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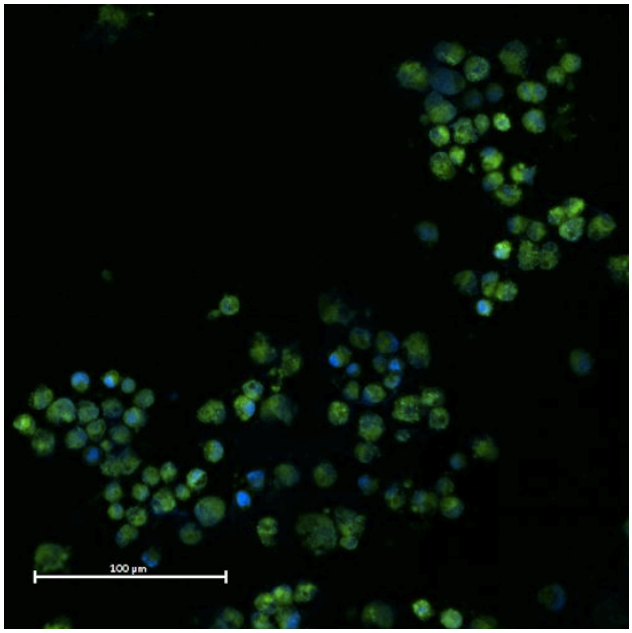

Effects on Chondrocyte Derived Induced Pluripotent Stem Cells Adhered to Nano Gradients Functionalized with Transforming Growth Factor Beta-1, -3 and Growth Differentiation Factor 5

Master Thesis in Biotechnology

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Department of Physics
CHALMERS UNIVERSITY OF TECHNOLOGY
Gothenburg, Sweden 2018

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Master Thesis, June 2018
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Cover: Aggrecan expression visualized in chondrocyte derived induced pluripotent stem cells adhered on a nano gradient functionalized with TGFβ-3 using fluorescence imaging at 40X magnification. Green fluorescent protein (aggrecan expression) is visualized using a FITC filter and cell nuclei are visualized using DAPI. Scale bar 100 μm.

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The Master thesis covers 30 credits and has been executed by Linnea Andreasson during the period January 2018 until June 2018. It has been executed at Cline Scientific AB, Gothenburg, which produces nanoparticle surfaces and gradients for mainly stem cell research and cancer diagnostics, and at Clinical Chemistry, Sahlgrenska University Hospital. The Master thesis is included in the program plan for the Biotechnology master program, 120 credits, Chalmers University of Technology.

I would like to send a big thank you to Hanne, Niklas and Patrik at Cline Scientific for having me and giving me the opportunity to perform this work at the company. A special thank will I send to Hanne Evenbratt, my supervisor at Cline Scientific, for all support and guidance along the way. I would also like to send a big thank you to my supervisor Stina Simonsson at Clinical Chemistry Sahlgrenska University hospital. Also, thank you, Camilla Brantsing and Josefine Ekholm for your time and experimental guidance and to Sandra Johansson, Histocenter, for taking care of my samples.

Linnea Andreasson, Gothenburg, June 2018

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Abstract

Osteoarthritis (OA) is the most common joint disease, causing pain for the patient due to cartilage degeneration and a lot of costs to the society. Due to lack of knowledge regarding disease mechanisms, there is no existing drug based modifying therapy. In this project, the impact of TGFβ-1, TGFβ-3 and GDF5 signaling on the expression of aggrecan in chondrocyte-derived iPSCs, were elucidated by using a concentration gradient to gain information regarding OA disease mechanisms. The overall aim of the project was to use gold nanoparticle gradients and surfaces to optimize formation of cartilage producing cells from iPSCs where the focus was to induce a high aggrecan expression in the cells. Also, cells were stimulated on gradients and surfaces and then removed from the surfaces and formed to a 3D structure for further differentiation and visualization of the components. Particle gradients were produced by attaching gold nanoparticles to a glass surface with different spacing between particles and the gradients were then functionalized with signaling molecules and seeded with c-iPS cells, differentiated for 5 days and thereafter visualized using fluorescence imaging. Due to the protein concentration gradients, cells were stimulated differently over the gradient and by visualization of the cell behavior at the gradient, an area of interest was observed and new uniform surfaces were produced.

No specific aggrecan expression was observed on surfaces differentiated for 5 days, especially not for GDF5 and TGFβ-1. TGFβ-3 indicated an elevated expression at high concentrations. Despite this result, 5 days of differentiation was found to be insufficient time and this result was also confirmed by the study were iPSCs were further differentiated for 6 weeks after removal from surfaces where there was a significant elevation of the aggrecan expression after 3 weeks of differentiation and it is suggested that the time should be prolonged in order to yield high aggrecan expressions. Even though the aim of the project to stimulate the cells to express a high expression of aggrecan was not fully fulfilled, other reactions of interest were observed on the surfaces. GDF5 stimulate cells tended to form a condensed structure where cells clustered together. In low concentrations of TGFβ-1, the effect in cells was indicated to be knocked out by laminin and higher concentrations were indicated to be sufficient for a cellular response of TGFβ-1. For cells on TGFβ-3 surfaces, aggrecan expression was indicated to be elevated in high TGFβ-3 concentrations. It can be concluded that the nanoparticle coated surfaces are effective substrates to use to study the effect of signaling molecules on cells and to optimize the differentiation process in stem cells.

Keywords: Osteoarthritis, cartilage, Aggrecan, Transforming Growth Factor β-1, Transforming Growth Factor β-3, Growth Differentiation Factor 5, nano gradient

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Introduction

Disease model

Osteoarthritis (OA) is the most common joint disease and causes a lot of suffering for the affected as wells as major economic costs for the society every year. OA causes irreversible degradation of cartilage which induces both pain and joint dysfunction for the patient [1]. Primarily, catabolic cytokines are induced to signal for degradation of matrix proteins that are important for cartilage structure [2]. Current treatments of the disease are focused on pain reducing operations or entire joint replacements [3]. Due to lack of knowledge regarding disease mechanisms, there is no existing drug-based disease-modifying therapy and no specific treatment to halt the degradation of cartilage. A high potential cell source for revealing disease mechanisms of OA is human induced pluripotent stem cells (iPSCs). Borestrom et al. have shown that autologous chondrocytes can be reprogrammed into iPSCs using a footprint-free method based on mRNA delivery with the possibility to differentiate the cells back into chondrocytes [4]. Signaling molecules that dependent on concentrations induce different cellular responses are called morphogens. Molecules such as transforming growth factor beta 1- and 3, (TGFβ-1, TGFβ-3) and growth differentiation factor 5 (GDF5) are thought to be concentration dependent proteins that play an important role in fundamental pathways in early development, in the signaling pathway of chondrocytes, and in the mechanism of OA [4].

This project will involve studies of the concentration dependence of TGFβ-1, TGFβ-3 and GDF5 on aggrecan expression in chondrocyte-derived iPS cells (c-iPSCs). Aggrecan is a major cartilage-specific proteoglycan that is an important component for the matrix structure of cartilage [5]. Aggrecan gene (ACAN) expression is consequently highly relevant for the formation of aggrecan which in turn is essential for the structure and function of cartilage [6].

Nanotechnology as an enabler in Life Science

Nanotechnology is a major reason for many revolutionary changes in life science. Drug delivery, biomaterials, medical materials, food, and diagnostics are important fields where nanotechnology has been of importance and still is, in ongoing research and development with great potential. Today, nanotechnology has been of importance within cell biology research where nanotechnology-based platforms are used for studies in e.g. controlled cell culturing, intracellular signaling, drug delivery and for monitoring complex cellular environment [7].

Nano gradients within stem cell therapy development

Nanoparticle gradients and surfaces provide a possibility to mimic tissue functions at the molecular level for *in vitro* applications, primarily aimed for stem cell research and cancer diagnostics. Stem cell research for therapeutic applications, such as tissue regeneration, require defined cell culture protocols to be able to control the cells for differentiation,

proliferation but also to maintain cell properties and to mimic *in vivo* conditions at the same time. Stem cells cultures requires a high cell quality and a homogeneous cell population for differentiation and traditionally 2D cultures tend to get a limited expansion and differentiation capacity [8]. Nano gradients and surfaces produced by Cline Scientific's manufacturing protocols provides a chemically defined 2D surface for controlled stem cell culture system for homogenous cell populations with a high reproducibility capacity.

Cline Scientific's surfaces are produced by attachment of gold nanoparticles on a plain glass surface. The surfaces are produced as a gradient which increases in the concentration of nanoparticles over the surface, illustrated in figure 1. The gold nanoparticles can be functionalized for a specific purpose with different biomolecules such as e.g. drugs and proteins. After biomolecules are attached to the nano gradient, it is possible to culture cells on the gradient where the cells will be stimulated differently by the concentration gradient of biomolecules, making it possible to observe where on the gradient the cells grow best, illustrated in figure 2.

Figure 1. *Illustration of a Cline Scientific gradient. The purple color is indicating the gold nanoparticles coated on a glass surface. There is an increase of particles over the surface, indicating the gradient. To the left in the illustration, particles are attached with a larger spacing and to the right, the particle spacing is smaller.*

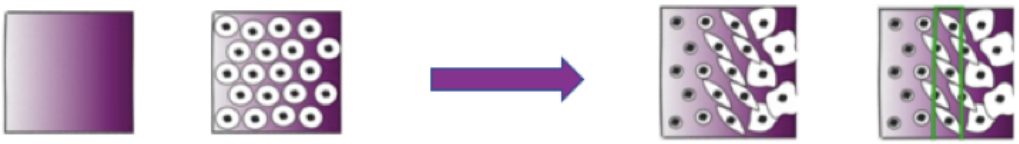


Figure 2*. Illustration of how cells are seeded on a Cline Scientific nano gradient. Cells are stimulated differently from biomolecules due to the concentration gradient and an optimal surface composition for further research can be determined.*

New surfaces with uniform nanoparticle spacing can be manufactured when an area of interest is detected on the gradient surface. The nano surfaces produced, based on the chosen particle density, can be functionalized to achieve optimal conditions for further high-quality cell cultivation. Important for the surfaces is the ability to bind any biomolecule and mimic nature's own way of controlling cell growth and cell migration. The surfaces are, as already mentioned, an implement in developing a precise and controlled stem cell cultivation and a possible step in developing treatments to serious diseases such as OA, Alzheimer's disease, Parkinson's disease and Diabetes type 1. Cline Scientific's nanoparticle surfaces are also suitable for cancer research and drug testing.

This nanotechnology-based method will be used in this project to enable studies of the concentration dependence of TGFβ-1, TGFβ-3 and GDF5 to try to induce a high production of the extracellular matrix (ECM) molecule aggrecan in c-iPSCs and to optimize the production of a homogenous cell population.

Aim

The aim of the project was to follow the effect of different concentrations of proteins (TGFβ-1, TGFβ-3, and GDF5) on cells adhered to a nano gradients and in 3D structures. The cells were genetically engineered to express green fluorescent protein (GFP) to visualize aggrecan expression. The intended outcome of the project was to try to induce a high aggrecan expression in iPS cells and to generate important information of aggrecan expressions dependent on different concentrations of proteins to try to reveal lacking disease mechanisms of osteoarthritis. It is known that TGFβ-1, TGFβ-3, and GDF5 plays an important role in development and structure of cartilage [9] [10]. Previous studies have also indicated the relevance of TGFβ and GDF5 in the production of aggrecan and other ECM proteins in chondrocytes [11] [12] [13]. Additionally, in order to show that there are proteins bound to the surface, though known chemical binding methods used to ensure it, an antibody staining approach will be undertaken within this work.

Approach

To be able to fulfill the aim, nano gradients were produced and functionalized with TGFβ-1, TGFβ-3, and GDF5 separately to study the concentration dependence to induce a high aggrecan expression in cells. Between the protein functionalized particles, laminin 521 was attached to promote adhesion of cells to the surface. After functionalization of gradients, cells were seeded on gradients and differentiated for five days. Thereafter, an interesting area on gradients was observed and determined using fluorescence imaging, and new uniform surfaces were produced with an optimal particle density for further culturing for a controlled and homogeneous cell population with a specific protein stimulus for a high cell quality.

In order to take the procedure of using nano gradients and surfaces one step further, cells (chondrocytes and iPS cells) were first seeded and cultured on surfaces for a specific protein

stimuli and then removed from the surfaces to form a 3D structure for further differentiation, also with the purpose to induce a high aggrecan expression. This part of the project was involving the use of both chondrocytes and iPS cells to form 3D structures (pellets and organoids, respectively) from gradients and uniform surfaces. Chondrocyte pellets were obtained to visualize the pellet's ECM composition using Alcian blue van Gieson and iPS cell organoids were obtained to visualize the aggrecan expression over six weeks using fluorescence imaging.

Additionally, two immunoassays were performed on the gradients were the first immunoassay was involving protein visualization using Diaminobenzidine (DAB) and the second immunoassay was involving a fluorophore for protein visualization.

Theory

Firstly, the structure and function of cartilage will be described as well as the three signaling molecules studied in the project. Secondly, methods and approaches used in the project will be presented.

Cartilage

Cartilage is a part of the connective tissue with the main purpose to maintain structure and for protection of the body and organs. Cartilage is both an avascular tissue without nerves and has a limited ability of self-renewal [14] [15]. It consists primarily of collagen fibers, proteoglycans, glycoproteins, hyaluronan and various elastic fibers which forms a dense ECM network, and chondrocytes, the specialized cell type within cartilage [16] [17]. The chondrocytes in adult cartilage are non-proliferating cells and the population of chondrocytes is relatively sparse with the focus to regulate synthesis and degradation of ECM [18]. Therefore, when a damage occurs in cartilage, the ability of regeneration is limited. Chondrocyte-derived iPSCs are thought to be an important cell source for regeneration of cartilage since the cells are reprogrammed into iPS cells and therefore have the ability to differentiate and proliferate. Important for differentiation into chondrocytes, proliferation and ECM production processes are growth factors such as TGFβ-1, TGFβ-3 and GDF5 [19].

Aggrecan

Aggrecan is an ECM protein produced by chondrocytes and encoded by the ACAN gene [20]. Aggrecan is the most abundant proteoglycan in cartilage and an important protein for structure and cartilage function [21]. Due to its linkage to hyaluronan, aggrecan provides a hydrated gel structure important for the structure of ECM. It is also important for chondroskeletal morphogenesis [17]. Proteoglycans, in general, are heavily glycosylated and consist of a core protein covalently attached to approximately 100-150 sulfated glycosaminoglycan (GAG) chains [6]. Aggrecan synthesis and degradation is regulated and

therefore not constant through life and the degradation of aggrecan is directly linked to cartilage erosion and diseases such as OA [6].

The cells that will be used in this project is genetically engineered to express GFP which is illustrated in figure 3. A GFP promoting sequence has been introduced right after the first aggrecan allele. Thereby, when an aggrecan is transcribed, a GFP will be directly transcribed as well, and therefore, when GFP is visualized in IN CELL microscope, using FITC filter to visualize aggrecan expression, it will be directly proportional to the amount of aggrecan present in the cells.

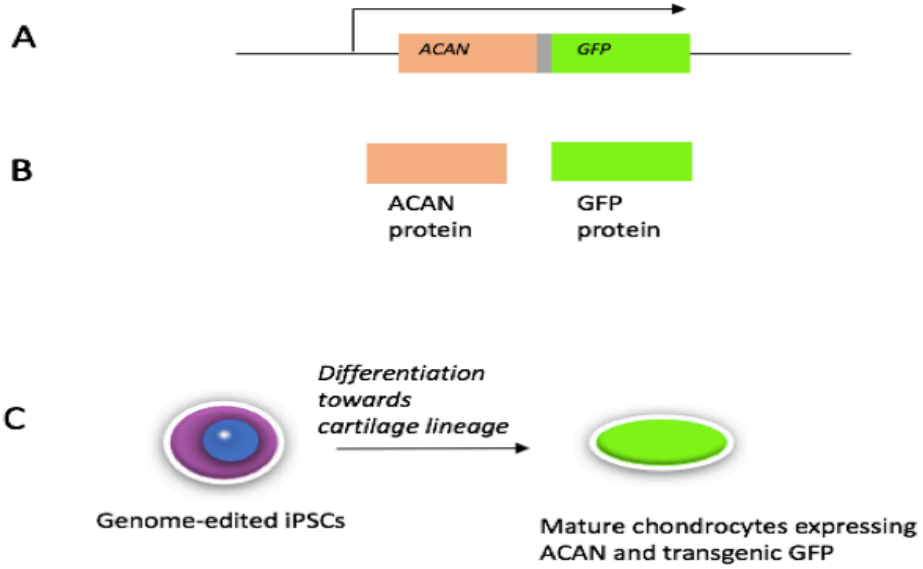


Figure 3. *Illustration of how the iPSCs are genetically engineered to express GFP.*

Transforming growth factor β

TGFβ is a group of cytokines belonging to the transforming growth factor superfamily of proteins with over 35 protein members where different members are distinguished due to the binding to different receptors on the cell surface to mediate a signal transduction. All members have a similar signaling cascade, that in combination with similar key molecules form a highly interconnected signaling network [22]. However, TGFβ signals through two different pathways, using the intracellular protein Smad or using a Smad independent pathway via ligand binding to two type II receptors that together with two type I receptors forms a heterotetrameric complex [23]. In mammals, three types of TGFβ are found; TGFβ-1, TGFβ-2 and TGFβ-3 [2]. TGFβ-1 and TGFβ-3 have shown to be important in early development and regulating a diverse number of cellular processes such as cell proliferation, differentiation, migration, apoptosis and has also been shown to be involved in regulation of

immune cells during e.g. inflammations [15] [24] [25] [26]. Van der Kraan et al. claims that alterations in TGFβ signaling cause changes in chondrocyte differentiation and OA development where an age-related decrease in signaling might be related to a loss of matrix proteins such as aggrecan and collagen type II [2].

