Project LaserImplant

D3.1 Images of osteoblasts on bone screws

Reporting period	from	**01.01.2021**	to	**31.12.2021**
Report completed and released		**11.04.2022** **Martina Muck, Johannes Heitz**		

1. Goals

The deliverable **D3.1** provides a collection of SEM images of osteoblasts on bone screw samples published on the **LaserImplant** web-site (www.laserimplant.eu).

2. Detailed Description

Introduction

JKU performs osteoblast cell experiments as follows:

Bone-forming cells (osteoblasts) of the commercially available human cell line SAOS-2 (provider DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) are used. The cells are cultivated in an established cell culture medium in an incubator with a water vapor saturated atmosphere with 5% CO_2 content at 37°C and are divided at a ratio of 1:10 once a week. The experiments are performed with typical culture times of about 3 weeks. After disinfection with ethanol, a set of samples is placed in a Petri dish with cells and culture medium, thus the samples are completely covered with liquid. After the chosen cultivation time in the incubator, the cells at the samples are fixated and dehydrated. In detail, the cells are initially fixed overnight with 6% glutardialdehyde (GA; Merck, Darmstadt, Germany) in phosphate-buffered saline (PBS) and subsequently dehydrated with the help of ascending ethanol series (30%, 40%, 50%, 50%. 80%, 90%, 96%, 3 x 100%) for 30 min each. The samples are repeatedly transferred 3 times into hexamethyl-disilazane (HMDS; Merck). After the overnight evaporation of HDMS, the samples are sputter-coated with gold and the cell density is evaluated by means of scanning electron microscope (SEM).

JKU

For the images in this deliverable **D3.1**, **JKU** uses a Yb-based femtosecond laser set-up (Spirit 1040-16 HE, Spectra Physics, wavelength λ = 1040 nm, pulse duration τ = 350 fs) to produce a laser beam that is guided through a system of five mirrors and focused by a lens (100 mm focus length) onto the sample stage, which can be moved linearly in two dimensions. The diameter of the focused laser beam is $2w_0$ = 75 µm ± 5 µm. To be able to produce conical structures with superimposed nanoripples (LIPSS), a parameter set (peak fluence F_0, scanning velocity v_{scan} and line distance ΔS) is determined beforehand based on parameters such as laser type (wavelength and pulse duration), focused beam diameter $2w_0$ and the desired laser frequency f.

For cylindrical Ti-based samples, a continuous rotation during laser structuring was chosen, which was tested for titanium cylinders of 8 mm diameter beforehand and was then adapted to other cylindrical samples such as bone screws and dental implants. A schematic of the spiral approach for area structuring on cylindrical sample is shown in Fig. 1. While rotating, the sample is moved along the y-axis with a velocity v_{scan} that leads to the desired line distance ΔS. Pulse repetition rate f and y-axis velocity can be chosen according to the desired rotation speed.

