (MSCGM™)
- Trypsin/EDTA Reagent
- PBS
- Low-attachment 24 well plates
- Maintenance medium (MEM medium with 1% Penicillin/Streptomycin (P/S) and 10% FBS)

#### Before seeding cells on scaffolds

- Remove media from cells and wash with 1XPBS. Discard the PBS.
   Note: The solution should cover the surface.
- Add Trypsin/EDTA and gently tilt to evenly distribute the solutions. Investigate cell detachment under a microscope to ensure that adhering cells are effectively detached before proceeding.

#### Preparing the cell suspension

- 3. Transfer the cell suspension into a centrifuge flask.
- 4. Centrifuge the flask at 500g/1200 rpm for 5 minutes. Discard the supernatant without disturbing the pellet. Resuspend the pellet in 1 –5mL media.



- 5. Determine cell density by calculating the number of cells/mL using a hemocytometer.
- 6. Prepare the final cell suspension by adding the appropriate amount of media to the centrifuge flash.
- 7. Finally, seed the cells on prepared P3D scaffolds in 24-well plates. **Note:** Seeding cell density should be  $3 5 \times 10^5$  cells/mL.

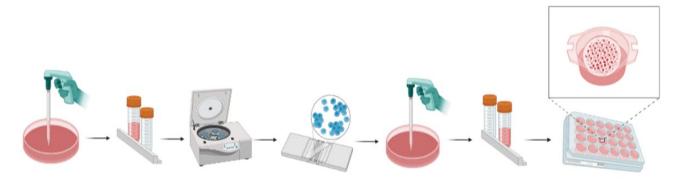


Figure 4: Process flow of cell suspension. Illustration demonstrating the process described in steps 3 -7.

**Note:** Seeded cells from the scaffold(s) may be retrieved as delineated in P3D Scaffold Cell Recovery by Trypsinization protocol available <u>here</u>.

## Culturing of cells on P3D Scaffolds

This protocol revolves around the culturing of human mesenchymal stem cells (hMSCs) on P3D Scaffolds. The content provides the user with guidelines for seeding and 3D culturing of hMSCs on P3D Scaffolds (Ø12 mm) in a 24-well plate. Also, the user will find guidelines on how to create 3D multicellular in vitro tissue constructs based on hMSCs and subsequently differentiation of hMSCs into osteoblasts to establish a 3D bone cell culture. We recommended downloading our Technical Data Sheet with indicated seeding densities, if using other sizes of P3D Scaffolds. Moreover, culturing of cells on P3D Scaffolds can be performed with mono- or co-cultures and enables different approaches for obtaining complex co-culturing depending on the user's research line. In the 3D environment, cell types may undergo separation by hydrogel encapsulation. For instance, embryoid bodies (EBs) were cultured in a collagen matrix indirectly in combination with cardiac fibroblasts, using well inserts to augment cardiomyocyte differentiation of the EBs (Ou et al., 2011). Indeed, indirect co-culture allows cells to reside in environments with similar architecture to native tissue, specifically tailored to each respective cell type, meanwhile maintaining the advantages of cell-to-cell communication (Paschos et al., 2015).

Prior to performing the culturing of cells on P3D Scaffolds:

I. All handling of the P3D Scaffolds should be performed using gloves, according to the standard aseptic methods.



II. The material and structure of the P3D Scaffolds are not compromised during sterilization or disinfection, however, disengaged particles may occur.

## Preparation of P3D Scaffolds

- 1. Unpack the low-adherence 24 -well plate.
- 2. Place one P3D Scaffold in each well of a low attachment 24 -well plate.
- 3. **Optional:** Wash the P3D Scaffolds in 500  $\mu$ L PBS x3 for one minute in order to remove any redundant particles.

## Preparation of cell suspension

This procedure uses cryopreserved hMSCs which needs to be thawed properly for effectively preserving the cell culture.

- 1. Follow your preferred method for cell thawing (1 vial of cells per culture flask)
- 2. Incubate the flasks at 37°C and change culture media 2-3 times per week. This allows cells to enter the growth phase.

Conduct the passaging of hMSCs to proliferate the cells until reaching confluency of 90%.

- 1. Wash the flasks with 1XPBS. The solution should cover the surface. Discard the PBS.
- 2. Add Trypsin/EDTA Reagent to the flask and gently tilt to evenly distribute the Trypsin/EDTA Reagent. Incubate for 5 minutes.

Ensure that cells are detached from the flask before proceeding.