Growth differentiation factor 5

GDF5, Growth differentiation factor 5, is a signaling molecule and a member of the BMP family and also included in the TGFβ superfamily. GDF5 is controlling chondrogenic cell growth and differentiation and is involved in both bone and cartilage formation by its regulation of cartilage anabolic genes such as COL2A1 and ACAN that encodes for collagen type II and aggrecan [27]. GDF5 is also found to be expressed in joint formation regions during early development. Mutations which leads to a decrease in GDF5 is suggested to be involved in diseases such as OA [28] [29] and previous studies indicate that an increased expression of GFD5 or an enhancement of its downstream signaling would possibly be an important key for preventing OA [27] [29].

Gold nanoparticles

For more than a decade, gold nanoparticles have been explored within biological research. High biocompatibility and unique optical properties are important characteristics which makes gold nanoparticles interesting for research [30]. Its chemical properties, such as no reaction with water, air, oxygen, hydrogen, nitrogen and organic acids during normal conditions are important qualities. But above all, gold nanoparticles have shown to be non-toxic for organisms and living cells [30] [31]. Gold nanoparticles are used in the production of Cline Scientific's nano gradients and surfaces. Via thiol bindings, it is relatively easy to attach biomolecules to the surface of gold nanoparticles, which will be utilized to functionalize gold nanoparticles on a surface in order to bind other biomolecules such as proteins. To easily functionalize a surface distributed with gold nanoparticles, biotinylation can be used for molecules with the purpose to attach the surface with strong interactions [32].

Biotinylation

To be able to functionalize the nanoparticles with proteins, the proteins will be biotinylated. The main reason to biotinylate proteins is due to biotins strong affinity to Streptavidin, which will create a strong covalent link between the proteins and Streptavidin. Thiolated Streptavidins will be used in a first step to functionalize the surfaces by binding Streptavidin to the gold nanoparticles since they are molecules modified with thiol groups, that easily binds to a gold nanoparticle which is illustrated in figure 4.

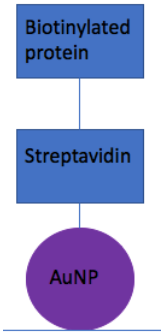


***Figure 4**. Illustration of a gold nanoparticle attached to a glass surface. A thiolated Streptavidin will be the linker to the biotinylated protein since biotin and Streptavidin has a strong affinity with each other.*

Since Avidins, in general, are molecules that allow easy purifications and detections of proteins and with immobilization properties that make proteins suitable for e.g. cell studies. Biotinylation of proteins, antibodies and other primary-amine containing molecules is a common requirement of immunoassay systems and the major reason is the strong interactions with Avidin, Neutravidin and Streptavidin [33]. N- Hydroxysuccinimide (NHS) esters of biotin is one of the most used reagents in biotinylation. The biotins are NHS-activated and react therefore primary in the side chain of the amines at lysine residues and the N-terminus of polypeptides [34] [35]. Because of the small size of biotin, a large range of proteins can be biotinylated and it does not significantly alter the biological activity [32]. By attaching biotin to the proteins, via biotinylation, it is possible to link the specific protein to Streptavidin and thereby create a functionalized surface.

Laminin

Laminin will be coated between the gold nanoparticles on the glass surface in order to promote adhesion of cells to the surfaces. Laminin is a large cross-shaped glycoprotein, with three different subunits, of the basal lamina which are sheet-like structures of the ECM. Laminins are important for physiological functions such as to maintain tissue structures and stability and to organize cells during embryonic development and organogenesis [36]. Besides the physiological properties, laminins are involved in functions such as cell differentiation, migration, and survival [37]. Laminins have a great ability to form self-assembled networks and adhere cells through interactions via receptors on the cell surface, where one of the important receptors is the Integrin receptor. They also have a great ability to bind other matrix proteins due to multiple binding sites [38]. There are many different types of laminins but in this project, Laminin 521 will be used since it is well suitable to use for iPS cells, for its cell adherent properties and capacity to drive cell proliferation of iPS cells [39].

Immunoassays for visualization of proteins on a functionalized gradient

Gradients and surfaces can be functionalized by using well-known chemical bindings. But due to the very low protein concentrations bound to the surfaces, it is hard to quantify the exact amount of proteins bound. Previous studies have been performed to try to determine

concentrations of proteins attached to gradients/surfaces but insufficient detection limits make quantification a problem. Also approaches such as Quartz Crystal Microbalance with Dissipation monitoring (QCM-D) and imaging Surface Plasmon Resonance (iSPR) has been used to successfully quantify proteins on gold nanoparticles on gold substrates but since the gold nanoparticles in this project is used on glass substrates, these methods is not working.

Two immunoassay coloring methods will, therefore, be performed to try to visualize protein binding to the gradients. In the first antibody staining procedure, Diaminobenzidine (DAB) will be used for visualization. A protein-specific primary antibody will be used and then a specific biotinylated secondary antibody will be incubated, seen in figure 5. After the attachment of the biotinylated secondary antibody, a highly sensitive streptavidin-horseradish peroxidase-conjugated complex (HSS-HRP) will be added and incubated to reaction completion. Visualization of antibody staining using DAB is based on an enzymatic conversion of DAB by horseradish peroxidase (HRP) which in turn will yield a brown colored precipitation where antibodies are located on the surface. Using a biotinylated secondary antibody will increase the sensitivity through the HSS-HRP complex where streptavidin will facilitate amplification due to its "four-armed" structure where to horseradish peroxidase is bound. When DAB is incubated on top of the molecule chain, an amplified signal is possible were an antibody is located.

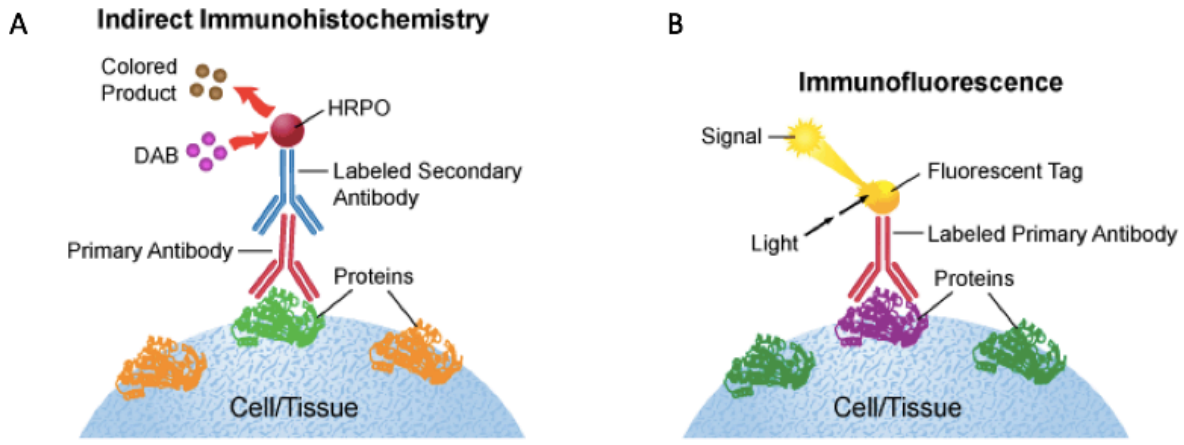


Figure 5. *A) An illustration of the antibody staining using DAB for visualization. A protein-specific primary antibody binds to the protein at the surface. When DAB is incubated on top of the surface it will be oxidized by HRP which in turn will yield a brownish precipitation. B) An illustration of the antibody staining using a fluorophore for visualization. A protein-specific primary antibody binds to the protein at the surface and allows biding of a secondary antibody labeled with a fluorophore [40].*

In the second antibody labeling procedure, the fluorophore Alexa Fluor 546 will be used. By attaching a specific primary antibody and then a secondary antibody with a fluorophore to the proteins on the gradient it might be possible to visualize the proteins using a fluorescent signal, illustrated in figure 6. The fluorophore will absorb light in a specific wavelength, and will be transferred from its ground state into a higher energy level and will then emit light in a longer wavelength, making it possible to detect it using a fluorescence s microscope [41]. Alexa Fluor 546 has an excitation maximum at 556 nm and an emission maximum at 573 nm [42].

Induced pluripotent stem cells

Induced pluripotent stem cells (iPSCs) are derived from somatic cells by the expression of different transcription factors and have the capacity of turning into cell types within all the three germ layers (ectoderm mesoderm and endoderm), called pluripotency.

Borestrom et. al. are describing how previous differentiation protocols have indicated that the matrix-forming capacity has been limited and a challenge for pluripotent human embryonic stem cells and human mesenchymal stem cells have tended to form hypertrophic cartilage and bone. Borestrom et.al. [4] have shown that chondrocytes can be used to efficiently be reprogrammed *in vitro* into footprint-free cartilage matrix-producing iPSCs using a method based on mRNA delivery with no genome-sequence modifications. The results from the method used are suggesting that the RNA-based technology is eliminating risks of integrations or aberrations of the genome, which is an important step in the procedure to find a potential cell source for regenerative medicine such as treatment of cartilage defects and OA. The mRNA reprogramming was performed (with some minor modifications) using the Stemgent mRNA Reprogramming Kit according to the manufacturer's protocol. The Oct4 variant used in the protocol was Oct4A, which is the isoform responsible for the pluripotency properties of embryonic stem cells. The cells were then differentiated into a 3D structure *in vitro* [4].

The cells that will be used in this project are c-iPSCs derived using the protocol mentioned above.

Removal of cells from gradients/surfaces for further cell culturing in a 3D structure

It is possible to use Cline Scientific's nano gradients and surfaces to seed cells, culture, then remove the cells from the surfaces and form a 3D structure for further culturing and differentiation. Concentration gradients occurs naturally in the body and the protein functionalized gradient surface might drive the cells to start a reaction or a response, as it also works in the body. The use of a uniform surface, on the other hand, will provide a uniform and a homogeneous cell population. The main reason to centrifuge the cells to form a 3D structure is to mimic the composition and organization of cells occurred in early development of limbs to form a miniature version of a knee where some key features of the representing

organ is involved. A high cell density will also contribute to the intercellular signaling between cells which is an essential step in chondrogenesis [4] [43].

Alcian blue van Gieson staining

For visualization of the composition of the cells removed from a gradient and formed to a 3D structure, Alcian blue van Gieson was used which is a staining technique commonly used to visualize different ECM components of a tissue section. Alcian blue is a water-soluble and cationic stain that is used for staining of molecules such as extracellular glycoproteins and GAGs which is visualized with a blue color. Due to its positive charge, it binds to negative sites at the stained molecule. The blue color formed when it binds to its target molecule and is induced by the presence of copper in the molecule [44]. Alcian blue is often combined with other staining methods such as van Gieson staining. To visualize collagen and other connective tissue, van Gieson stain can be used which are visualized by a pink/red color and cytoplasm is stained yellow [45].

Scanning electron microscopy

To analyze the nano gradients and surfaces produced to determine the number of particles per area but also to confirm the gradient pattern, Scanning Electron Microscopy (SEM) was used. Instead of using a beam of light as in optical microscopy SEM uses a beam of electrons to detect the sample. SEM is particularly used to observe a surface or a solid sample for high-resolution studies of primarily biological and physical samples [46]. Electrons have a shorter wavelength than light which makes it possible to study very small objects, down to a few nanometers. The setup of a scanning electron microscope (illustrated in figure 6) is constituted of an electron gun that provides electrons as a beam directed at the sample. There is also a condenser lens and an objective lens which are aimed to focus the electron beam at the sample. An electron detector is collecting electron signals generated from backscattered electrons as well as secondary electrons generated from interactions of the electron beam with atoms of the sample which provides information about the composition and the surface structure. The process is working in a vacuum that requires the samples to be dry [46] [47].

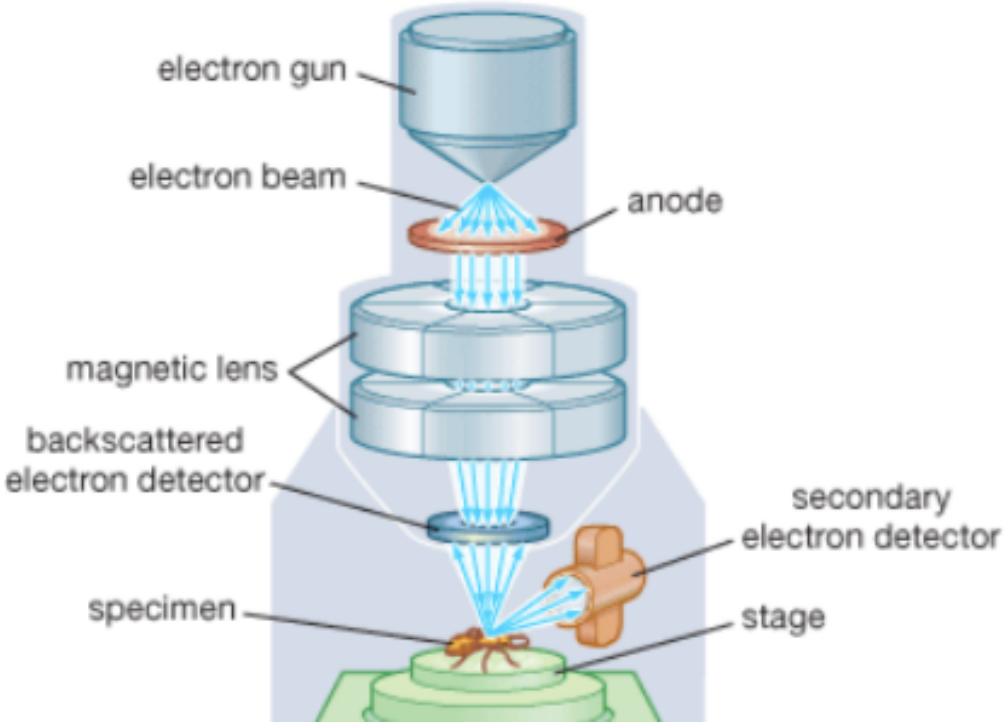


***Figure 6**. Scheme over a scanning electron microscope [47].*

Transmission electron microscopy

To confirm the shape and size of gold nanoparticles, Transmission Electron Microscopy (TEM) was used and the procedure is illustrated in figure 7. As in the SEM, TEM uses a beam of electrons that are focused in a similar way as SEM via a condenser lens to detect the sample. TEM is used due to its possibility to see through a sample or cells and to visualize internal cellular structures. In comparison to SEM, which scans the surface of a sample, electrons are transmitted through a sample to create an image [48].

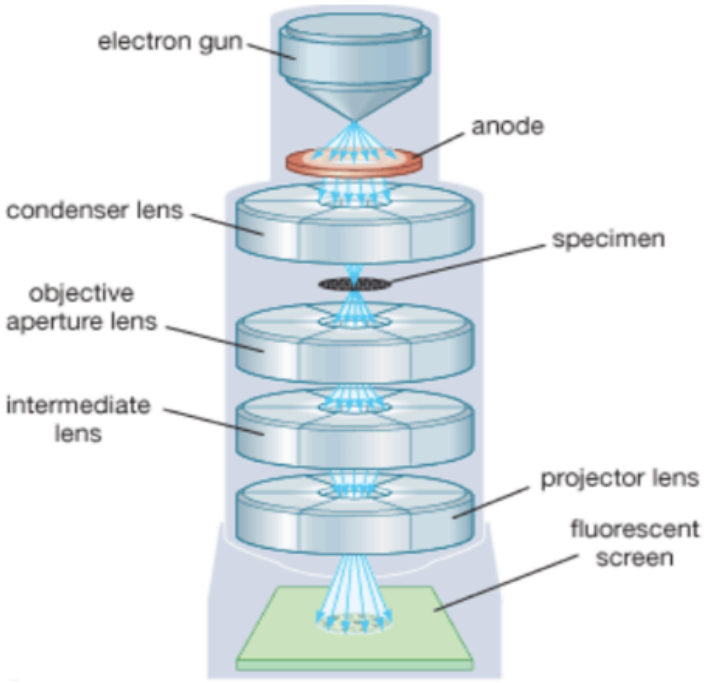


Figure 7*. Scheme of a transmission electron microscope* [49].

Methods

Preparation of nano gradients

A 10 nm gold nanoparticle solution was prepared according to Cline Scientific's manufacturing protocols and applied on a plain 18x18 mm glass surfaces (Menzel Gläser, Thermo Scientific, USA) for manufacturing of nano gradients. Gradients with the highest concentration of particles in the bottom of the gradient and a decrease in concentration of gold nanoparticles over the surface were also produced according to Cline Scientific's patented technology and their manufacturing protocols. After manufacturing of the gradient, the particles were functionalized with different biomolecules; TGFβ-1, TGFβ-3, GDF5. Between the nanoparticles, Laminin 521 was attached to the glass surface which creates a reverse laminin gradient in comparison to the particle gradient. The particle gradient is spanning from about 170-3000 particles/μm^2.

To functionalize the surfaces, thiolated Streptavidin (SH-Streptavidin, Nanocs, USA) was applied to the manufactured nano gradient. After sufficient incubation, superfluous Streptavidin was rinsed off with Phosphate Buffered Saline (PBS) (Phosphate Buffered Saline, Amresco, USA). Thereafter, laminin (Laminin 521, BioLamina, Sweden) was applied with the purpose to attach it to the glass surfaces between the particles. The major role of laminin between the particles is to utilize adhesion of cells and promote cell proliferation and differentiation. After sufficient incubation, superfluous laminin was rinsed with PBS.