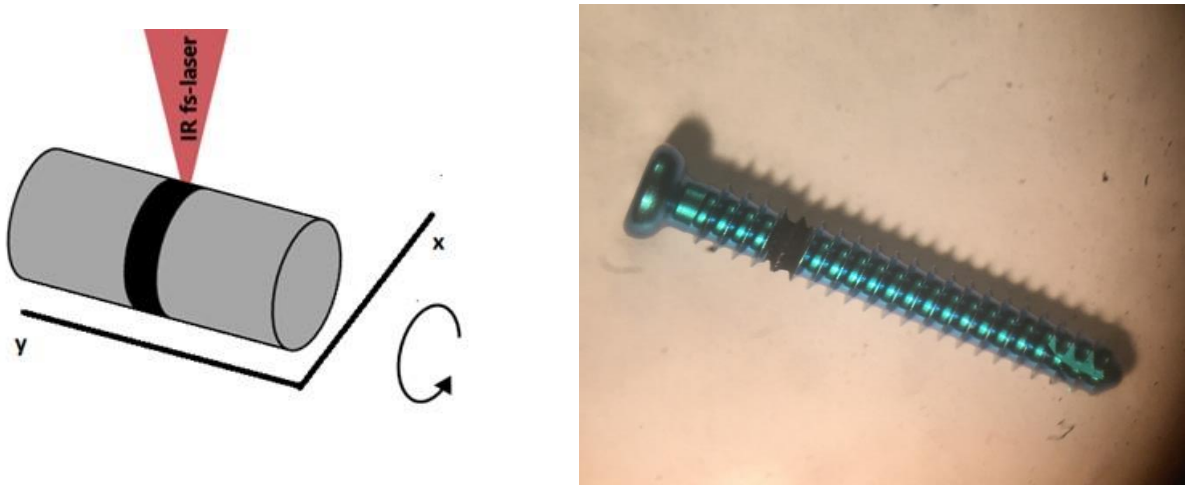

Fig. 1: The left image shows the schematics of continuous laser processing of cylindrical samples. The laser is focused onto the highest point of the cylindrical sample. For continuous laser processing, the sample is irradiated while being rotated and moved along the y-axis, leading to a spiral around the cylinder. The right image shows a bone-screw (length 17 mm, diameter 2.5 mm) with a laser-processed ring in black (the blue color of the screw results from the pre-anodization). Figure adapted from [1].

It was shown in [1], that laser treatment of pre-anodized bone screws leads to micro- and nano-sized structures that have a cell-repellent effect. On the unstructured areas of the screw, osteoblasts grow in dense confluent layers, which start to grow into the third dimension at the rim of the screw windings. Many laser-treated areas remain cell-free and the laser-induced structures become visible. At some areas the cells tend to grow together instead of adhering to the surface underneath, which is also an indication for the cell-repellent effect of the laser-treated surface. Figure 2 demonstrates the cell-repellent effect of the laser-induced structures on the osteoblasts.

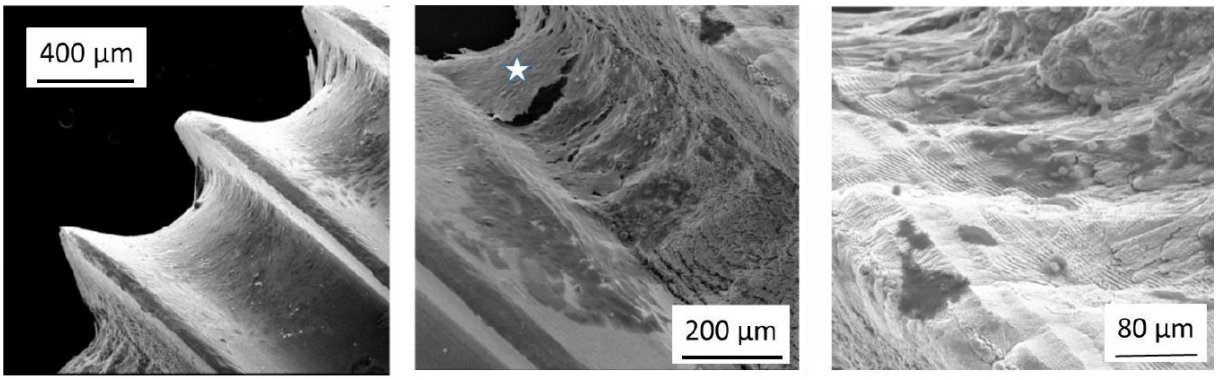

Fig. 2: SEM images of pre-anodized Ti-6Al-4V-ELI bone screw after 3 weeks in osteoblast cell culture. The left image shows the unstructured bone screw surface, where the osteoblasts grow in dense confluent multilayers and start to grow into the third dimension at the rim of the screw windings. The middle image shows the laser-induced structures between two windings. The cells tend to grow together and loose contact to the surface underneath (position marked with star). The right image shows a higher magnification of only a few cells left at the windings, where nano-sized ripples become visible. Figure taken from [1].

For the new osteoblast cell tests, Ti-6Al-4V-ELI bone screws with gold and blue pre-anodization were ordered from **HOFER** (length 30/26 mm, diameter 2.5 mm) (Fig. 2). Two gold and two blue bone screws were structured with two different peak fluence values F_0 = 0.935 J/cm^2 and 1.2 J/cm^2 (4 screws in total with five rings each (Fig. 3)). The remaining parameters were chosen according to the tests on titanium cylinders: repetition rate f = 33 kHz, rotation speed v_{rot} = 2500 ms/round trip, v_{scan} = 8 µm/s to achieve a line separation ΔS = 20 µm. The achieved hierarchical structures on a bone screw for a peak fluence F_0 = 0.935 J/cm^2 are shown in Fig. 4.