- 1. Transfer cell suspension into a centrifuge tube, and centrifuge at 500 g/1200 rpm for 5 minutes.
- 2. Discard the supernatant without disturbing the pellet. Resuspend the pellet in 1-5 mL media.
- 3. Count the cells and calculate number of cells/mL to determine cell density using a hemocytometer.
- 4. Prepare final cell suspension by adding the appropriate amount of media to the centrifuge flask. Note: Seeding cell density in the 24-well plate should be  $3.5 \times 10^5$  cells/Ø12 Scaffold.

#### Plating cells

- 1. Add one sterilized or disinfected P3D Scaffold to each well of a 24-well plate.
- 2. Add 500  $\mu$ L culture media to each well and let the scaffold soak. The scaffold must be completely submerged in medium.



3. Incubate the plate in a humidified incubator for at least 30 minutes, at  $37^{\circ}$ C and 5% CO<sub>2</sub>. Next, add  $500 \,\mu$ L of the final cell suspension prepared in step 2., to each well by placing the cells on top of the scaffold.

Important: Avoid touching the scaffold.

- 4. Replace the medium and evaluate cell status after 24 and 48 h with maintenance medium.
- 5. In order to differentiate the hMSCs into osteoblasts, replace the maintenance medium with osteogenic medium after 48 h. Change the medium twice weekly. The organoids are now ready for analysis and downstream experiments.

Visit our <u>YouTube channel</u> to find interactive protocols, and find more protocols at <u>Ossiform:</u> Protocols

## Recovery of cells

In order to retrieve cells from the P3D Scaffolds, an additional centrifugation step is incorporated to ensure that any cells ensured within the scaffold are retrievable. Find our video protocol delineating this procedure at Video protocol: <u>Cell recovery by trypsinization</u>.

Prior to performing the recovery of cells cultured on P3D Scaffolds:

- All handling of the P3D Scaffolds should be performed using gloves, according to the standard aseptic methods.
- In general, 3D cultures require longer incubation and more rigorous shaking compared to 2D cultures.

#### Materials

- Enzymatic agent such as trypsin, accutase, collagenase (in case of long-term culturing) or other appropriate enzymes
- Media
- Falcon tube
- Centrifuge

#### Procedure

- 1. Retrieve scaffolds from the incubator.
- 2. Aspirate medium and add enzymatic agent (Trypsin or Accutase) to the scaffolds.

Note: Ensure that the enzymatic agent completely covers the scaffolds (minimum 300  $\mu L$  pr well for 24-well plates)

**Important**: Long-term culturing of osteoblasts is often more complicated to trypsinize due to the extensive amount of collagen. Therefore, we recommend using collagenase during long-term culturing.



- 1. Incubate the plate for 3-5 minutes.
- 2. Terminate the trypsinization process by adding media and transfer liquid and scaffold to a clean 50 mL Falcon tube.
- 3. 5. Centrifuge for 5 minutes at 1200 -1400 rpm to retrieve dislodged cells inside the scaffold.
- 4. Finally, remove scaffold and resuspend cell pellet by pipetting up and down.

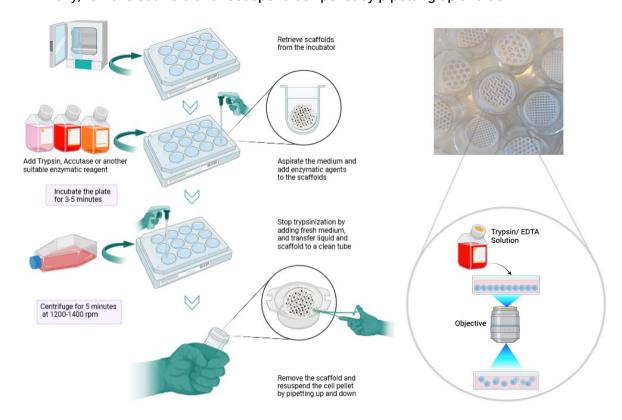


Figure 5: (Left) Process of recovery of cells. Illustration demonstrating the process described in steps 1-6. (Right) Cell detachment by Trypsini-zation/EDTA. Illustration demonstrating the process of successful trypsinization occurring within the scaffolds.

#### Downstream procedures

Single cell analyses, such as single cell sequencing, single nuclei sequencing, and FACS or flow cytometric cell sortingare compatible with our P3D Scaffolds.

#### **RNA Harvest**

This protocol provides the basic procedure of harvesting RNA from cells cultured on P3D Scaffolds. Find our video protocol delineating this procedure <u>Video protocol</u>: <u>RNA harvest of P3D Scaffolds</u>.

#### Materials

- Qiazol (or other RNA harvesting agent)
- Shaking table
- RNA-free Eppendorf tubes
- Vortexer
- Ice

Prior to performing the culturing of cells on P3D Scaffolds:



- All handling of the P3D Scaffolds should be performed using gloves, according to the standard aseptic methods.
- II. The P3D Scaffolds are supplied sterile by dry heat sterilization and remains sterile until unpacked.