TGFβ-1, TGFβ-3, and GDF5 were biotinylated to be able to bind to Streptavidin. Biotinylation of TGFβ-1, TGFβ-3, and GDF5 were performed with a superfluous amount of biotin (EZ-Link™ Sulfo-NHS-LC-Biotin, Thermo Fischer Scientific, USA) to make sure that biotin was attached in all available sites of the proteins. The excess amount of biotin that was not attached was discarded using repeated wash and centrifugation according to Thermo Fisher's biotinylation protocol. The success of the biotinylation was controlled using a HABA test (HABA/Avidin Reagent, Sigma Aldrich, USA) which is a dye reagent that enables detecting the mole-to-mole ratio between proteins and biotin in a solution. The HABA test ensured that the number of biotins attached to the proteins were within specified limitation according to the producer of the test. Finally, the biotinylated proteins with a known concentration, 40 nM in suspension, was attached to the Streptavidin coated gold nanoparticles and incubated at 4 °C overnight. As in the previous steps superfluous proteins were rinsed off with PBS. After functionalization, chondrocyte-derived iPSCs were seeded on the gradients and the growth pattern were studied.

Each protein functionalized gradient was produced in duplicates which resulted in a total of 4 gradients for each protein. Two repeats of gradient batches (30102, 30104) were produced were all the gradients (6 in total) were produced at the same time which means that gradients in batch 30102 and 30104 respectively has the same gradient. GDF5, TGFβ-1 and, TGFβ-3 were functionalized on the nano gradients and they were also acting as a control against each other since the surfaces are functionalized with different proteins and different responses are to be expected. After analysis of gradients functionalized with different proteins, a specific area of interest from the gradients will be produced on new surfaces, without gradient and instead with the specific particle density of interest. Two uniform control surfaces with a particle density higher or lower than the particle density of interest was also produced as references against the newly produced surfaces. To be able to compare the effects of the three molecules functionalized to the surfaces, surfaces with only Streptavidin and laminin will also be produced.

Cell culturing

The cells used for experiments were chondrocytes provided from three combined anonymous male donors and iPS cells derived from chondrocytes from an anonymous female donor with genotype protective against OA, occurred by mutations in the genome. Two 25 cm^2 plastic culture flasks were coated (DEF-CS 500 Coat-1, Cellartis, Sweden) and seeded with newly thawed c-iPS cells and chondrocytes respectively. The iPS cell line used was genetically engineered to express GFP for visualization of aggrecan as described above. Cells were cultured in cell flasks for monolayer culturing and placed in a 37°C humidity chamber with 90% humidity and 5% CO_2. Cell medium (DEF-CS 500 Basal Medium, Cellartis, Sweden) was changed every day and cell passage was performed every third day. After the first passage of cells, the cells were transferred to two 75 cm^2 plastic culture flasks. All handling of cells was performed in a sterile cell lab area.

Experiments on nano gradients and surfaces

Gradients were manufactured while cells were cultured and the gradients were stored in a 6-well plate and kept as sterile as possible in 4 °C until cells were seeded. At the first gradient batch (30102) 50 000 cells/cm^2 were seeded on each nano gradient and incubated in 37°C in a humidity chamber for two hours. After two hours of incubation in a 37 °C humidity chamber, 2 ml cell medium was applied to the gradients in the 6-well plate. The next day, 2 ml differentiation medium according to *table 1* was added to each well, to which different growth factors according to *table 2*, for different gradients were added in the medium. In the second gradient batch (30104) only 25 000 cells/cm^2 was seeded on gradients instead of 50 000 cells/cm^2 and that was because the cell density might have been a little too high for the previous experiment where cells tended to fall of the gradient during fixation, see figure 22 B). All uniform surfaces were seeded with 25 000 cells/cm^2.

Table 1*. Components and concentrations for differentiation medium. The first six components were always included in the medium. Component 7 (TGFβ-1) and component 8 (TGFβ-3) was added depending on which molecule was functionalized on the surface. Component 7 (TGFβ-1) was not included in the medium for a TGFβ-1 gradient or surface and component 8 (TGFβ-3) was not included in the medium for a TGFβ-3 gradient or surface. Both TGFβ-1 and TGFβ-3 was added in the medium for GDF5 gradients and surfaces.*

Stock conc	**Volume taken from stock concentration**	**Solutions (Final concentrations)**	**Company and Lot number**
1x	12 ml	DMEM, high glucose (1x) with PEST	Gibco, 1929008
100x	120 µl	Insulin-Transferrin-Selenium (1x)	GIbco, 1929185
8 mM =1.4 mg/ml	120 µl	Ascorbic acid, 14 µg/ml	Sarstedt, SLBB7986V
20 µg/ml	24 µl	Dexamethasone 10^{-7} M	Sarstedt, BCBL6479V
0.5 mg/ml	120 µl	Linoleic acid, 5 µg/ml	Sarstedt, 95.064.997
0.1 M	13 µl	Sodium Pyruvate, 1 mM	Sarstedt, 95.064.981
1 µg/ml	120 µl	TGFβ-1, 10 ng/ml	Sarstedt, 95.064.997
20 µg/ml	0.5 µl	TGFβ-3, 10 ng/ml	Sarstedt, 95.064.981

Different compositions of differentiation medium were added to the different surfaces depending on which protein the surface was functionalized with and this was based on previously (unpublished) performed studies in chondrocytes that have indicated that the

combination of TGFβ-1 and TGFβ-3 in the differentiation medium has the strongest effect on inducing aggrecan expressions in chondrocytes. Therefore, this combination was also used in this experiment since a high expression of aggrecan was sought for. Though, this combination has previously not been tested for iPS cells. On gradients functionalized with TGFβ-1, only TGFβ-3 was added in the differentiation medium according to table 1 and on gradients functionalized with TGFβ-3, only TGFβ-1 was added to the differentiation medium. GDF5 had both TGFβ-1 and TGFβ-3 in the medium.

Table 2. *Different growth factors for different gradients were added in the differentiation medium. Concentrations of each molecule added to the medium is indicated in table 1.*

Biomolecule attached to the gradient	**Biomolecule added to the medium**
TGFβ-1	TGFβ-3
TGFβ-3	TGFβ-1
GDF5	TGFβ-1, TGFβ-3

Table 3. *Information about the gradient batches, how many gradients that were produced in each batch and for each protein. Cell number seeded on gradients differs between the two batches.*

Gradient batch number	Batch 1 (batch number 30102)	Batch 2 (batch number 30104)
Number of cells seeded	50 000 cells/cm^2	25 000 cells/cm^2
Number of gradients produced per batch	2 gradients for each protein (6 in total)	2 gradients for each protein (6 in total)
Proteins functionalized	GDF5, TGFβ-1 and TGFβ-3	GDF5, TGFβ-1 and TGFβ-3
SEM analysis	Gradient 1	Gradient 2

Cell analysis on nano gradients/surfaces

After 5 days of differentiation on the nano gradient in differentiation medium, the cells were fixed with Formaldehyde (4% Histofix, Histolab, Sweden). They were also stained with the fluorophore DAPI (ProLongTM Gold Antifade Mountant with DAPI, Thermo Fisher Scientific, USA) to enable detection of all cells. DAPI stains the nuclei in the cells and all cells will, therefore, emit blue light in the microscope. The genetically modified cells that express a significant amount of aggrecan will appear green and the cell response, dependent on the presence and amount of TGFβ-1, TGFβ-3 and GDF5, can be studied. Cell analysis was performed using the fluorescence imaging microscope IN Cell 6000 (IN Cell 6000, GE Healthcare, United Kingdom).

Experiments based on nano gradient results - removal of cells for continued growth

When an area of interest was identified on the nano gradients, new surfaces were produced with a specific particle density for further cell culturing. As already described, gradients and surfaces were mounted with DAPI on slides to be visualized in IN CELL 6000. Gradients and surfaces were also produced in order to remove live cells from the surface for longer differentiation times in a 3D structure. Two gradients functionalized with GDF5 were used to culture chondrocytes and two surfaces functionalized with GDF5 with a particle density at 700 particles/μm^2 was used to culture iPS cells. The GDF5 density on uniform surfaces corresponded to a particle density at the end of the gradient. When the cells had been cultured for 5 days on the surfaces, the cells were released from the surfaces by 5 minutes of incubation in Gibco TrypLE Select 10X (ThermoFisher Scientific, USA). The reaction was then quenched with human serum and the cells were collected with a pipette. Cells from each gradient/surface were transferred into 15 ml tubes and centrifuged for 5 minutes at 700g in 2 ml differentiation medium (*table 1*) to form 2 pellets and 2 organoids. The pellets gained from the centrifugation was stored in a 96 well plate in 200 µl medium, according to *table 1,* in a 37°C incubator. The pellets gained from chondrocytes were cultured in a 96 well plate during two weeks and the iPS organoids were differentiated in a 96 well plate for six weeks. The medium was changed every third day. In order to compare results from the chondrocyte pellets derived from GDF5 gradients to evaluate the impact of the gradient, one pellet was also derived from chondrocytes without using a gradient, cultured for two weeks in differentiation medium. Also, a pellet was obtained from chondrocytes without a GDF5 gradient and cultured for two weeks in differentiation medium plus 5% human serum.

After two weeks of culturing of chondrocyte pellets, they were fixated in formaldehyde (4% Histofix, Histolab, Sweden) overnight and then stored in 70% ethanol. Thereafter, pellets were sent to Histocenter (Gothenburg, Sweden) for sectioning and to be stained with Alcian blue van Gieson. After sectioning, the pellet compositions were analyzed in a light microscope (Eclipse 90i, Nikon Instruments, Japan) for visualization of aggrecan expressions.

iPS organoids were differentiated for six weeks in differentiation medium in a 96 well plate. Every week after week 2, organoids were visualized by fluorescence imaging using IN CELL 6000 to visualize aggrecan expressions.

Immunoassays for visualization of proteins on a functionalized gradient

Gradients were analyzed using two immunoassays to visualize the attached proteins to the nanoparticles on the surfaces. GDF5 was used as a model protein to validate if the methods could be used.

Immunoassay using DAB

Three gradients were produced according to the same manufacturing protocol as already mentioned, but no cells were seeded. One gradient was incubated with a GDF5 specific primary antibody and a secondary antibody, specific to the primary antibody. A second gradient was incubated with an anti-GDF5 isotype control antibody and a secondary antibody. The isotype control antibody was used to function as a type of negative control where the isotype antibody was not supposed to be specific to GDF5 functionalized at the surface. The third gradient was incubated without the primary antibody or isotype but with a secondary antibody to function as a negative control. Gradients were labeled using Cell & Tissue Staining Kit (HRP-DAP System, R&D Systems, USA) and the procedure was performed according to the following protocol:

The first step after functionalization with GDF5 was incubation with 300 µl Peroxidase Blocking Reagent for 5 minutes. After incubation, the gradients were rinsed and then washed in PBS for 5 minutes. Thereafter, the gradients were incubated, firstly, in Serum Blocking Reagent for 15 minutes, secondly, in Avidin Blocking Reagent for 15 minutes and, thirdly, in Biotin Blocking Reagent for 15 minutes. All reagents were applied in a volume of 300 µl to each surface. After mentioned incubations, the gradients were rinsed with PBS. A primary polyclonal specific GDF5 antibody (Abcam AB115678 rabbit), 0.01 µg/µl, was added to one of the gradients and incubated at 4°C, overnight. The second gradient was incubated with an isotype antibody control (Rabbit IgG polyclonal isotype control, Abcam AB27478) and the third gradient was incubated without a primary antibody/isotype. Next day, gradients were rinsed in PBS and washed three times for 15 minutes per wash. After the washing steps, a Biotinylated Secondary Antibody was added and the gradients were incubated for 60 minutes and then again rinsed and washed in PBS for 15 minutes per wash. The next incubation used 300 µl HSS-HRP for 30 minutes and the rinse- and wash procedure in PBS was reduced to 2 minutes per wash. The last step was to incubate with DAB for 20 minutes and wash in distilled water for 5 minutes. The gradients were then mounted with Pertex (Pertex Mounting Medium, Histolab, Sweden) on a glass slide for visualization in a microscope (Eclipse 90i, Nikon Instruments, Japan).

Additionally, to the gradients functionalized with GDF5, one gradient functionalized only with laminin, without GDF5, was incubated according to the above protocol to exclude binding of the secondary antibody to laminin.

Immunoassay using Alexa Fluor 546

Three gradients were prepared and firstly, gradients were rinsed and washed two times with PBS for 5 minutes per wash. After washing the gradients were incubated with blocking medium (2 % BSA, 0.1 % tritonX-100, 100 mM glycine and 5% goat serum solved in PBS) for 15 minutes in room temperature. Next, one gradient was incubated in 300 µl of the GDF5 specific primary antibody (Abcam AB115678 rabbit), 0.01 µg/µl, was incubated on one of the gradients at 4°C overnight. A second gradient was incubated with 300 µl an anti-GDF5

isotype antibody control (Rabbit IgG polyclonal isotype control, Abcam AB27478) at 4°C overnight. A third gradient was incubated in blocking medium without antibody or isotype at 4°C overnight. The next day, gradients were rinsed and washed with PBS three times for 3 minutes. Thereafter, all gradients were incubated with a secondary antibody (Alexa Fluor 546) for 2 hours in a humidity chamber and after 2 hours, gradients were rinsed and washed five times for 5 minutes in PBS. After washing, gradients were mounted with ProLong TM Gold Antifade Mountant media (ProLong TM Gold Antifade Mountant with DAPI, Thermo Fisher Scientific, USA) and were then visualized in a fluorescence microscope.

Results

TEM

Cline Scientific's manufacture protocol was used to produce gold nanoparticles with 10 +/- 1.5 nm in diameter and TEM analysis was performed to confirm the shape and size of the manufactured gold nanoparticles, which can be seen in figure 8. 100 nanoparticles were measured using ImageJ to determine the diameter of the particles. Median value from 100 measurements was 9.916 nm and the standard deviation was determined to 1.2 nm.

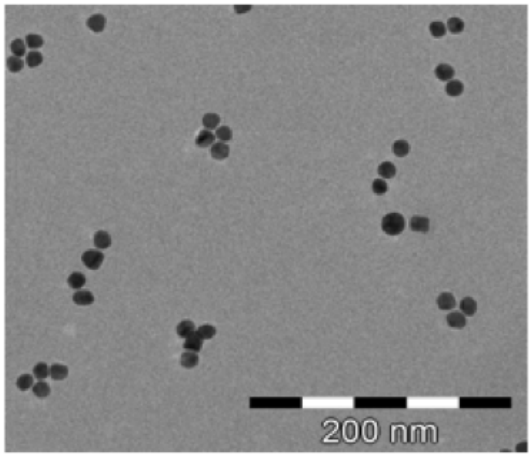


***Figure 8**. 10 nm gold nanoparticles analyzed in a transmission electron microscope to confirm size and shape of the particles.*

SEM

Cline Scientific's manufacture protocol was used to produce nano gradients and surfaces. To confirm the structure of the gradient and to calculate the particle density over the surface, SEM (Zeiss Ultra 55, Germany) analysis of all gradients and surfaces was performed. The analysis consisted of counting the number of particles observed in the SEM images and divided by the physical area of the field of the view. Figure 9 is illustrating the distribution of particles on three different sites at the gradient. Analysis at the gradients was performed at three points along the gradient in steps of 1 mm, illustrated in figure 10 and the gradient

pattern is indicated in figure 11. Analysis of uniform surfaces was performed at five random sites at the surface to calculate the particle density.