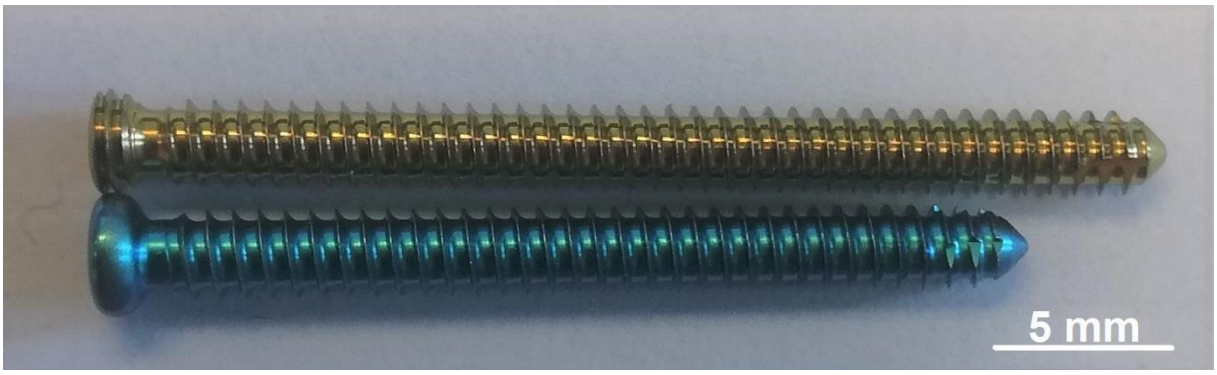

Fig. 3: Image of Ti-6Al-4V-ELI bone screws from **HOFER** with gold (top) and blue (bottom) pre-anodization.

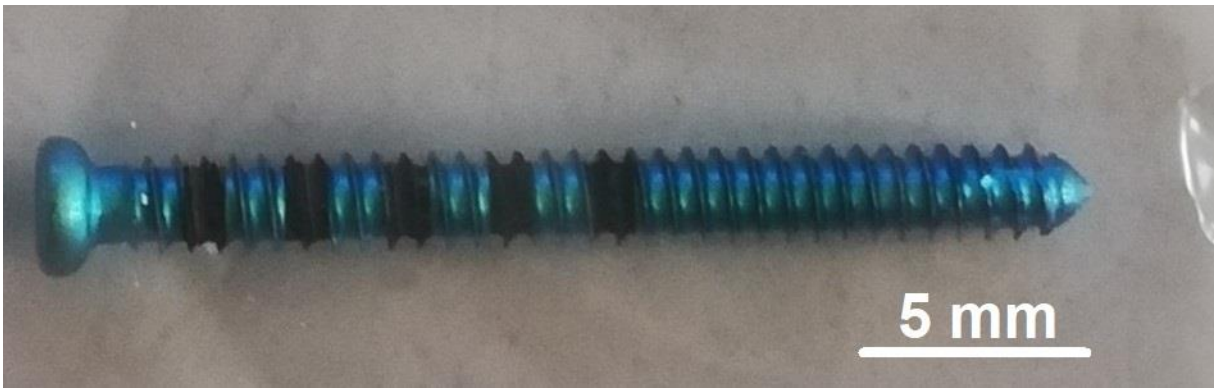

Fig. 4: Image of structured Ti-6Al-4V-ELI bone screw with blue pre-anodization. The five processed rings are shown in black.

Though the structures on the screws are irregular, which results from partial blocking/reflection of the laser beam on the windings, most of the irradiated surface is covered by a surface roughness in the micro- and nano-size range. On one side of the windings ripple patterns can be found and on the top of the windings micro-sized cones and nanosized ripples are induced by laser irradiation. The other side of the winding is tilted in a way that the laser irradiation is blocked, leading to almost no structure formation (Fig. 5). The structures look similar to the ones that can be seen in Fig. 2.