#### Procedure

- 1. Aspirate cell medium.
- 2. Add 450-500 µL Qiazol to each well.
- 3. Incubate for 2–10 minutes at RT on shaking table at 900 rpms. Increasing the incubation time can markedly increase the yield.
- 4. Flush the scaffolds surface by pipetting and transfer lysate to RNA-free Eppendorf tubes on ice.
- 5. Mix the solution by pipetting the full volume up and down.
- 6. Vortex each sample for 10 seconds and incubate at RT for 5 minutes.
- 7. Finally, store samples at -80°C or continue with downstream procedures.

#### Downstream procedures

Transcriptomic analyses, such as quantitative PCR (qPCR) and bulk RNA sequencing, are compatible with our P3D Scaffolds.

#### Protein extraction

This protocol provides the basic procedure of performing protein extraction on P3D Scaffolds. Find our video protocol delineating this procedure at <u>Video protocol</u>: <u>Protein extraction on P3D Scaffolds</u>.

#### Materials

- PBS
- Lysis buffer
- Centrifuge
- Ice

Prior to performing the culturing of cells on P3D Scaffolds:

- I. All handling of the P3D Scaffolds should be performed using gloves, according to the standard aseptic methods.
- II. The P3D Scaffolds are supplied sterile by dry heat sterilization and remains sterile until unpacked.

#### Procedure

- 1. Aspirate cell medium.
- 2. Wash with 10–20x in prewarmed PBS for 1 minute per wash. When washing, alternate between dripping on the top of the scaffold and beside the scaffold.

**Important:** Remove all traces of medium before proceeding to the next step.



- 3. Cover scaffolds in lysis buffer.
- 4. Shake scaffolds at 300 rpms for 30 minutes.
- 5. Flush scaffolds and collect lysate in Eppendorf tubes.
- 6. Centrifuge samples at 16.000g for 20 minutes at 4°C.
- 7. Place samples on ice and pipette the supernatant, containing proteins, into clean prelabelled Eppendorf tubes.

Finally, store samples at -80°C or continue with downstream protein applications.

## Cell viability — Live/dead stain using Invitrogen LIVE/DEAD Cell Imaging Kit

This protocol explains how to perform Cell viability using Invitrogen LIVE/DEAD Cell Imaging Kit with P3D Scaffolds. For more protocols, please visit our Resources Platform.

#### Materials

- Invitrogen LIVE/DEAD cell imaging kit
- · Fresh media
- Microscope with FTIC/GFP and Texas Red filter
- Tinfoil for covering the samples during incubation time

#### **Flowchart**

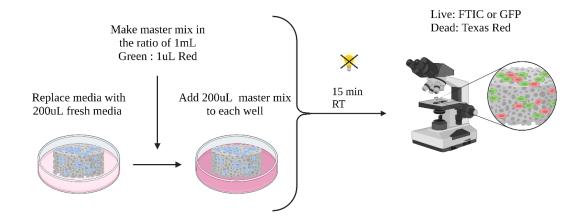


Figure 6: Workflow for evaluating cell viability using the LIVE/DEAD cell imaging kit by Invitrogen

#### Notes before starting and general advice on material handling

- All handling of The P3D Scaffolds products should be performed using gloves, according to the standard aseptic methods.
- The scaffolds are supplied sterile by dry heat sterilization and remains sterile until opened.



#### Procedure

- 1. Thaw the cell kit at the room temperature.
- 2. Aspirate the media from the scaffold.
- 3. Add 200 µL fresh media to each scaffold.
- 4. Mix the Live/Dead stain master mix by adding 1 mL green staining solution to the 1 μL red staining solution enough for 5 Ø12mm P3D Scaffold samples. Please upscale the master mix according to your experimental setup.
- 5. Add 200 µL from the Live/Death cell kit solution to each scaffold.
- 6. Cover with tinfoil paper to prevent it from the light damage.
- 7. Incubate for 15 min at room temperature.
- 8. Make the analyses using by fluorescent microscope.
  - a. For the live cells, use FTIC or GFP filters.

For the dead cells, use the Texas Red filters.

## Evaluation of cell viability

This protocol explains how to evaluate cell viability using CellTiter-Glo 3D cell viability assay with P3D Scaffolds. For more protocols, please visit our Resources Platform.

This assay lyses all cells and measures the amount of ATP present. It works by mixing cells in media 1:1 with Assay Reagent and measuring output luminescence. The output requires the use of black/opaque 96-well plates with clear bottoms. This procedure describes how to use this kit with scaffolds. Please also refer to manufacturer Promega's protocol.