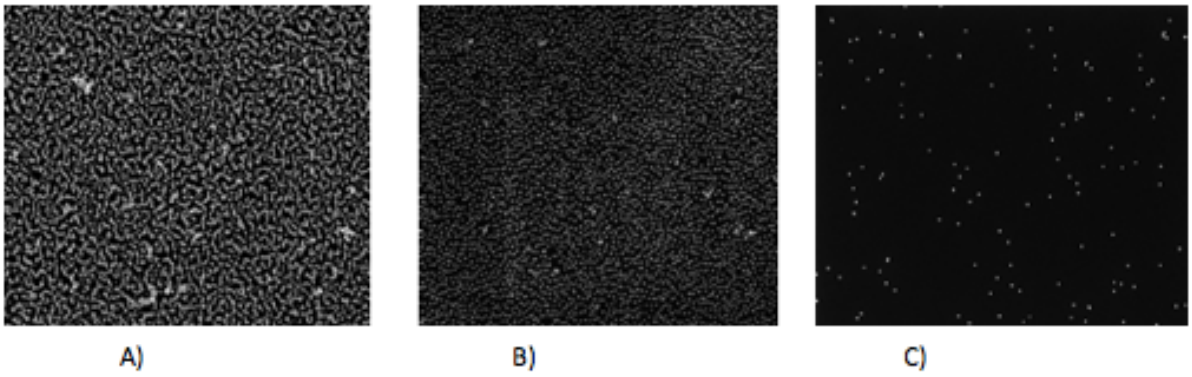


Figure 9. *Nano gradient 1 analyzed using SEM. Pictures are taken from the bottom of the gradient at three different sites on the gradient. A) 1 mm in from the start of the gradient. B) 3 mm in from the start of the gradient. C) 5 mm in from the start of the gradient.*

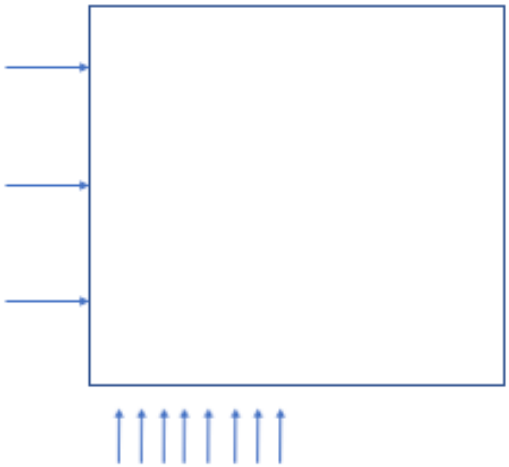


Figure 10. *Illustration of an 18x18 mm gradient surface which shows a schematic illustration of where in the gradients the SEM images were taken, starting from 1 mm to 8 mm of the surface at three different sites.*

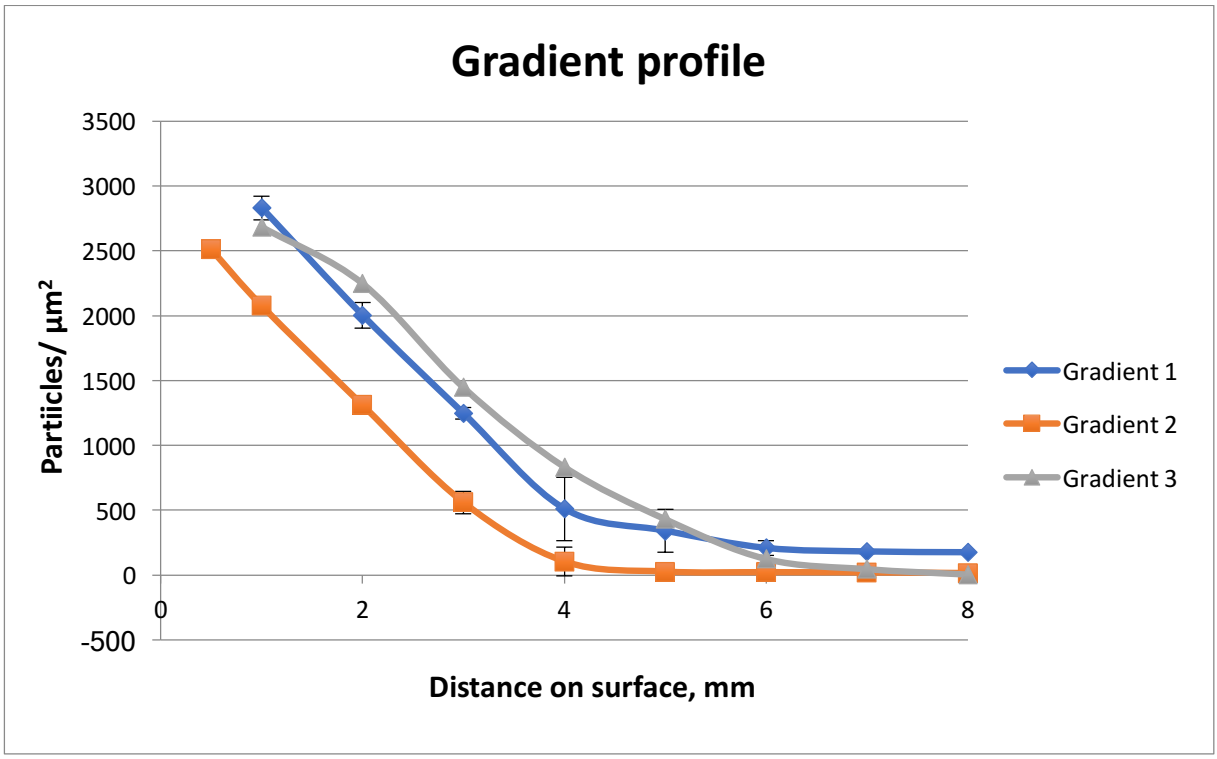


***Figure 11**. Gradient profile for the manufactured gradients. Gradient 1 was used for gradients in batch 1 (30102). Gradient 2 was used for gradients in batch 2 (30104). Gradient 3 was used for control gradients with only laminin.*

Fluorescence imaging of cells on nano gradients/surfaces

The nano gradients with cells that express GFP was analyzed by fluorescence imaging using IN CELL 6000. Firstly, the gradients were scanned in 4X, 10X or 20X magnification to visualize the entire surface. When an area of interest was detected the gradients were scanned further to visualize the significant area with higher magnification.

In total, 4 functionalized gradients with genetically engineered cells were screened using IN CELL. Notably, the number of cells on the two batches of gradients differs since gradients from batch 1 were seeded with twice as many cells, information regarding this is presented in table 3.

Using SEM, gradients were analyzed to determine particle density over the surface. This knowledge was used to manufacture surfaces with the same particle density over the entire surface. The desired densities were chosen depending on cell results from the gradient surfaces experiments. Two uniform surfaces were also produced to function as negative controls. Results from gradients and surfaces functionalized with a separate protein are presented below.

GDF5

Gradients from batch 1 (Batch 30102, A and B, figure 12) were functionalized with GDF5 and resulted in an interesting cell response in an area ca 3.5 mm from the highest

concentration of the gradient to about 8 mm. Within this area, small buds were found in between larger cell clusters. Worth noticing is not only the highlighted area but also the clusters with higher cell density over the entire surfaces. Gradients from batch 2 (Batch 30104, C and D, figure 12) resulted in an interesting clustering phenomenon where all cells moved into one spot about 5 mm from the highest concentration of the gradient. Pictures in a higher magnification are visualizing the specific cell reaction (figure 13) where the cells tended to form a tightly packed and condensed structure. Figure 14 is visualizing the aggrecan expression from the condensed structure in figure 13.

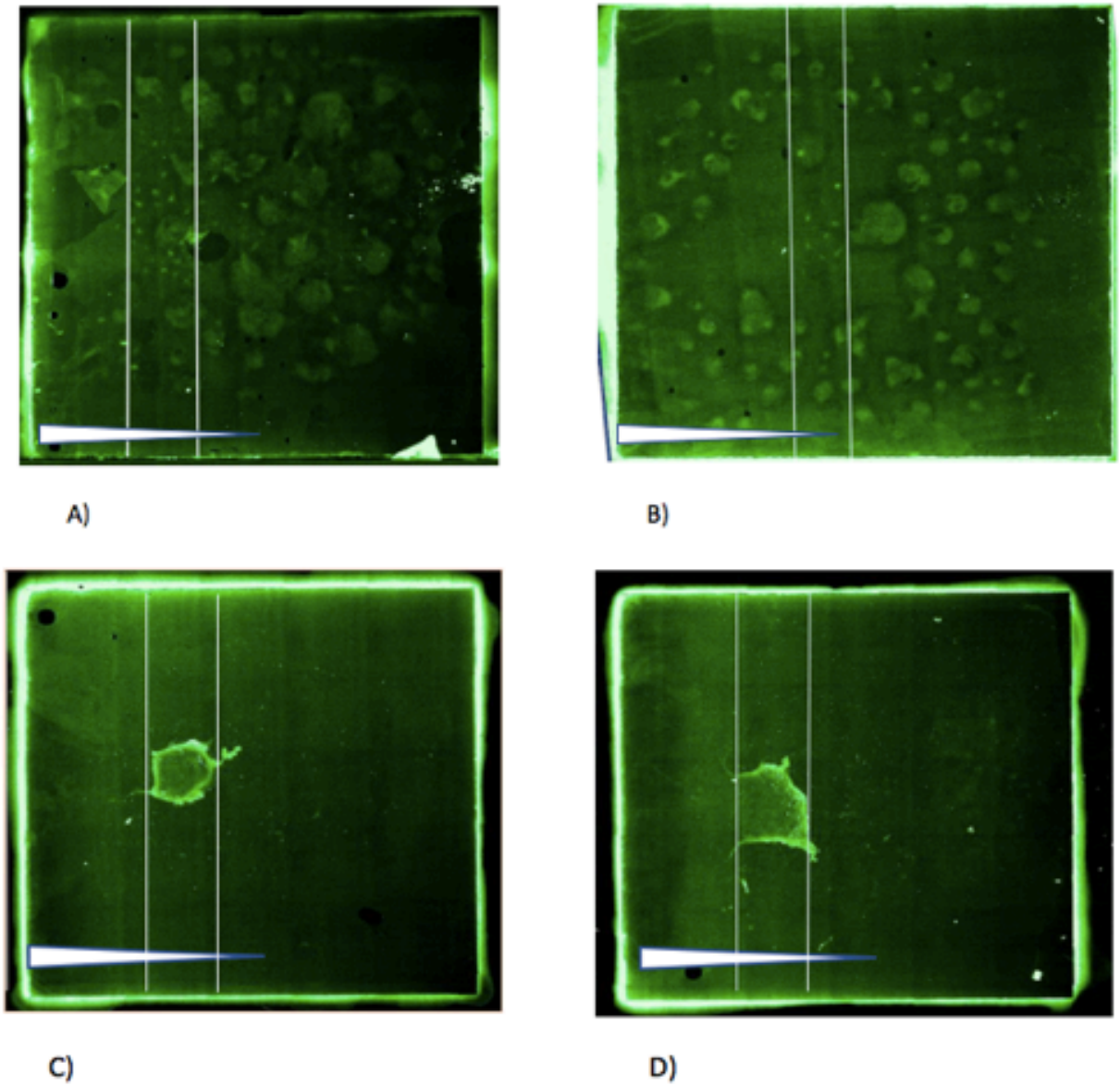


***Figure 12**. Gradients 18x18 mm visualized in IN CELL 6000. The concentration gradient starts from the left which corresponds to the highest concentration of* GDF5 *which decreases towards the right. The white lines indicate a highly relevant area to study further. All pictures were taken at 10X magnification using a FITC filter to visualize the aggrecan expression.*

Gradients from batch 1 were seeded with 50 000 cells/cm^2 and gradients from batch 2 were seeded with 25 000 cells/cm^2. *A) Nano gradient from batch 1 functionalized with GDF5 B) Nano gradient from batch 1 functionalized with GDF5. C) Nano gradient from batch 2 functionalized with GDF5. D) Nano gradient from batch 2 functionalized with GDF5.*

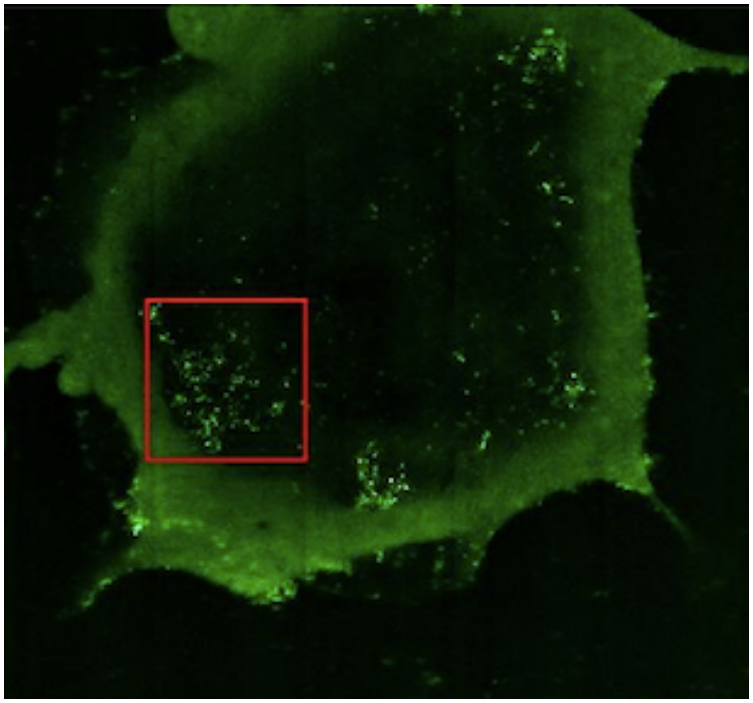


Figure 13. *Specific area of interest from GDF5 gradient batch 2 (30104), figure 12C) scanned in 20X magnification. The higher intensity in green indicates an area with higher expression of aggrecan. The area within the red square is visualized in more detail in figure 14.*

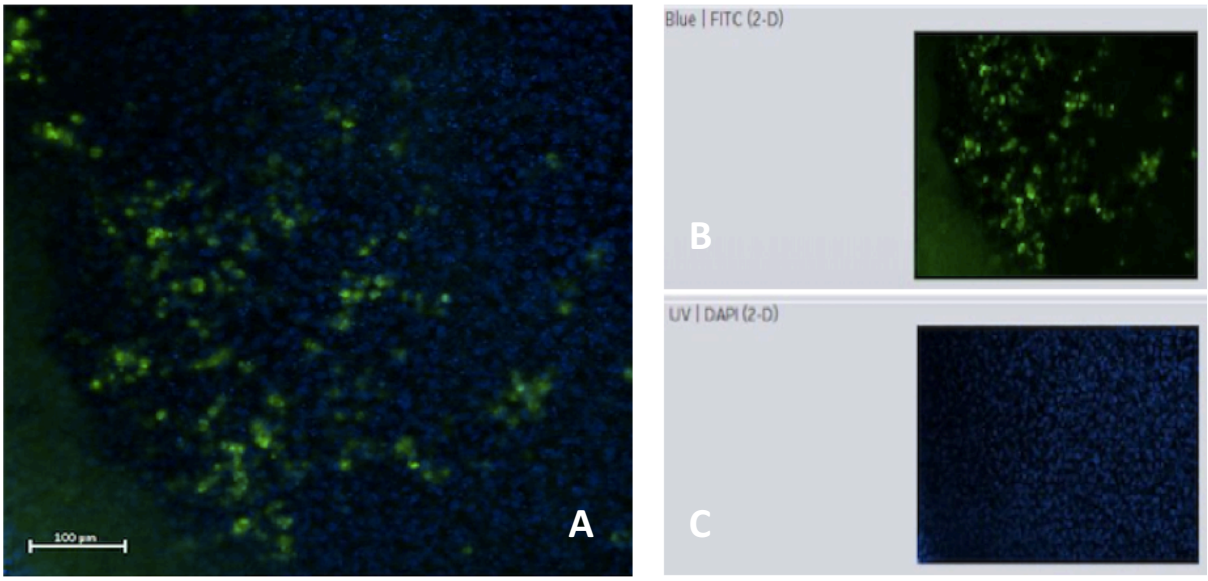


Figure 14. *Detailed picture from the area shown in figure 13, scanned in 20X magnification. A) FITC and DAPI merged. The blue color is visualizing cell nuclei and the green color is visualizing aggrecan. Scale bar 100 µm. B) FITC, 20X magnification. C) DAPI, 20X magnification.*

Cells on gradients functionalized with GDF5 were reacting in approximately the same area corresponding to a quite low particle concentration (700 particles/µm^2). Two new uniform surfaces were produced at that particle density, thus creating surfaces with one specific GDF5 density, see figure 15. An area with a higher green intensity at the edge of the condensed structure from figure 15B) can be observed in figure 16.

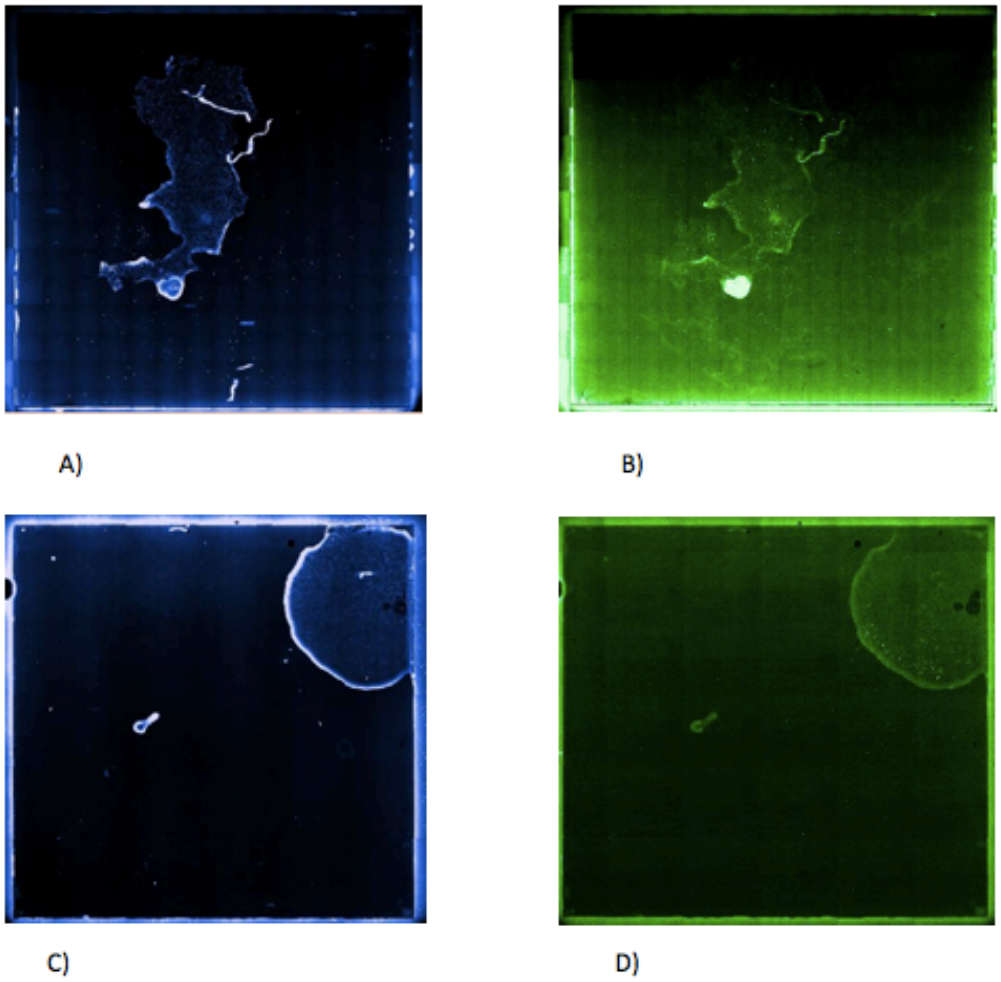


Figure 15. *Surfaces 18x18 mm visualized in IN CELL 6000 with a particle density of 700 particles/µm^2. All pictures were taken at 10X magnification. Both surfaces were seeded with 25 000* cells/cm^2. *A) Cell nuclei visualized on the surface using DAPI. B) The same surface as in A) but scanned using FITC filter to visualize aggrecan. C) Cell nuclei visualized on the surface using DAPI. D) The same surface as in C) but scanned using FITC filter to visualize aggrecan.*