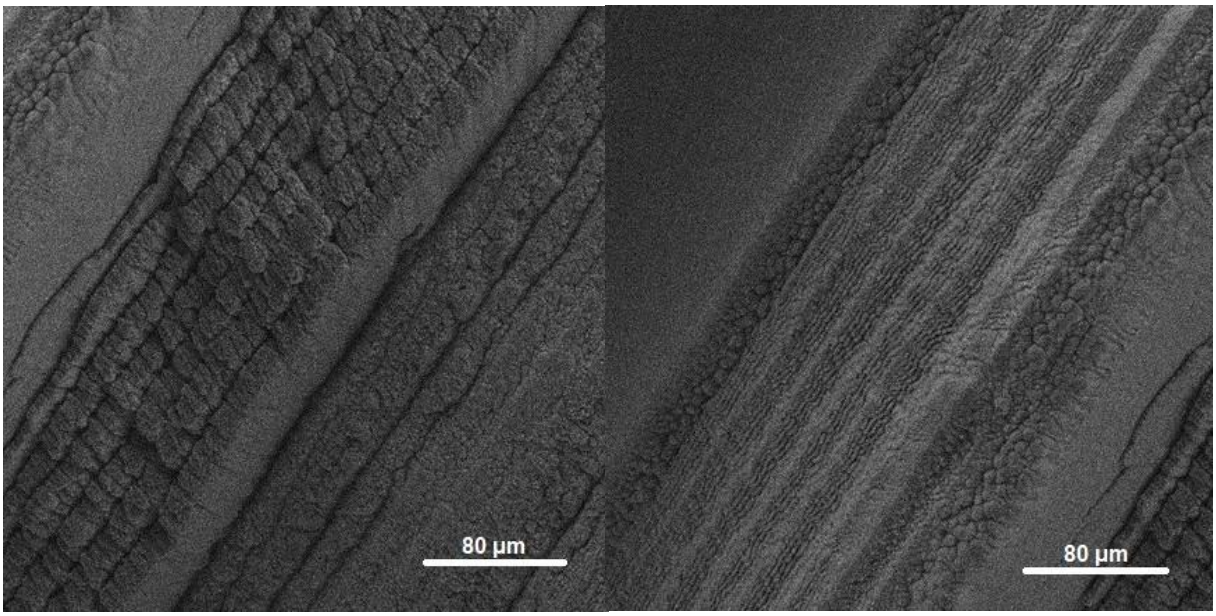

Fig. 5: SEM images of laser-structured ring on Ti-6Al-4V-ELI bone screw. On the left image micro- and nano-sized structures are shown. The irregularities result from partial blocking/reflection of laser light at the windings. On the right side the ripples on one side of the winding are shown. The laser arrives at the top of the windings, inducing both micro-sized cones and nanoripples. The other side of the winding is tilted in way that the laser light is partially blocked, leading to no structure formation.

These four screws were then put into cell culture for three weeks as described above in the introduction. After cell growth the screws were investigated under the SEM. Figure 6 shows the osteoblasts on the bone screw with blue pre-anodization. The overview of the bone screw shown in Fig. 6 (top-left image) indicates that the osteoblasts grow in dense multilayers on the unstructured areas, while on the laser-processed rings only a few osteoblasts can be found. The top-right and the left-bottom image of Fig. 6 show a higher magnification of the osteoblasts on the laser-processed rings. The laser-induced micro- and nanostructures can be seen, where only a few of them are covered with osteoblasts. The bottom-right image of Fig. 6 shows the unstructured ring surface with dense multilayers of osteoblasts and extracellular matrix, which indicates high activity of cell growth, and several osteoblast cell clusters.

As intended, reduced osteoblast cell growth was achieved by using laser-processed rings on Ti-6Al-4V-ELI bone screws with blue pre-anodization. The osteoblasts on bone screws with a golden pre-anodization also show reduced osteoblast cell growth, although not as efficient as the ones with blue pre-anodization (Fig. 7). This can be mainly described by the reduced oxide layer thickness for golden-colored screws. The laser-processing of the oxide layer for blue screws, which has a thickness in the range of 200 nm, shows a more effective reduction of osteoblast cell growth, which is an effect resulting both from surface roughness and surface chemistry.