Plate type	Media volume	Assay Reagent volume
96 well plate	100 uL	100 uL
24 well plate	200 uL	200 uL
12-well plate	500uL	500uL
6-well plate	1500uL	1500uL

Always include cell-free scaffolds as a negative control.

## Materials

- CellTiter-Glo 3D cell viability assay
- Plate shaker
- Opaque plate
- Plate reader





Figure 7: Workflow for evaluating cell viability using CellTiter-Glo 3D cell viability assay

Notes before starting and general advice on material handling

- All handling of The P3D Scaffolds products should be performed using gloves, according to the standard aseptic methods.
- The scaffolds are supplied sterile by dry heat sterilization and remains sterile until opened.

#### Procedure

The day before performing the procedure: place Assay Reagent at 4C to allow for slow thawing.

## Before you start

- Place Assay Reagent and cell plates at RT to equilibrate for at least 30 min.
- Gently invert assay reagent bottle to mix before use.
- Make aliquot of assay reagent by calculating the amount needed.
- If necessary, remove media from wells to achieve volumes listed in table above.
- 1. Add Assay Reagent to each well, using multichannel pipette if possible. Volume of Assay Reagent should be equal to the volume media already present in wells.
- 2. Place plate at RT on a plate shaker at 300 rpms for 5 min
- Remove plate from plate shaker and incubate plate at RT for 25 min.
- 4. Pipette 200 uL lysate to opaque plate as quickly as possible
- 5. Measure luminescence using a plate reader with integration time 0,25-1 seconds. Settings may vary between instruments.



## **ANALYSIS**

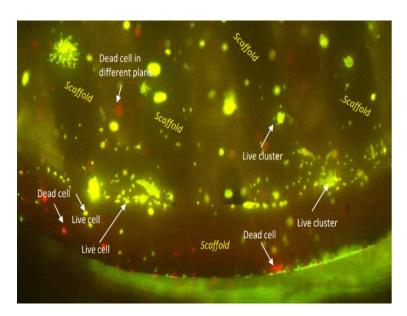
## Cell Viability Assays

## Quantitative CellTiter-Glo® 3D Cell Viability Assay

The CellTiter-Glo® 3D Cell Viability Assay enables the evaluation of cell viability and is compatible with P3D Scaffolds, only requiring one additional step compared to the manufacturers (Promega) protocol This assay determines the number of viable cells in 3D cell culture based on the quantification of ATP, which is a marker for the presence of metabolically active cells. This assay works by lysing all cells, measuring ATP content, and mixing cells in media 1:1 with the CellTiter-Glo® 3D Reagent and subsequently measuring output luminescence. Please refer to our protocol at Evaluation of Cell Viability Using CellTiter-Glo 3D Cell Viability Assay.

## Qualitative Invitrogen™ LIVE/DEAD™ Cell Imaging Kit

This kit allows for discrimination between live and dead cells using two probes that measure recognized parameters of cytotoxicity and cell viability. In dying and dead cells bright, red fluorescence is generated upon DNA binding. Thus, we recommend utilizing the Invitrogen™ LIVE/DEAD™ Cell Imaging Kit in combination with P3D Scaffolds, in order to evaluate cell viability.



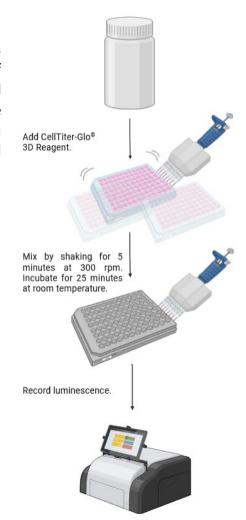


Figure 8: Overview of Cell Viability Assays compatible with P3D Scaffolds. (Right) Overview of the CellTiter-Glo® 3D Cell Viability Assay. (Left) Illustration of LIVE/DEAD cell activity within P3D Scaffolds with indicative arrows representing dead cells, live cells, live clusters, and scaffold microporous structures.



# Qualitative Analysis Alkaline Phosphatase Staining

Alkaline Phosphatase (ALP) staining is a stem cell detection method that allows the user to monitor, visualize, and to differentially stain pluripotent stem cells (PSCs). Alkaline Phosphatase (AP) is a phenotypic marker of PSCs and many other types of stem cells. While AP is widely expressed in various cell types, its expression is highly elevated in PSCs. Therefore, due to stem cells/PSCs exhibiting increased AP activity, we have utilized the Abcam ALP Staining Kit to visualize the presence of ALP, indicative of hMSCs undergoing differentiation into osteoblasts (Figure 9). Moreover, ALP plays an essential role in osteogenesis and is one of three identified osteogenic marker genes. At Ossiform, we have demonstrated novel preliminary data which suggests that osteoblasts cultured on P3D Scaffolds indicate an upregulated expression of the ALP gene (ALPL), when compared to traditional cell culturing standards (Figure 10, right). Find more information at News From The Lab. Additionally, find more information on how to verify osteogenesis and investigate osteoblast differentiation using ALP Staining on P3D Scaffolds at Protocol: How to verify osteogenesis on P3D Scaffolds.