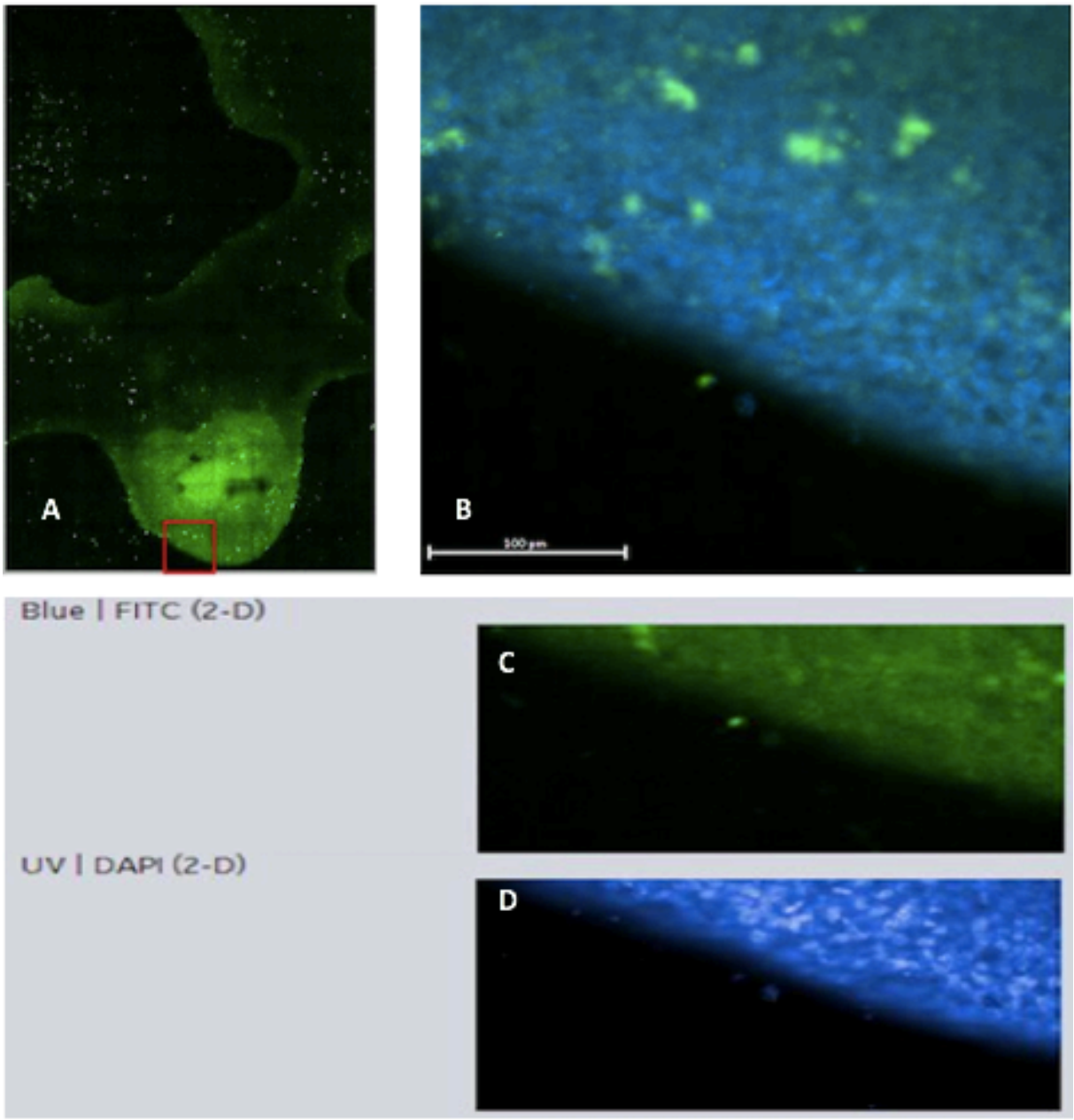


Figure 16. *A) Area of interest from figure 15B), scanned in 40X magnification to visualize aggrecan expression. The higher intensity in green indicates an area with higher expression of aggrecan. B) A zoomed area within the red square in A), is visualized in more detail. FITC and DAPI merged. The blue color is visualizing cells and the green color is visualizing aggrecan. Scale bar 100 µm. C) FITC, 20X magnification. D) DAPI, 20X magnification.*

For comparison, two surfaces with a high particle density (2500 particles/μm^2) corresponding to the start of the gradient, were also produced, see figure 17. Cells on surfaces produced, both with a lower and a higher particle density, tended to move towards each other. Cells were evenly distributed on the surface when seeded on the surface, but after 5 days of differentiation, cells tended to form a condensed structure, visualized in figure 15 and 17.

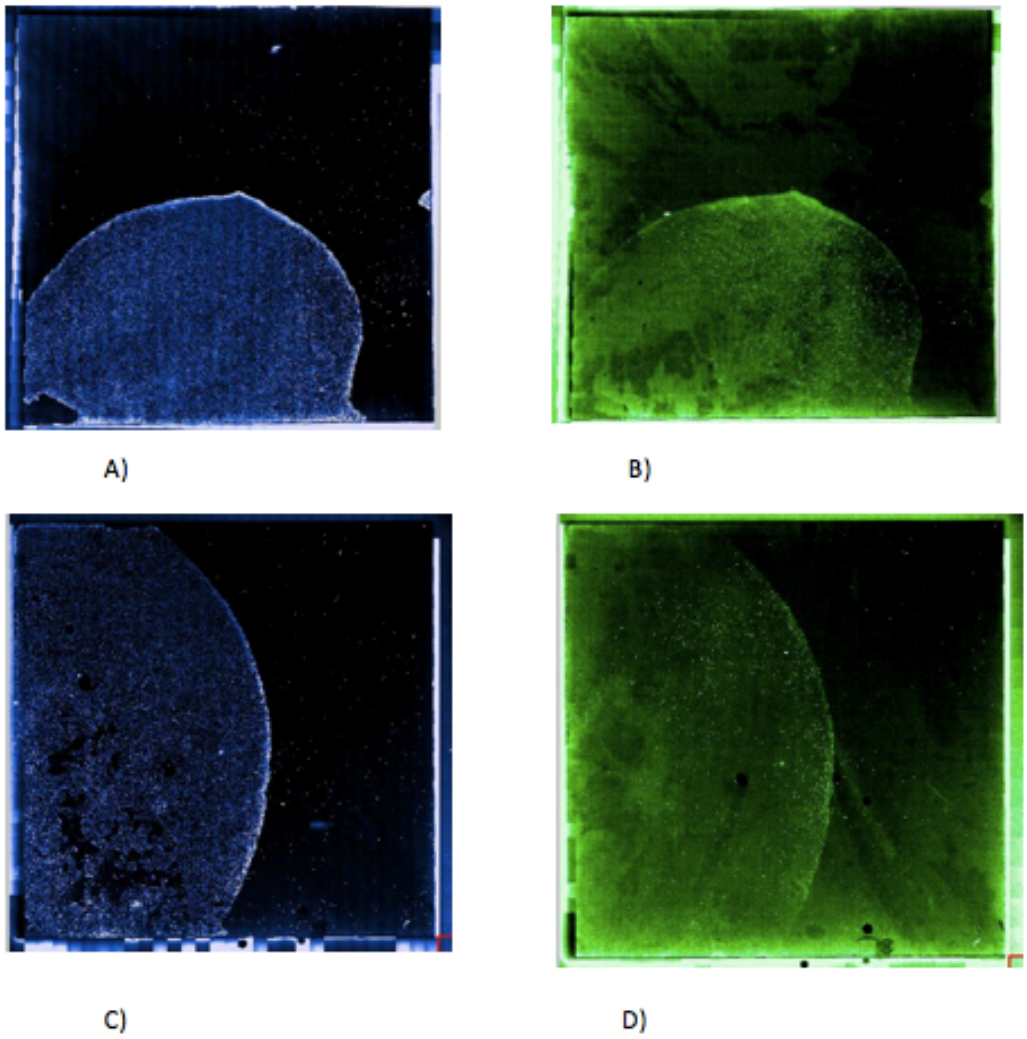
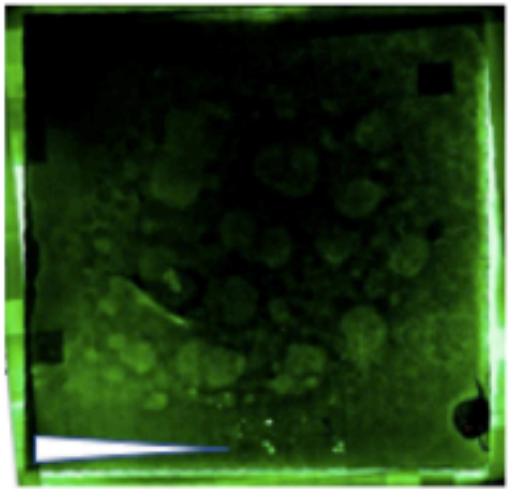


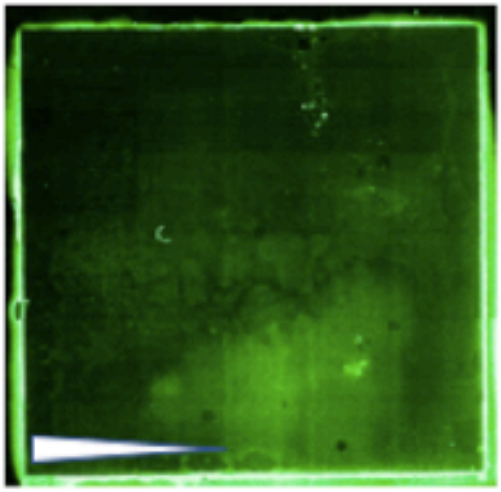
Figure 17. *Surfaces 18x18 mm visualized in IN CELL 6000 with a particle density of 2500 particles/µm^2. All pictures are taken at 10X magnification. Both surfaces were seeded with 25 000* cells/cm^2. *A) Cell nuclei visualized on the surface using DAPI. B) The same surface as in A) but scanned using FITC filter to visualize aggrecan. C) Cell nuclei visualized on the surface using DAPI. D) The same surface as in figure C) but scanned using FITC filter to visualize aggrecan.*

TGFβ-1

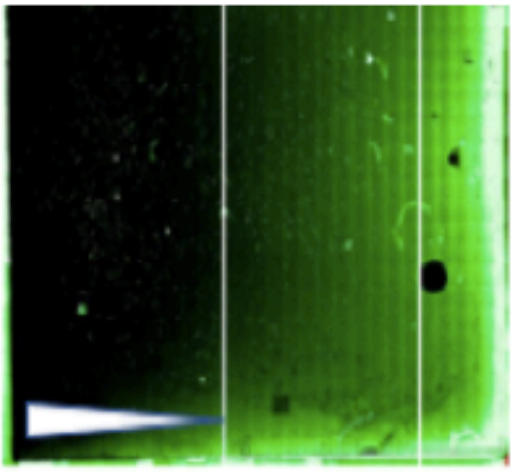
Gradients from batch 1 (30102) were relatively hard to identify a specific area of interest. Gradient A in figure 18 visualizes dense cell clusters over the entire surface. Gradient B in figure 18 indicate cells over the entire surface but not as many dense cell clusters as in A. Gradients from batch 1 (30102) were seeded with twice as many cells compared to gradients in batch 2 (30104). Gradients from batch 2 (Batch 30104, C, E and D, F figure 18) resulted in an interesting area from 8.5 mm from the highest concentration of the gradient to 18 mm which are corresponding to low densities of particles. Since gradients in batch 2 (30104) were the gradients that indicated any kind of specific reaction, at a low particle density, two homogenous surfaces were produced in a low particle density corresponding to the end of the gradient and two surfaces with a high particle density corresponding to the start of the gradient.



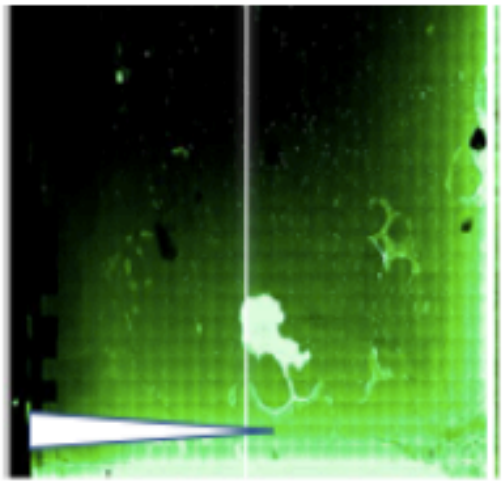
A)



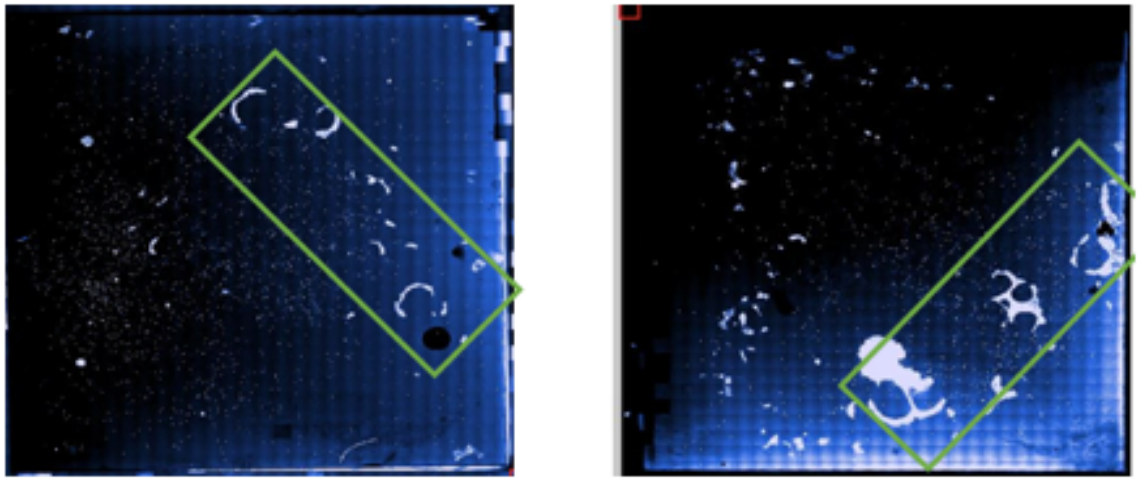
B)



C)



D)



E) F)

Figure 18. *Gradients 18x18 mm visualized in IN CELL 6000. The concentration gradient starts from the left which results in the highest concentration of TGFβ-1 and a decrease in TGFβ-1 concentration to the right. Pictures A and B are taken at 10X magnification and pictures C-F are taken in 20X magnification. The area between the white lines indicate an interesting area to study and the green box is indicating a specific reaction of the cells. Gradients from batch 1 were seeded with 50 000* cells/cm^2 and gradients from batch 2 were seeded with 25 000 cells/cm^2.
A) *Nano gradient from batch 1 functionalized with* TGFβ-1. *B) Nano gradient from batch 1 functionalized with* TGFβ-1. C) *Nano gradient from batch 2 functionalized with* TGFβ-1. *D) Nano gradient from batch 2 functionalized with* TGFβ-1. *E) Gradient C visualized with DAPI to visualize all cells present on the surface. F) Gradient D visualized with DAPI to visualize all cells present on the surface.*

Cells on gradients functionalized with TGFβ-1 were reacting in the same area corresponding to a quite low particle concentration (600 particles/μm^2). Two new surfaces were produced at that particle density, thus creating surfaces with one specific protein density, see figure 19. For comparison, two surfaces with a high particle density (2500 particles/μm^2) corresponding to the start of the gradient, were also produced, see figure 20. Figure 19 visualize surfaces with a lower particle density, functionalized with TGFβ-1. Cavities without cells are formed all over the surface and no significant aggrecan expression was found. Figure 20 visualize surfaces with a higher particle density and thus higher TGFβ-1 concentration. Cells on these surfaces are still, after 5 days of differentiation, evenly distributed on the surface. No significant aggrecan expression was observed which can be observed in figure 21.