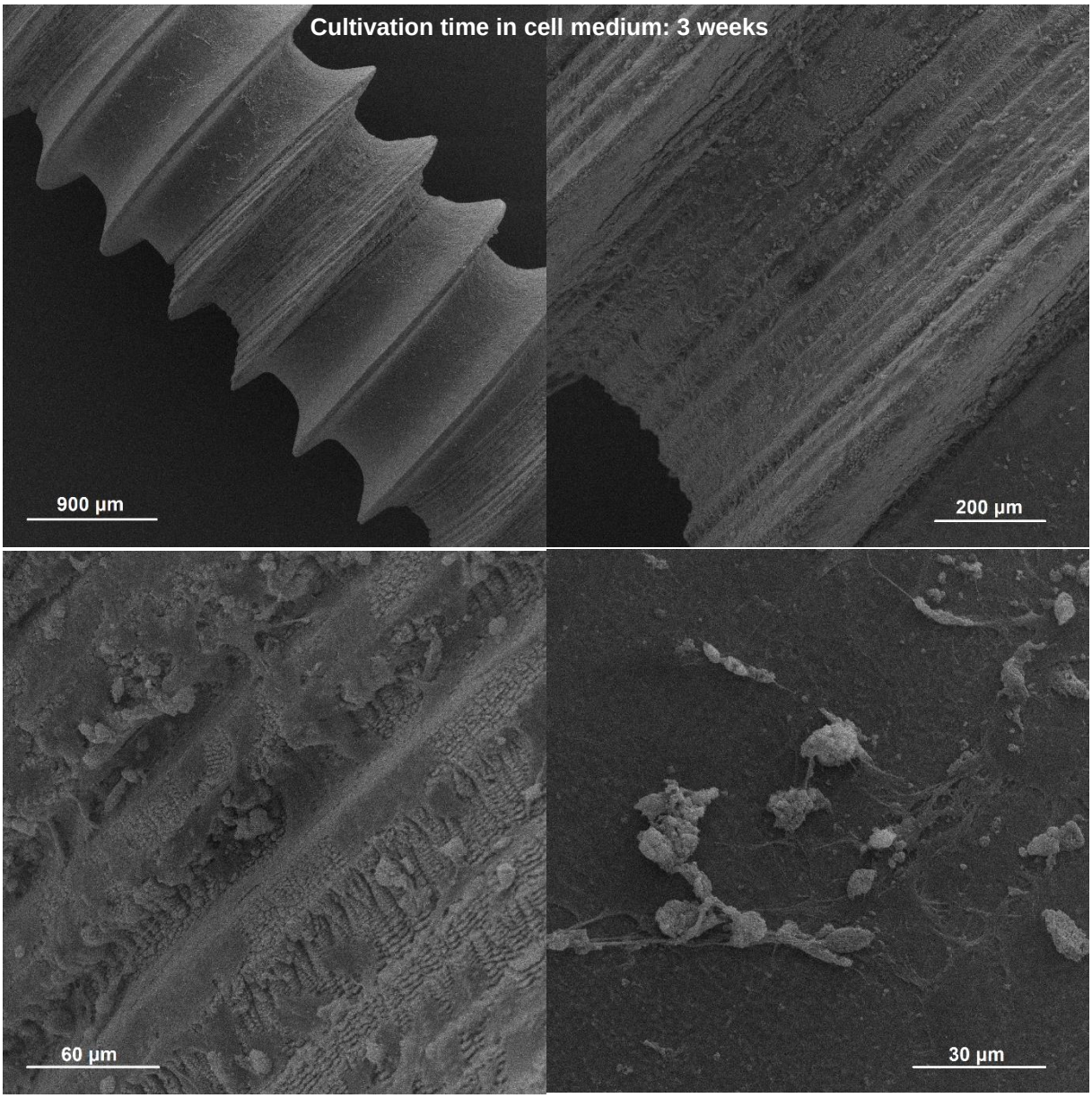

Fig. 6: SEM images of osteoblasts on Ti-6Al-4V-ELI bone screw with blue pre-anodization. The cultivation time in the SAOS-2 cell line was 21 days. The top-left image shows an overview of the bone screw with osteoblast cell growth. One of the laser-processed rings is located in the middle. While the ring itself is only covered with a few cells, the unstructured areas surrounding the ring show dense confluent multilayer cell growth. The top-right and bottom-left image show a higher magnification of the osteoblasts on the laser-processed ring, where it is clear that only a few cells cover the structures and most of micro- and nanostructures can be determined easily. The bottom-right image shows dense multilayers of osteoblasts and extracellular matrix, which indicates high activity of cell growth. A few osteoblast cell clusters can be seen on top of the layers.