## Alkaline Phosphatase Assay

In order to evaluate the presence of osteoblasts cultured on the scaffold in a more quantitative manner than the ALP Stain, we here recommend the Colorimetric Alkaline Phosphatase Assay Kit (Abcam). This kit enables the user to acquire estimated values proportional with the ALP activity in stem cells, from which the user can easily calculate statistical significance. Importantly, using P3D Scaffolds does not interfere with the manufacturer's instructions. The Assay Kit utilizes p-nitrophenyl phosphate (pNPP) as a phosphatase substrate which yields a yellow precipitation when dephosphorylated by ALP.

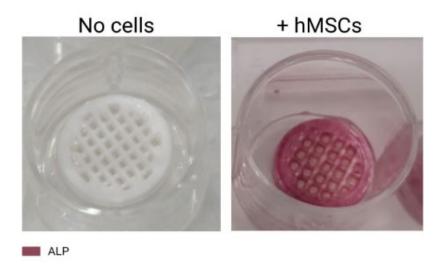


Figure 9: Qualitative evaluation of osteoblast differentiation 20 days after seeding of hMSCs on P3D Scaffolds. (Left) blank P3D Scaffold as control. (Left) Scaffold harboring hMSCs culture. No unspecific binding of the ALP Stain was detected on the scaffolds. Red staining indicates ALP presence.



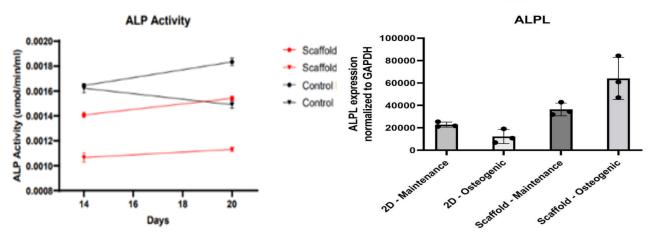


Figure 10: (Left) ALP Activity of samples with P3D Scaffolds and Controls harboring hMSCs. (Right) Upregulated expression of ALPL with P3D Scaffolds as a 3D cell culture system.

#### Alizarin Red Staining

Alizarin Red Staining (ARS) is an anthraquinone dye widely used to stain for calcium deposits, which are indicators of the presence of mature osteocytes. The dye interacts with calcium and forms a complex during the process of chelation resulting in birefringence (1). Conversely, since the P3D Scaffolds contain large amounts of calcium, the ARS will seldom dye the scaffolds themselves (Figure 11). Therefore, we recommend using ARS as a measurement of calcium-containing cells and to compare before- and after images to effectively achieve staining. In terms of quantitative data, we recommend using an image-generating tool for determining the size of the non-red area within the macroporous structure of the scaffolds. Indeed, by comparing these measurements with blank scaffolds, the user will obtain an estimate of the calcification process within the respective cells (Figure 11).

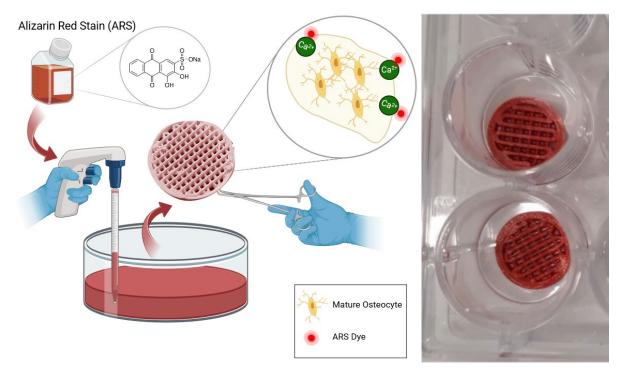


Figure 11: Representative images associated with Alizarin Red Stain. (A) Overview of the ARS process with added P3D Scaffold. (B) Blank P3D Scaffolds after the onset of ARS.