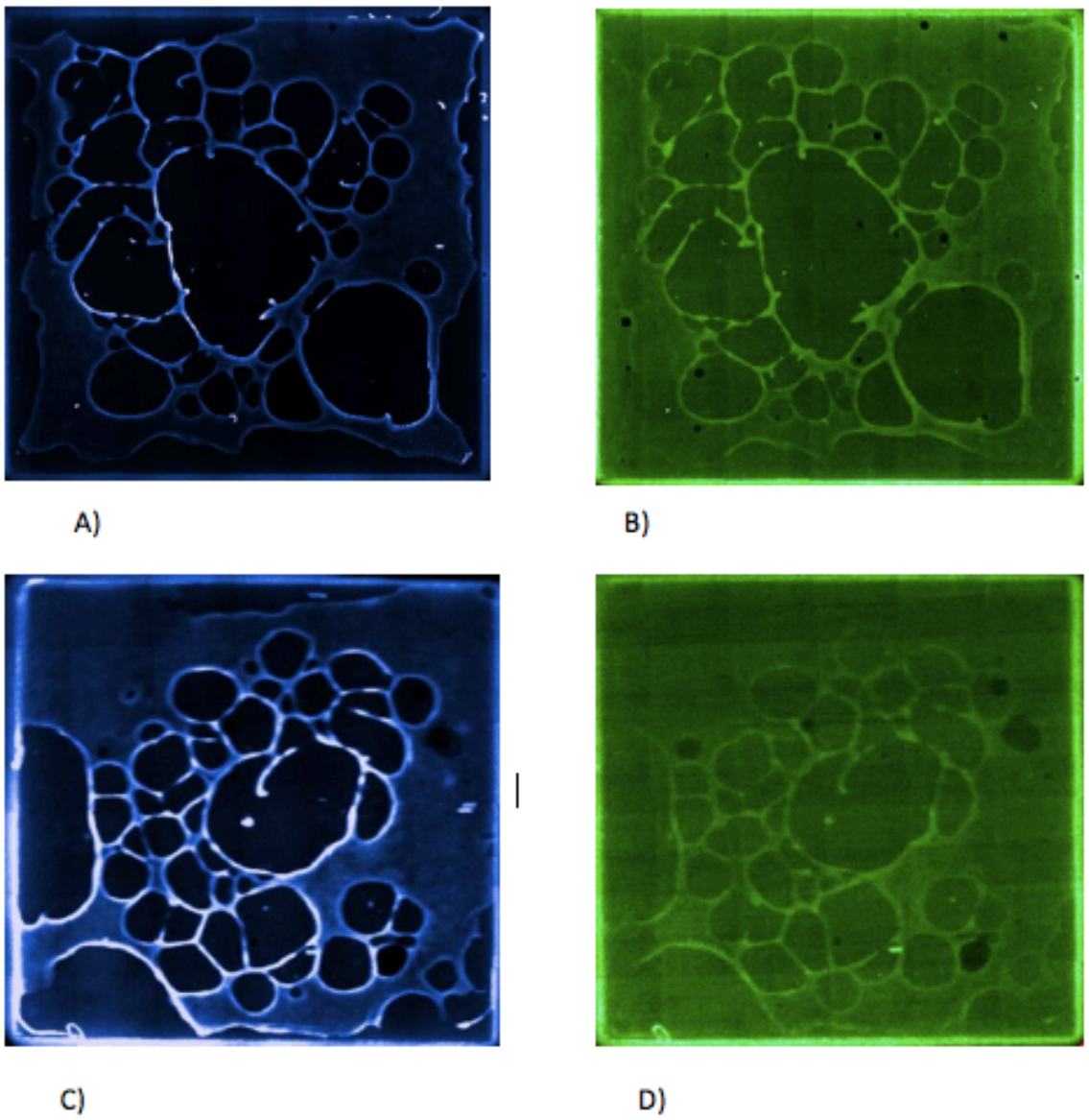


Figure 19. *Surfaces 18x18 mm visualized in IN CELL 6000 with a particle density at 600 particles/µm^2. All pictures are taken at 4X magnification. Both surfaces were seeded with 25 000* cells/cm^2. *A) Cell nuclei visualized on the surface using DAPI. B) The same surface as in A) but scanned using FITC filter to visualize aggrecan. C) Cell nuclei visualized on the surface using DAPI. D) The same surface as in C) but scanned using FITC filter to visualize aggrecan.*

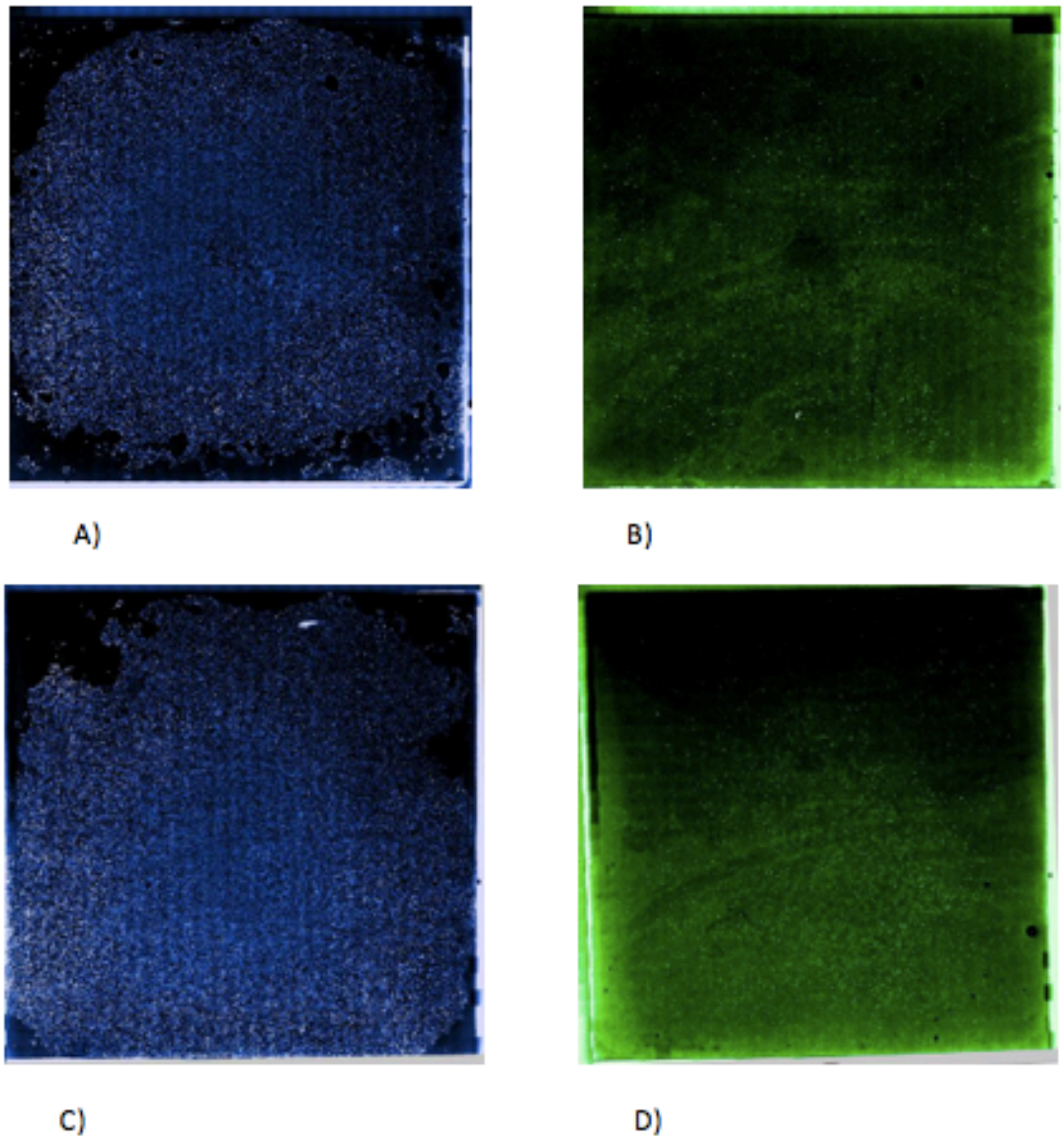


Figure 20. *Surfaces 18x18 mm visualized in IN CELL 6000 with a particle density at 2500 particles/µm^2 which corresponds to a higher density, used as negative controls. All pictures are taken at 10X magnification. Both surfaces were seeded with 25 000* cells/cm^2. *A Cell nuclei visualized on the surface using DAPI. B) The same surface as in A) but scanned using FITC filter to visualize aggrecan. C) Cell nuclei visualized on the surface using DAPI. D) The same surface as in C) but scanned using FITC filter to visualize aggrecan.*

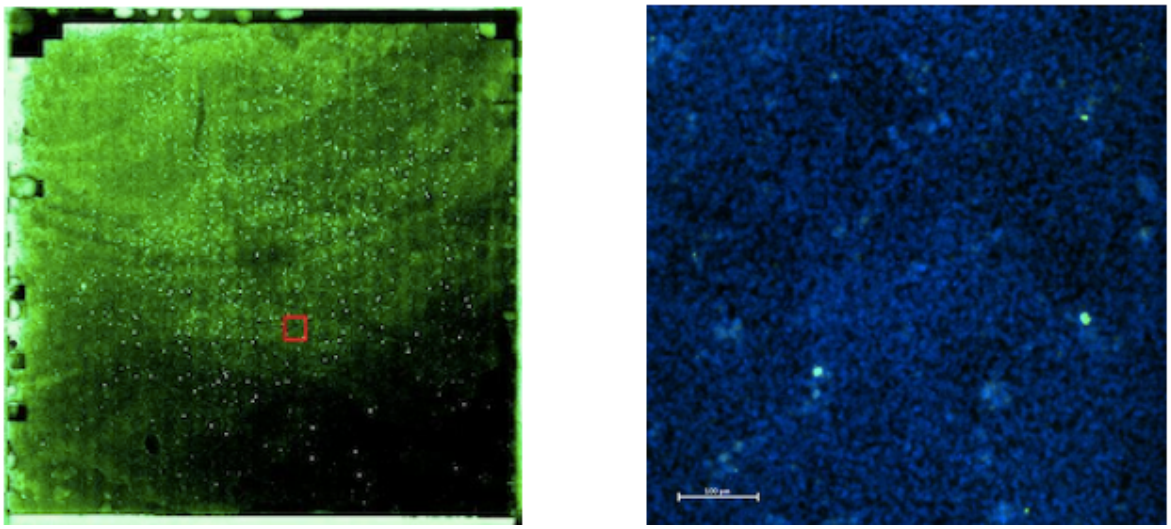


Figure 21. *Surface from figure 20D) visualized in 20X magnification to the left. White dots in the picture is indicating a background signal. The picture to the right is also scanned using 20X magnification and FITC and DAPI is merged. It is clearly shown that no significant aggrecan expression is observed since a very low green color is observed in the picture.*

TGFβ-3

Gradients from batch 1 (Batch 30102, A and B, figure 22) resulted in no observed area of interest on TGFβ-3. Using the information in figure 22A), no area of interest was observed. For the second gradient in batch 1 (30102) with TGFβ-3, cells fell off during fixation from a major area in the middle of the gradient. Therefore, this surface was not included in considerations. Notably, despite the hole in the middle of the gradient, cell clusters with higher cell density was observed the gradient. Gradients from batch 2 (30104, C and D, figure 22) resulted in an interesting area 3.5 mm from the highest concentration of the gradient to about 15 mm.

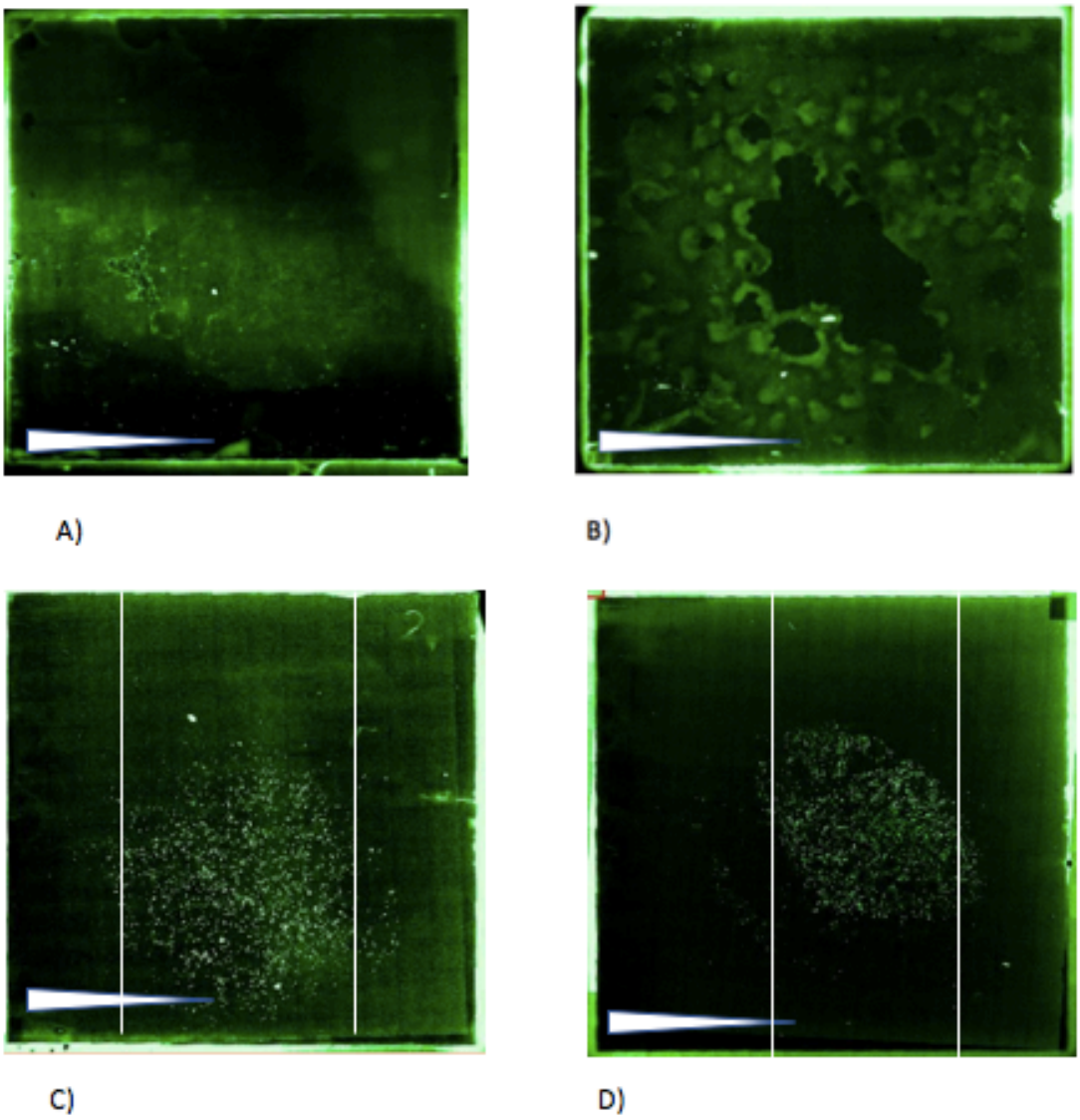


Figure 22. *Gradients 18x18 mm visualized in IN CELL 6000. The concentration gradient starts from the left which results in the highest concentration of TGFβ-3 which decreases to the right. The white lines indicate a highly relevant area to study further. All pictures are taken at 10X magnification. Gradients from batch 1 were seeded with 50 000* cells/cm^2 and gradients from batch 2 were seeded with 25 000 cells/cm^2. *A) Nano gradient from batch 1 functionalized with TGFβ-3. B) Nano gradient from batch 1 functionalized with TGFβ-3. The open area in the middle of the gradient visualizes how cells fell off the surface during fixation. C) Nano gradient from batch 2 functionalized with TGFβ-3. D) Nano gradient from batch 2 functionalized with TGFβ-3.*

Cells on gradients in figure 22, functionalized with TGFβ-3, was reacting in approximately the same area on the gradients which corresponds to a low particle density (600 particles/μm^2) in which two new surfaces were produced, see figure 23. Two surfaces were also produced with a high particle density (2500 particles/μm^2), which was used to compare surfaces too, see figure 24. Cells were, as earlier, seeded evenly over the entire surface and mostly maintained evenly over the surface after the five days of differentiation, which is visualized in

the blue pictures using DAPI to stain the nuclei. For pictures in green, aggrecan expression was indicated to be up-regulated in comparison to earlier visualizations of GDF5 and TGFβ-1 which is also confirmed in figure 25 and figure 26.