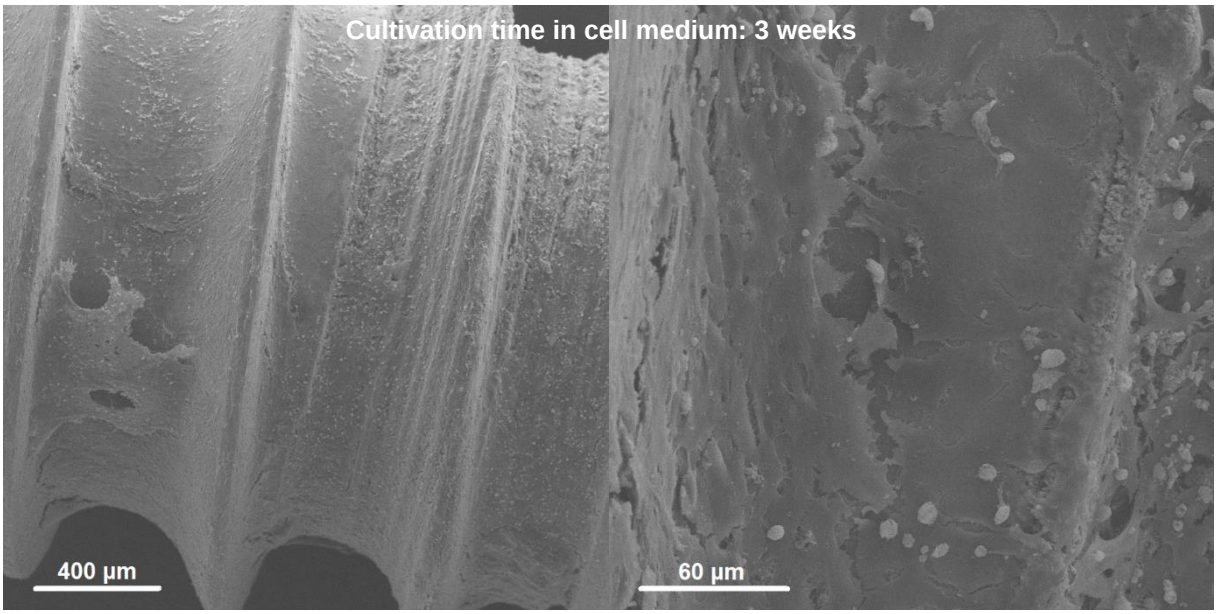

Fig. 7: SEM images of osteoblasts on Ti-6Al-4V-ELI bone screw with gold pre-anodization. The cultivation time in the SAOS-2 cell line was 21 days. The left image shows an overview of the unstructured and laser-processed areas on the screw. The osteoblasts grow in dense confluent multilayers on the unstructured areas and also grow into the third dimension at some places between the windings. The right image shows a magnification of the rim between the unstructured and laser-processed area. While the unstructured area is densely covered by the cells, the structured area to the right side of the image shows a cell-repellent effect, though not as effective as the cell-repellent effect seen on the screw with blue pre-anodization in Fig. 6.

References

[1] M. Muck, B. Wolfsjäger, K. Seibert, C. Maier, S. Ali Lone, A.W. Hassel, W. Baumgartner, J. Heitz: *„Femtosecond Laser-Processing of Pre-Anodized Ti-Based Bone Implants for Cell-Repellent Functionalization“*, Nanomaterials **11** (2021), 1342. https://doi.org/10.3390/nano11051342

3. Evaluation of Goals and Resulting Actions

The deliverable **D3.1 Images of osteoblasts on bone screws** was finalized in time by m9. A link to this report will be implemented into the Dissemination the **LaserImplant** web-site (www.laserimplant.eu).

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