#### Visualizing Osteoblast Activity on P3D Scaffolds

Collectively, every tissue and organ encompass its own spatial and temporal 3D extracellular matrix (ECM) organization. Cells within a 3D bioscaffold for tissue engineering will typically align new ECM components according to the provided bioscaffold. Thereby, scaffolds with a specific 3D structural design mimicking the actual ECM of a particular tissue may present itself with a great potential in tissue engineering (2). Although the P3D Scaffolds are not translucent, they remain highly compatible with confocal microscopy. The use of P3D Scaffolds in combination with confocal microscopy does not require alterations of the users existing protocols. Furthermore, several cell staining methods have proven to be compatible with the P3D Scaffolds, including Hematoxylin and Eosin (H&E), Human Vimentin (hVim), Pico Sirius Red (PSR), and DAPI-Staining, respectively (Figure 12, right). Intriguingly, our P3D Scaffolds were recently involved in a peer-reviewed study investigating the in vivo tissue development in bone scaffolds, monitored by Raman spectroscopy. In this study, the hMSC loaded scaffolds associated with collagen and bone development, and the spectral observations demonstrated excellent correlation with the histological analysis. Find more information about this study at Ossiform: Scientific Publications.

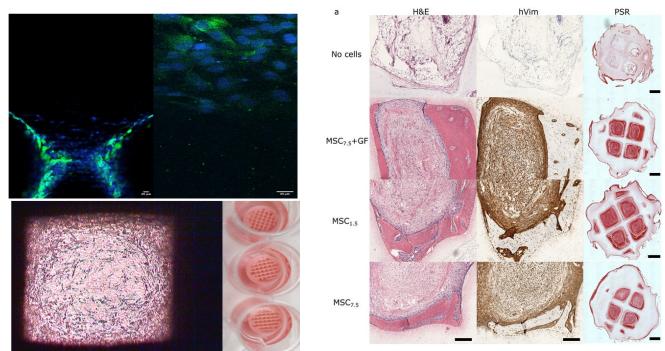
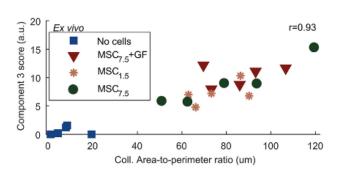


Figure 12: (Upper left) Confocal microscopy image of osteoblasts (blue, DAPI-stain) and their corresponding ECM (green, collagen type 1) on P3D Scaffolds. (Right) Representative micrographs of histological sections showcasing H&E, hVim, and PSR stains after 16 weeks of tissue development in subcutaneously implemented calcium phosphate scaffolds in mice. (Lower left). 10x magnification of osteoblasts residing in ECM within a macropore of P3D Scaffolds to the left and fully intact P3D Scaffolds to the right.

Additionally, our in-house scientists are thrilled to share their results on long term culturing of osteoblasts residing in culture on P3D Scaffolds for 20 weeks (53 weeks per April 2023), which has been captured using light microscopy (Figure 12, lower left). Based on the images, the cells are clearly proliferating and demonstrating that long term culturing of osteoblasts for bone formation studies is indeed plausible and simple to achieve using P3D Scaffolds.





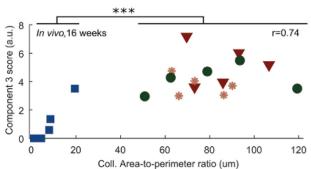


Figure 13: Quantitative analysis of collagen content from Raman spectroscopy and PSR images after 16 weeks of implantation. Collagen content from PSR histology is represented by a ratio of total PSR positive area divided by total scaffold cavity parameter. Pearson's correlation coefficient (r). Scale bars: 200 μm (H&E, hVim micrographs) and 1 mm (PSR micrographs). Indications: scaffolds without (No cells ) hMSCs, with hMSCs (MSC<sub>1.5</sub> \*, MSC<sub>7.5</sub> , MSC<sub>7.5</sub> + GF ) amount indicated by subscript (e.g.,  $7.5 = 7.5 \times 10^5$  cells per scaffold). hMSCs preconditioned with growth factor (+GF) 24h prior to implantation.

Similarly, another microscopic technique, scanning electron microscopy (SEM), is also highly compatible with the P3D Scaffolds. Indeed, the P3D Scaffolds do not require any chemical preparation for SEM; simply mount and cover the scaffolds using 10 nm gold coating. However, if cells are present within the macroporous structure of the scaffolds, follow the simplified process flow of optimal SEM sample preparation provided in Figure 14.

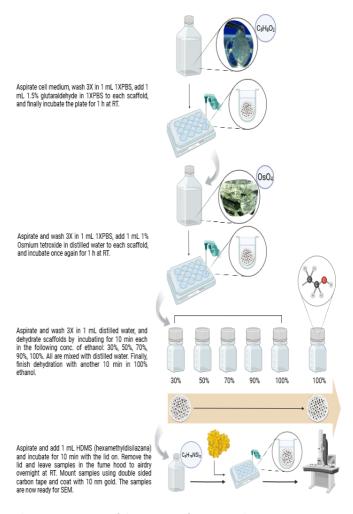


Figure 14: Schematic overview of the process of SEM sample preparation on P3D Scaffolds.