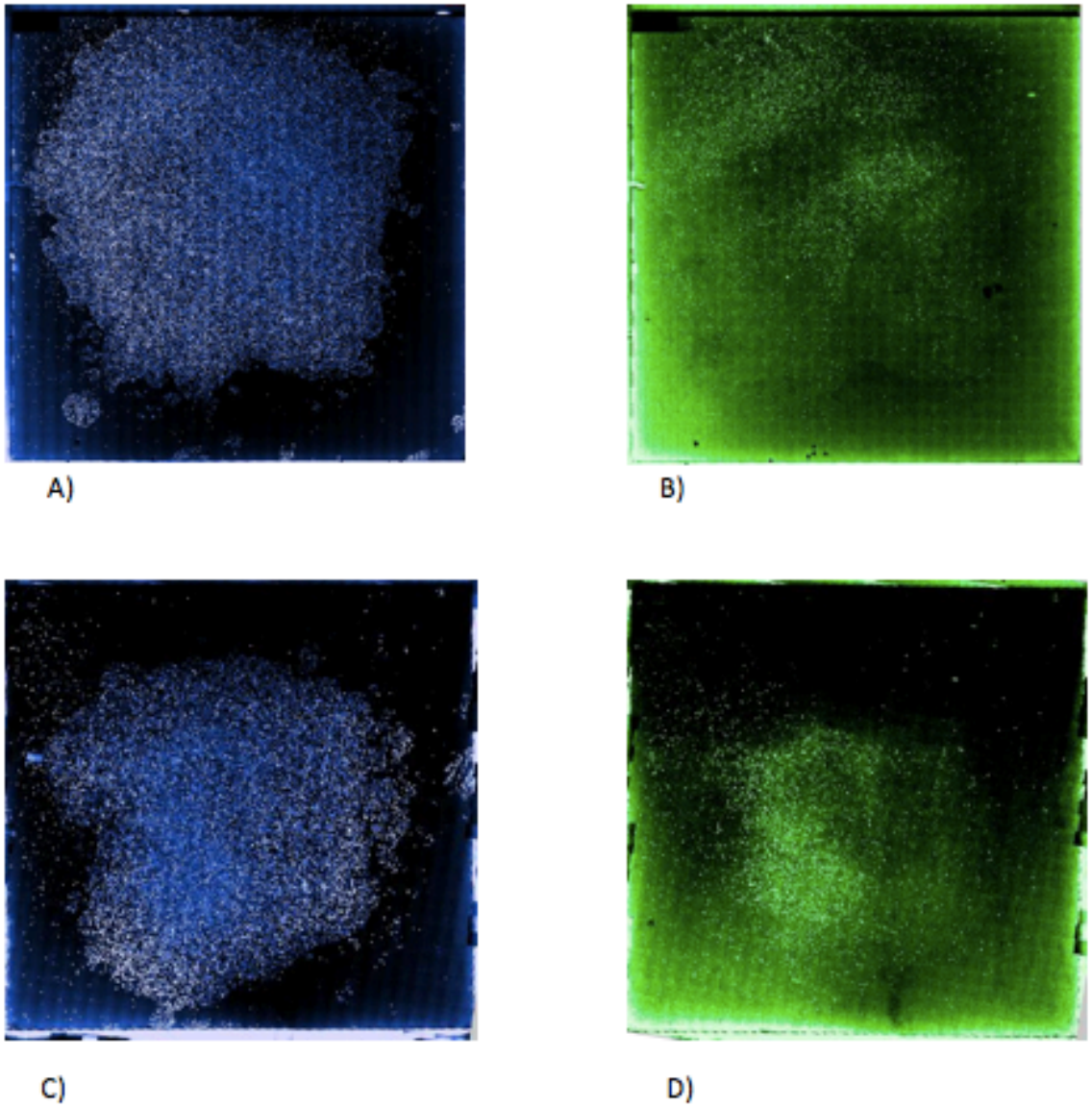


Figure 23. *Surfaces 18x18 mm visualized in IN CELL 6000 with a particle density at 600 particles/µm^2. All pictures are taken at 20X magnification. Both surfaces were seeded with 25 000* cells/cm^2. *A) Cell nuclei visualized on the surface using DAPI. B) The same surface as in A) but scanned using FITC filter to visualize aggrecan. C) Cell nuclei visualized on the surface using DAPI. D) The same surface as in C) but scanned using FITC filter to visualize aggrecan.*

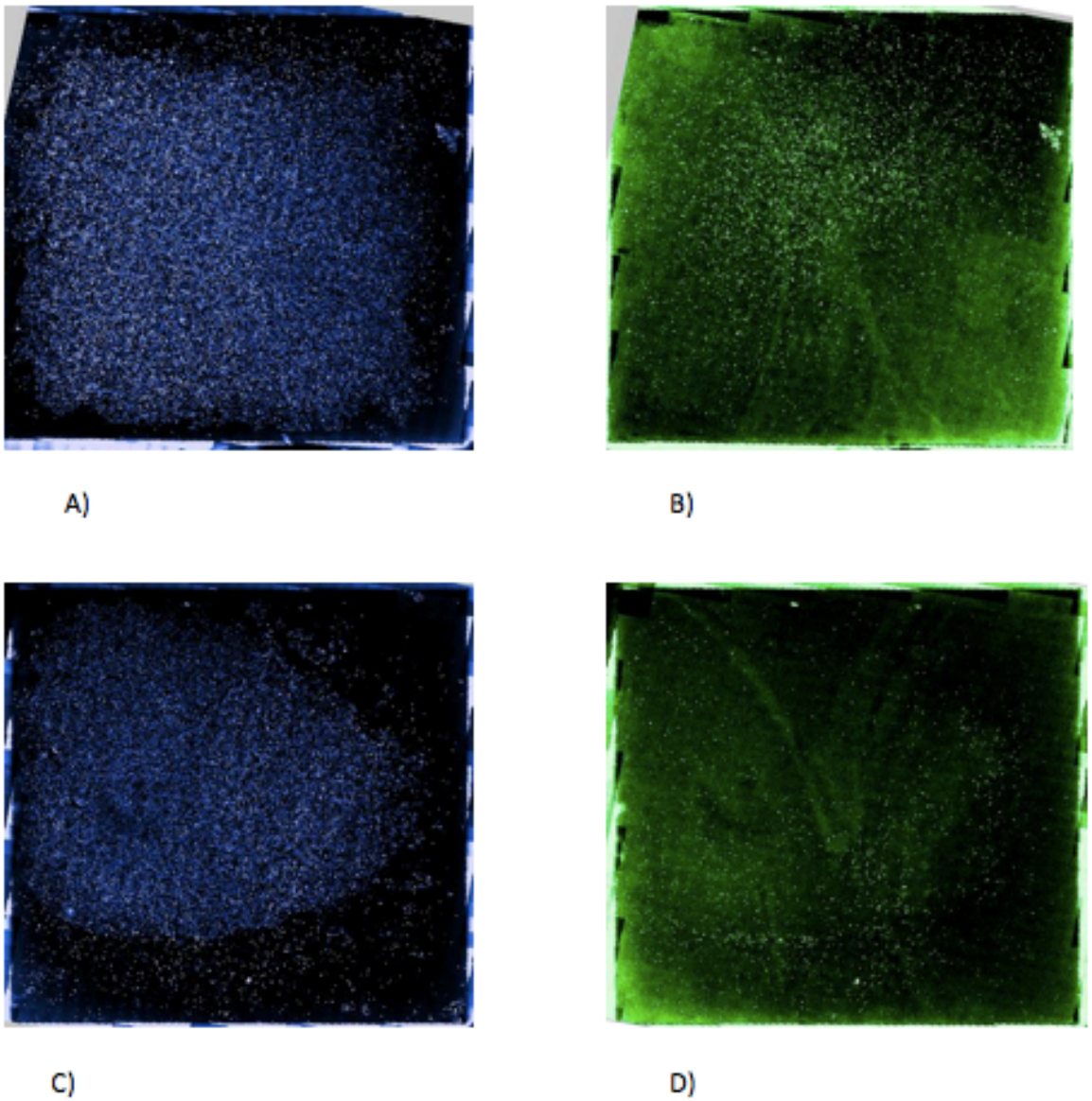


Figure 24. *Surfaces 18x18 mm visualized in IN CELL 6000 with a particle density at 2500 particles/µm^2. All pictures are taken at 20X magnification. Both surfaces were seeded with 25 000* cells/cm^2. *A) Cell nuclei visualized on the surface using DAPI. B) The same surface as in A) but scanned using FITC filter to visualize aggrecan. C) Cell nuclei visualized on the surface using DAPI. D) The same surface as in C) but scanned using FITC filter to visualize aggrecan.*

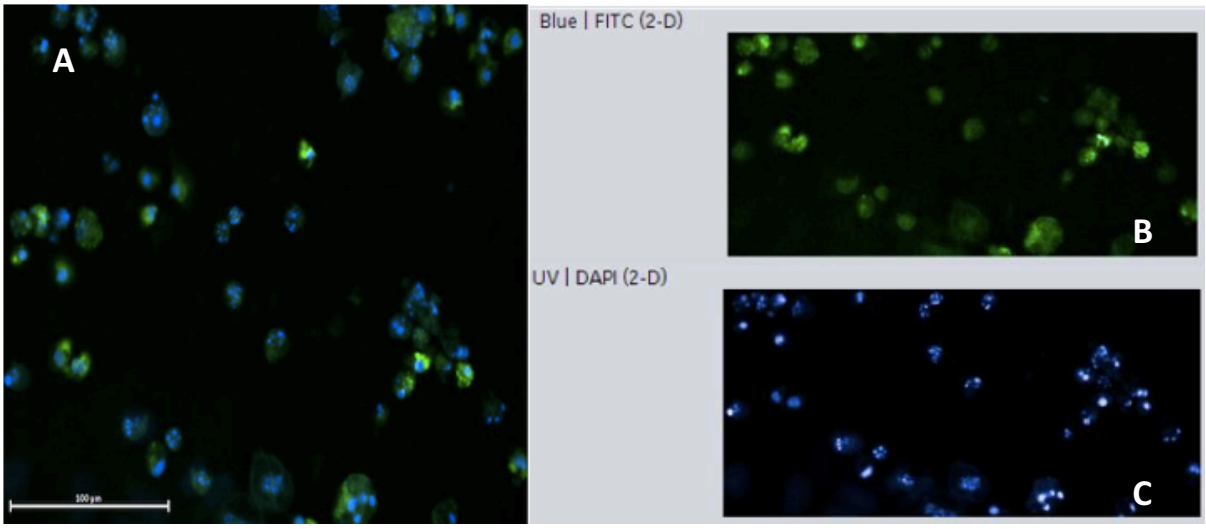


Figure 25. *Detailed picture from the area shown in figure 24B), from surfaces with a high concentration TGFβ-3, scanned in 40X magnification. A) FITC and DAPI merged. The blue color is visualizing cells and the green color is visualizing aggrecan. Scale bar 100 µm. B) FITC, 40X magnification. C) DAPI, 40X magnification.*

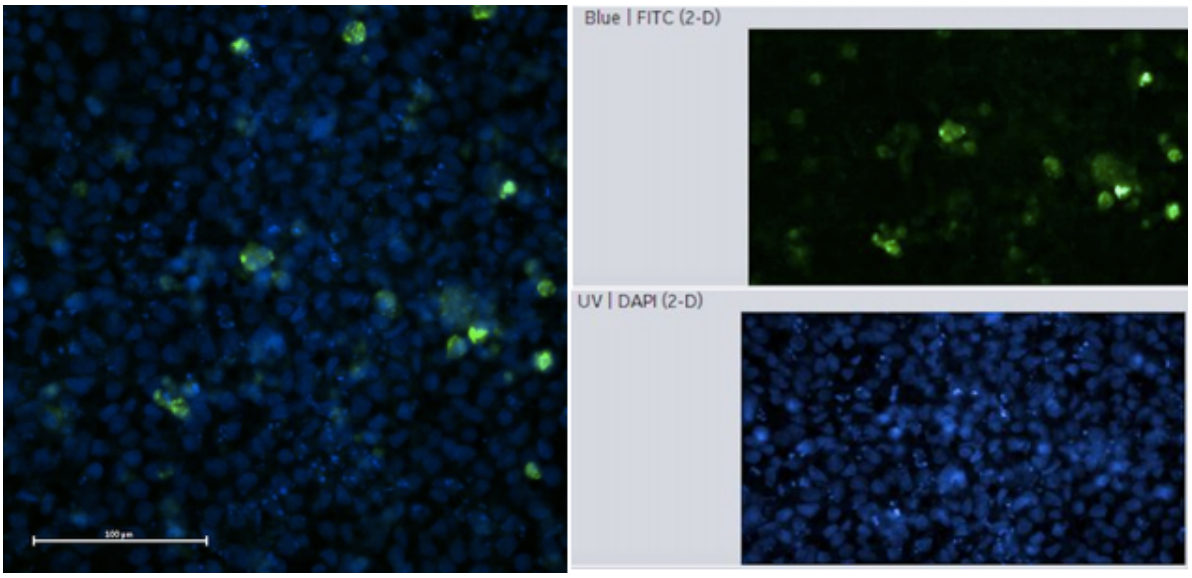


Figure 26. *Detailed picture from area shown in figure 23D), from surfaces with a low concentration TGFβ-3, scanned in 40X magnification. A) FITC and DAPI merged. The blue color is visualizing cells and the green color is visualizing aggrecan. Scale bar 100 µm. B) FITC, 40X magnification. C) DAPI, 40X magnification.*

Negative control surfaces, nano gradients with streptavidin and laminin

Three nano gradients were produced and functionalized with only laminin to be compared to gradients functionalized with GDF5, TGFβ-1, and TGFβ-3. All three of the gradients are visualizing the same tendency, to form cell-free cavities on the surface, indicated in figure 27. Since the gradients with the highest particle density start from left, the tendency is to form more cavities at lower particle densities and higher concentrations of laminin. Gradients do not visualize any specific fluorescent area of interest which are indicating no significant production of aggrecan.

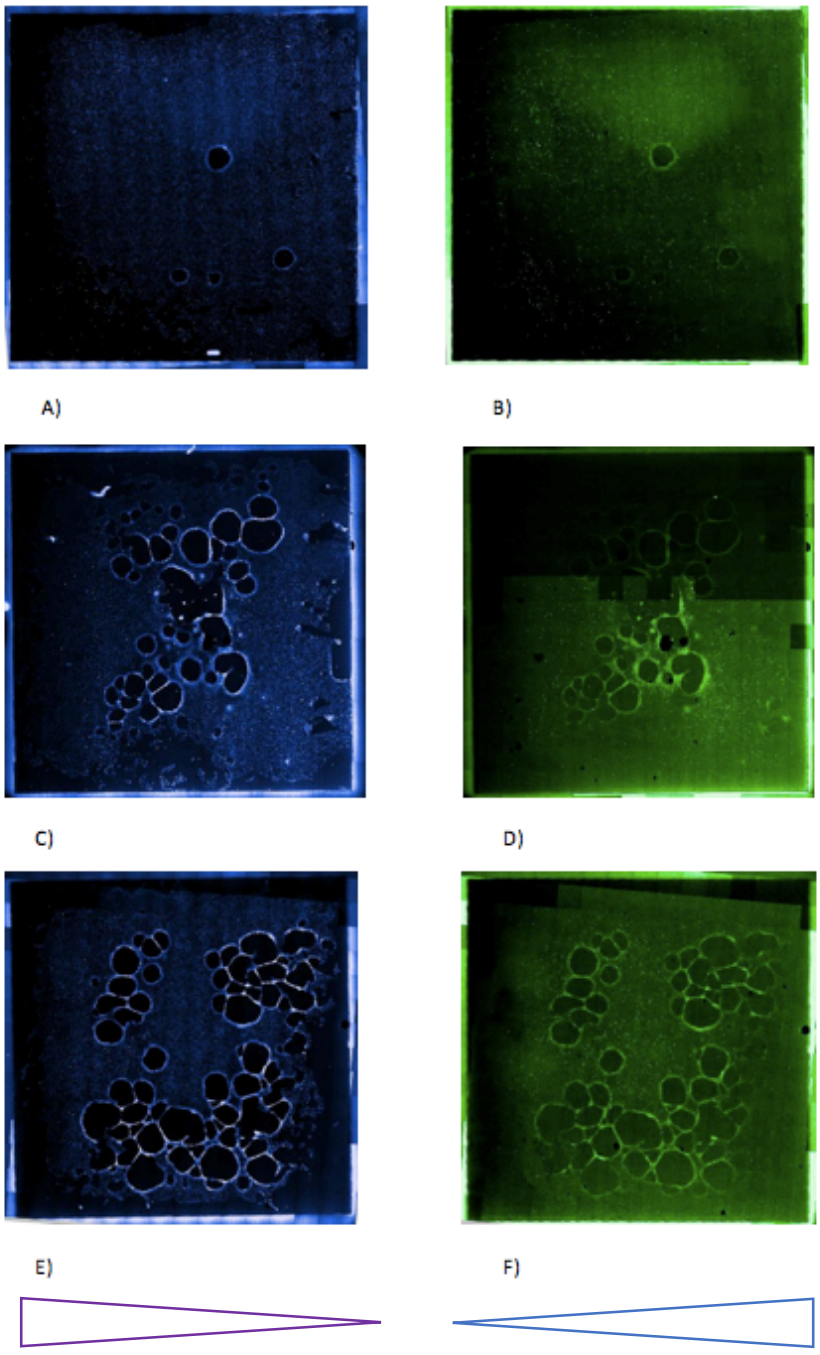


Figure 27. *Gradients 18x18 mm visualized in IN CELL 6000. Three gradients were produced without GDF5, TGFβ-1 or TGFβ-3. The particle gradient with the highest particle*

concentration is starting from the left, indicated by the purple arrow. The laminin gradient is starting from the right, since laminin is bound in the space between particles, indicated by the blue arrow. All three gradients are scanned in 10X magnification. A) and B) are pictures of the same gradient visualized with DAPI in blue and FITC in green. C) and D) are pictures of the same gradient visualized with DAPI in blue and FITC in green. E) and F) are pictures of the same gradient visualized with DAPI in blue and FITC in green.

Experiments based on nano gradient results - analysis of cells removed from surfaces

Cells were cultured and then removed from GDF5 functionalized gradients and surfaces. The cells were then centrifuged to form either chondrocytes pellets or organoids from iPS cells. The reason of using only a GDF5 gradient (and not a TGFβ-1 or a TGFβ-3 gradient) to form chondrocyte pellets was because of the condensed structure that appeared on the GDF5 gradients and surfaces where it is possible that the GDF5 condensed structure is linked to the formation of limb buds and the observations wanted to be confirmed in chondrocyte pellets as well. The organoids were derived from a uniform GDF5 surface and the reason of using only a GDF5 surface (and not a TGFβ-1 or a TGFβ-3 surface) was due to the hypothesis that if aggrecan expression was elevated in GDF5 there are reasons to believe that the aggrecan expression is elevated in the other two molecules as well. Chondrocyte pellets were cultured in a 3D structure for two weeks and organoids from iPS cells were differentiated for six weeks.

Pellets

Two chondrocyte 3D structured pellets were formed from a GDF5 functionalized gradient and cultured for 2 weeks and after two weeks the pellets were sectioned and stained with Alcian blue van Gieson performed by Histocenter. One of the two pellets that were sent back could not be sectioned since it was too small for handling but one pellet could be sectioned into three and analyzed in a microscope. The GDF5 gradient derived pellet observed in the microscope were visualizing a combination of a blue, pink and red/brown color. The blue color was tended to be visualized in a specific area of the pellet while a red/purple color was indicated to be expressed around the blue color and mostly in the edges of the pellet. The blue color is indicating the formation of GAGs and the red/purple color is indicating the formation of collagen, visualized in figure 28.