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View our scientific publications using SEM at <u>Ossiform: Scientific Publications</u>. Also, find our post investigating whether osteoblasts, cultured on the P3D Scaffolds, are able to produce collagen at <u>Ossiform: News From The Lab</u>. This investigation, performed by our in-house scientist, utilized SEM imaging to uncover the large amounts of collagen being produced within the scaffolds (Figure 15, left).



Figure 15: Representative SEM images showing collagen produced within P3D Scaffolds. (Left) ECM formed on a P3D Scaffold. (Right) Osteoblast produced collagen on a P3D Scaffold.

Additionally, Ossiform has recently compared our P3D Scaffolds to bone slices. Here, we established that the P3D Scaffolds provide an obvious alternative to traditional 2D cell culturing and hydrogel-based 3D cell culture systems, due to the scaffolds' physical resemblance with bone and ability to induce collagen production in osteoblasts (Figure 15, right). Find more information on the entire study at Ossiform: News From The Lab.

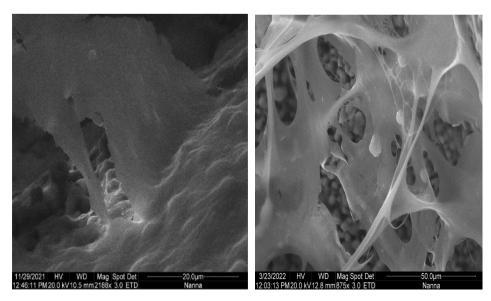


Figure 16: Collagen covering the porous surface of a P3D Scaffold. Cells are clearly distinguished within the formed collagen layer.



## SHORTENING THE GAP BETWEEN IN VITRO AND IN VIVO

The fabrication and development of *in vitro* tissue and organ models are paving the way for breakthroughs in regenerative medicine and drug screening. In recent years, several *in-vitro*-cell-based and organ/tissue models have been evaluated for their potential usage in drug development, pharmaceutical agents, chemicals, personal care, and cosmetics, together with other biomedical application areas. Indeed, in vitro testing models demonstrate a novel platform to provide markedly cheaper, more rapid, and precise, and more reliable test results compared to animal models. Historically, animal models have proven to be poor biomimics of the physiological environment and conditions of the human body, and subsequently generating predictive pathophysiological disease models. Thus, newer research in the field of regenerative medicine revolves around refining animal models with more effective in vitro testing models, in order to achieve a more rapid approval of available drugs and vaccines against menacing diseases.

*In vivo* models constitute the internal environment of a living organism. The results of *in vivo* studies are often considered more reliable and more relevant than those of *in vitro* studies.

Besides providing a platform for replacing animal testing, in vitro research also bestows numerous advantages over in vivo research, such as regulated control of chemical and physical environment, reduced cost, higher throughput, and predominantly, reduced animal testing. Conversely, in vitro testing harbors a substantial weakness in failing to effectively replicate the conditions of cells in an organism, such as the fact that isolated and cultivated primary cells strongly distinguish themselves from the corresponding cell type in an organism, henceforth limiting the value of in vitro data to predict in vivo behavior (Graudejus et al., 2018). In vitro models do not utilize living organisms as subjects. However, they are unable to capture the inherent complexity of organ/tissue systems and the internal environment of the human body. Hence, in vitro studies are typically followed by animal testing (in vivo testing). Aside from the ethical concerns over the use of animal testing, the remaining issue of transability persists due to the considerable physiological differences between humans and animals, drug absorption, distribution, excretion, and efficacy of a pharmaceutical agent may significantly differ and consequently induce unwanted adverse toxic side effects (Uttekar & Allarakha, 2021). Intriguingly, since the P3D Scaffolds do not contain any materials invasive to the body, the scaffolds resemble native tissue to a greater extent, thus providing a well-suited environment for in vivo animal trials as well as in vitro diagnostics.

#### Guidelines for animal studies using P3D Scaffolds

To date, there has been several opinion polls demonstrating the public desire for more accessible information on animal testing. In fact, according to a survey of six EU countries (UK, France, Germany, Italy, Sweden, and the Czech Republic) conducted by You GOV in 2009, prior to the establishment of <a href="Directive 2010/63">Directive 2010/63</a> on the protection of animals used for scientific purposes, an astounding 80% collectively that all information regarding animal testing should be publicly available. Conversely, prior to the creation of the Directive, only limited statistical information was obligated to be published periodically by member states and provided in three-yearly reports to the European Commission (Taylor et al., 2018).



Importantly, under the new Directive 2010/63/EU, member states must publish non-technical summaries (NTSs) of the projects involving animals that they claim to authorize. These summaries should demonstrate compliance with the 3Rs: a set of established guidelines for more ethical use of animals in scientific research.

## I. Replacement

State why animals must be used and why non-animal alternatives cannot be used

#### II. Reduction

Explain how the use of minimum numbers can be assured

#### III. Refinement

Explain the choice of species and why the animal model(s) used are the most refined, having regard for the scientific objectives. Explain the general measures to be taken to minimize welfare costs (harms) to the animals.

Figure 17: Definition of the 3Rs principle is acquired from Taylor et al., 2018.

Authorization is required for all establishments of animal testing. Authorization is only granted or renewed when establishments fulfil the requirements of the Directive. Aside from following the guidelines set in motion by the Directive, and demonstrating that the 3Rs have been considered, an outline of the number of animals that will be used and the level of suffering they are expected to undergo has to be provided through a project proposal, in the shape of a harm-benefit analysis by the competent authority in each EU Member State, which then decides whether to grant the license (European Animal Research Association).

The guidelines instigated by the European Commission and the new Directive 2010/63 are in direct compliance with the user instructions for using Ossiform's P3D Scaffolds in animal testing. Moreover, we recommend considering the following guidelines for successfully conducting animal testing with our 3D cell culture system.

Ossiform is a member of <u>The North American 3Rs Collaborative (Na3RSC)</u> as a provider of commercially available microphysiological system technology, having a core mission focused on microphysiological systems.

THE NORTH AMERICAN
3Rs COLLABORATIVE

The P3D Scaffolds constitute a relevant micro physiological system (MPS) which provide translational biochemical responses to represent organ and tissue function, with the potential to replace animal testing in research. Find more information on this feature <a href="here.">here.</a>



#### P3D Scaffolds in animal testing: Instructions of use

- Protocols must be performed by trained personnel (e.g., surgeons, animal keepers)
- Critical size defects (e.g., calvarial defects with a diameter of 5.0 mm could be considered as a critical size defect, Vajgel et al., 2013).
- Non-load bearing the implants must not bear any weight and should be stabilized in alternative ways.
- P3D Scaffold preparation the scaffolds are supplied sterile and therefore do not require further preparation procedures unless the scaffolds must be suited for specific requirements.
- Wound healing & recovery depends on the individual users' requirements of evaluating the state of the animal post-surgery. However, we strongly recommend using pain-relieving drugs and antibiotics.

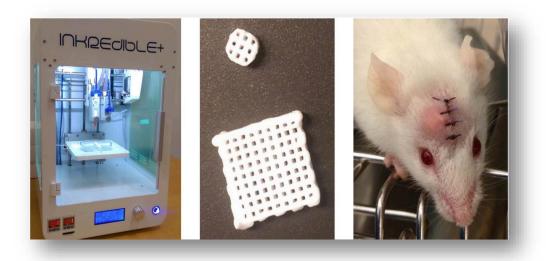


Figure 18: Use case: Treating mouse skull defects with 3D-printed fatty acid and tricalcium phosphate implants

The study shown in Figure 16, performed by Ossiform and collaborators from The University of Southern Denmark (SDU), investigated the performance of 3D printed bone implants in a mouse model of craniectomy with the aim of producing biodegradable porous implants that can be applied to a patient's anatomy.

The study concluded that the investigated implants could be seeded with hMSCs that engrafted, proliferated, and contributed to bone formation by differentiating into osteoblasts and osteocytes. Find the entire publication <a href="here">here</a>.

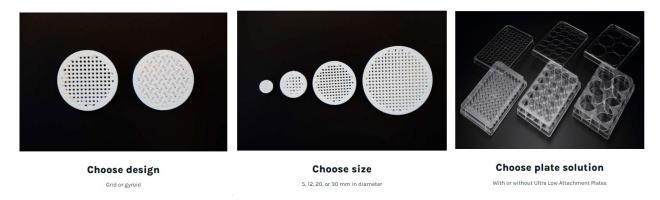


## GENERAL INFORMATION AND CONTACT

Ossiform Research Line offers you both bioceramic 3D printed cell culture systems, the P3D Scaffolds, and high quality Ultra Low Attachment Plates best suitable for the scaffolds.

## P3D Scaffolds solutions

Explore the P3D Scaffolds and full plate solutions.



Choose between the standard options shown above, or request for customizable features. Special forms, sizes and designs of the scaffolds can be requested for a fully customized set of cell culture systems.

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Get a non-committal quote today. Tell us about your research needs, and we will help find the best solution for you.



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