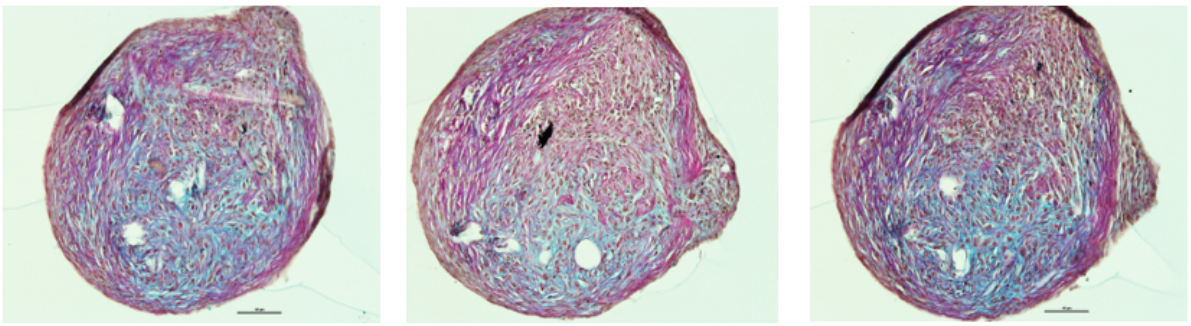


Figure 28. *Chondrocyte pellet from GDF5 gradient cultured for 2 weeks, scanned in 10X magnification. Scale bar 100 μm.*

One pellet was also obtained without using a GDF5 gradient to function as a control to the gradient pellet and can be observed in figure 29. One pellet was also obtained without a GDF5 gradient, figure 30, but additionally to the differentiation medium 5% human serum was added.

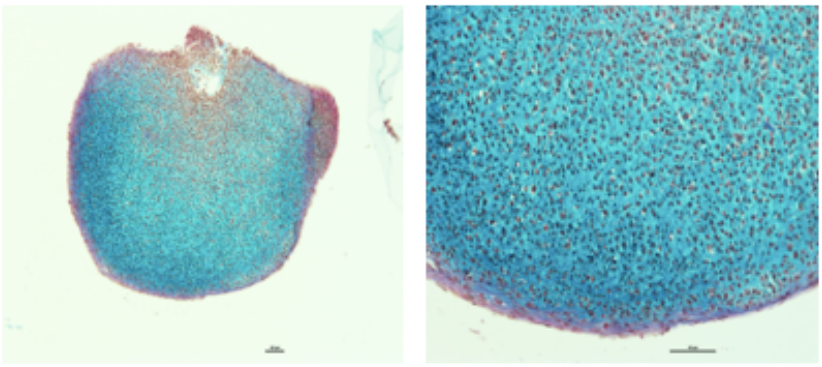


***Figure 29**. Chondrocyte pellet obtained without GDF5 gradient cultured for 2 weeks in differentiation medium, scanned in A) 4X magnification. B) 10X magnification. Scale bar 100 μm.*

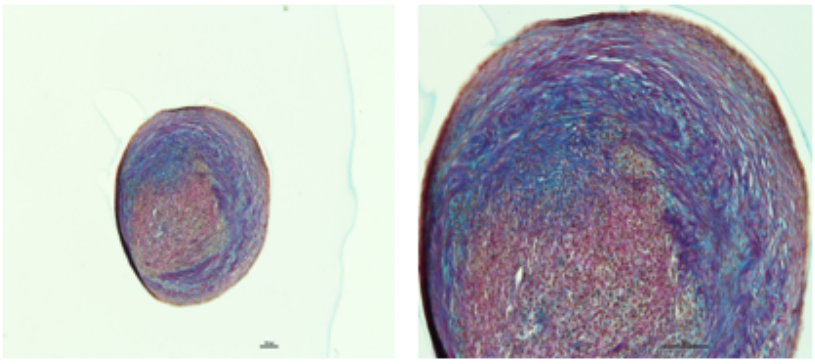


***Figure 30**. Chondrocyte pellet obtained without GDF5 gradient cultured for 2 weeks in differentiation medium plus 5% human serum, scanned in A) 4X magnification. B) 10X magnification. Scale bar 100 μm.*

Organoids

Two iPSc organoids were formed from a GDF5 surface with a particle density at 700 particles/μm^2 which corresponds to a particle density at the end of the gradient. Organoids were imaged every week from week 2 to week 6 to evaluate the aggrecan expression, visualize in figure 31. The green color intensity was observed to be elevated after week 2.

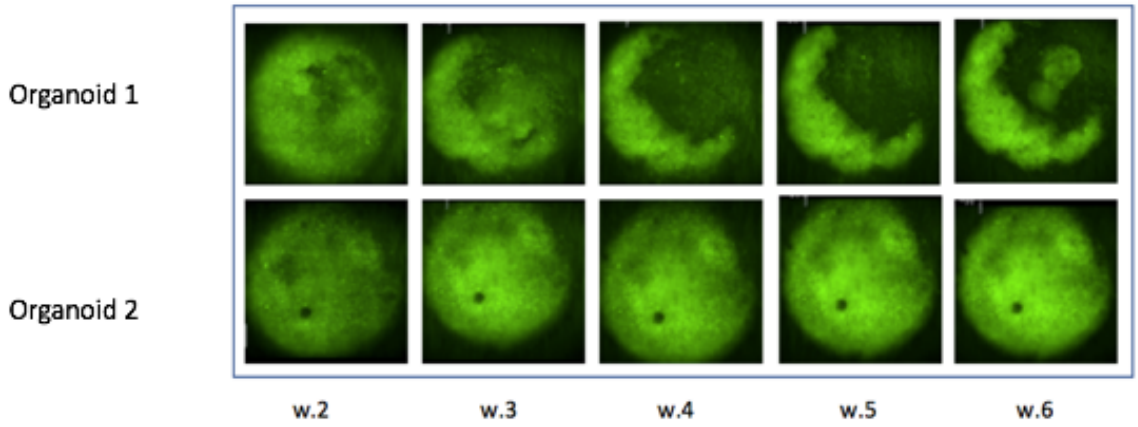


***Figure 31**. Fluorescence imaging of organoids visualized using IN CELL 6000 in 4X magnification to visualize the aggrecan expression over 5 weeks. Aggrecan expression is followed from week 2 until week 6.*

Immunoassays for visualization of proteins in a functionalized gradient

Gradients functionalized with GDF5 was produced for two different antibody immunoassays to visualize attachment of proteins to the nanoparticles on the gradient. Three gradients were included in the experiment where one was incubated with a GDF5 specific primary antibody. The second gradient was incubated with an anti-GDF5 isotype control antibody and a third gradient was a negative control and was treated in the same manner except exclusion of the primary antibody/isotype.

Analysis of immunoassay using DAB for visualization of proteins on GDF5 functionalized gradient

The GDF5 specific primary antibody incubated gradient resulted in a brown color change over the entire surface, seen by the eye. The brown color is visualized when DAB is oxidized by HRP and might indicate binding of the primary antibody to GDF5 thus showing if there are GDF5 bound to the surface, and in such case, where. Using microscopy, the higher detail revealed that the gradient was darker brown in the bottom of the glass surface, where the gradient was located. The gradient incubated with an anti-GDF5 isotype control antibody resulted also in a color change over the entire surface into brown. In the microscope, the gradient was not as dark as the gradient with an antibody specific to GDF5. The brown color might indicate a non-specific binding of the isotype control to GDF5 or another surface molecule. The negative control gradient without primary antibody and isotype also showed a light brown color by visualization in microscopy, primarily around the gradient.
Results from all three gradients are visualized in figure 32.

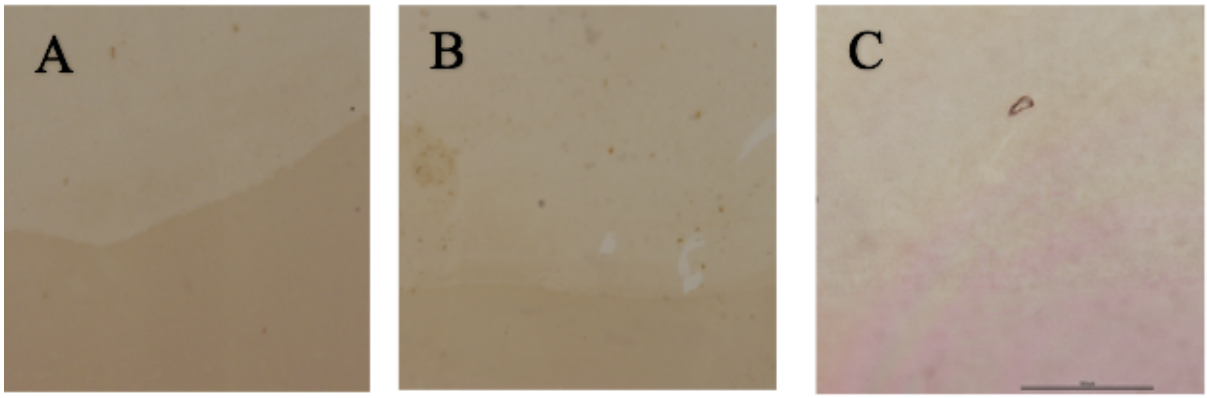


***Figure 32**. Visualization of proteins on a GDF5 functionalized gradient. The darker brown color in the bottom is visualizing the end of the gradient which also can be seen by the eye. A) Gradient stained with a GDF5 specific primary antibody and a biotinylated secondary antibody visualized by DAB in the microscope. B) Gradient incubated with an isotype control antibody visualized by DAB in the microscope. C) Gradient incubated without isotype or GDF5 specific primary antibody visualized in the microscope. The purple color shows the end of the particle gradient which is visualized by eye. Scale bar 500 μm.*

A) B) C)

***Figure 33.** A) Gradient labeled with a specific primary antibody and a brown color is observed by the eye. B) Gradient incubated with isotype control antibody and a brown color is observed by the eye. C) Gradient incubated without primary antibody. No dark brown precipitation is observed by eye.*

Additionally, one gradient without GDF5 was incubated to exclude binding of the secondary antibody to laminin. No indications of binding to laminin were observed since no brown precipitation was observed on the surface.

Analysis of fluorescence immunoassay for visualization of proteins on GDF5 functionalized gradient

During incubation of antibody and isotype in the fridge overnight, the gradients were almost dried out the next day. However, the entire gradients were not dried out so if the method is working, fluorescence might be possible to detect anyway. When gradients were visualized in a fluorescence microscope, no detection of any fluorescence was observed.

Discussion

The Cline nano gradients provide a great tool for cell culture experiments in the sense that they represent “many experiments in one”. Using Clines nano gradients eliminate, as an example, the use of dilution series to find an optimal medium composition with the perfect protein stimuli of live cells. Dilution series are very time-consuming to validate and run and lack the possibility to stimulate cells with precision with maintained low variance between experiments. Also, every medium composition used would also require a validation, which also might be a very time-consuming work. Due to the concentration gradient of biomolecules enabled by the nanoparticle gradient technology, the cells will be affected differently over the surface which enables determination of a composition of biomolecules that stimulate the cells optimally for further high-quality studies.

Experiments on nano gradients and surfaces

Two gradient batches were produced, batch 1 (30102) and batch 2 (30104) and functionalized separately with GDF5, TGFβ-1, and TGFβ-3. The cells used in the project was genetically engineered to express GFP and using the IN CELL 6000 FITC filter, aggrecan expression could be visualized. The cell nuclei were also stained with DAPI to visualize all cells in the microscope. By merging the two different filtered pictures it is possible to determine if the green signal is present in cells (blue signal) or a background signal. The two gradient batches were hard to compare to each other mainly due to the difference in amount cells seeded and the significant difference in appearance. Gradients from batch 1 (30102) were seeded with twice as many cells than seeded on gradient batch 2 (30104) and on other surfaces. The decision to seed a lower number of cells is based on that the cells were loosened from one TGFβ-3 gradient during fixation and it was hard to handle the number of cells on the surface, see figure 22 B.

To function as a control to compare to gradients functionalized with GDF5, TGFβ-1, and TGFβ-3, three gradients with only laminin was produced. Laminin 521 was adhered to all glass surfaces and gradients (between particles) mainly since it is an iPSc stimulating- and adhesion promoting protein. The results from the gradients with only laminin, (Figure 27) showed that cell-free cavities formed on the surface. This indicates that laminin has an effect by itself on the cells. However, the three proteins do influence the cells more, especially GDF5 and TGFβ-3. TGFβ-1, on the other hand, tended to form the same pattern at low concentrations as with only laminin present. Most likely, laminin has a stronger effect than low concentrations of TGFβ-1. Higher concentrations of TGFβ-1 did though not influence the cells to respond in the same manner as low concentrations which might be an indication that the cells are not as sensitive to lower concentrations of TGFβ-1 than GDF5 and TGFβ-3.

GDF5

GDF5 gradients generated cell clusters with higher cell density over the entire gradient. Both gradients also generated a specific area of interest with an overlap around 6.5 mm from the highest concentration of the gradient (white lines are indicating the specific area in figure 12). Within this area, small buds were found that showed a higher intensity in green. If the small clusters are more important than the larger clusters, is hard to tell without other methods to analyze cells, but larger clusters were formed without any concentration dependence.

The two gradient batches were hard to compare to each other mainly due to the difference in amount cells seeded and the significant difference in appearance. It is hard to tell if there were too few cells seeded on gradient batch 2 (GDF5, 30104) where all the cells tended to form a large cluster instead. Cells were seeded over the entire surface but after 5 days of differentiation, all cells were lumped together at a specific spot. Cells on both gradients in batch 2 (GDF5, 30104) formed a cluster at the same area of the gradient which corresponds to a low particle density at the end of the gradient. Even though it was hard to compare gradient batch 1 (GDF5, 30102) to gradient batch 2 (GDF5, 30104), the mutual gradients from the same batch were showing an interesting response at a similar area over the gradient. Both surfaces with low and high density, created in order to see whether aggrecan was more expressed in a high or low GDF5 density, have an impact on the cells to form the clusters found on the gradients.

In the surfaces produced, the cells showed a tendency to behave in the same way and attract to each other, to condense, which is an important event in cartilage and bone formation. Cellular condensation, meaning a reduction of intercellular spaces, is an important part of the process during chondrogenesis and limb skeletogenesis. Also, important for condensation is the cellular microenvironment such as responses from different surrounding molecules [50].

Comparing the three different proteins, GDF5, TGFβ-1, and TGFβ-3, there was clearly shown on the surfaces that GDF5 tend to condense cells much more than TGFβ-1 and TGFβ-3 after 5 days of differentiation, indicating an impact of GDF5 to the condensation process. According to the surfaces with a uniform particle density, condensation tends to appear on both surfaces produced, with different particle densities, indicating that condensation will proceed regardless concentration. Though, it seems condensation proceeds faster in lower concentrations of GDF5.
The surfaces with a lower particle density tended to form smaller, condensed structures in comparison to the cells on surfaces with a higher particle density. Possibly, cells on a surface with a lower particle density and thus a lower concentration of GDF5, condense faster. This conclusion is based on the more dense and compact structure found than comparing to surfaces with a higher concentration of GDF5. GDF5 is known to play a role in the formation of cartilage and joints and the appearance of cells on both gradients and surfaces functionalized with GDF5 seem to indicate formations of limb bud structures.

Even though cells tended to form a condensed structure, aggrecan expression was not significantly expressed overall, though, some parts of the condensed structure were though

further investigated and indicated a higher expression of aggrecan. An elevated aggrecan expression is seen in Figure 16, was found in some regions of the condensed structure. At the edges of the cell clusters, the aggrecan expression was elevated than in comparison to the middle of the structure. If this is because that the expression is higher or if it is due to a higher cell density at the edges has not been determined in this project. Indications (which are discussed more in the "Removal of cells from gradient/surface for further cell culturing in a 3D structure" part in the discussion) are that 5 days of differentiation is too short to induce a high aggrecan expression.

TGFβ-1

From gradient batch 1 (TGFβ-1, 30102) it was hard to identify any specific area of interest. Denser particle clusters can be observed on gradients but not as much and as defined as when comparing with GDF5. Gradients from batch 2 (TGFβ-1, 30104) showed a specific area (C, E, and D, F figure 18) from around 8.5 mm from the highest concentration of the gradient to18 mm. At the lower TGFβ-1 concentration of the gradient, cells formed smaller clusters in a semicircle appearance. Since the results were hard to compare between batches and the gradients in batch 2 (TGFβ-1, 30104) were the gradients that indicated any kind of specific reaction, homogeneous surfaces were produced based on the cellular reaction from gradient batch 2 (TGFβ-1, 30104). Two surfaces with a high particle density were also produced as a comparison.

The cells on the surfaces with a low particle density formed cavities, without any cells, all over the surface. The behavior of the cells is comparable to the results from the surfaces functionalized with only laminin. Most likely, the effect of laminin on the cells induced this behavior overriding the effect of TGFβ-1 at low concentrations. Surfaces produced with a higher concentration did not respond as the surfaces with a low concentration of TGFβ-1. Cells on surfaces with a higher concentration of TGFβ-1 were still after 5 days of differentiation, evenly distributed on the surfaces and no cavities were formed.

Although different cell behavior was observed due to different concentrations, no specific aggrecan expression was observed after 5 days of differentiation.

TGFβ-3

Despite that cells fell of one gradient (Figure 22 B), it was possible to observe denser cell clusters from gradient batch 1 (TGFβ-3, 30102). No specific area of interest was found in batch 1 (TGFβ-3, 3012). When a less number of cells were seeded on the gradients and differentiated for 5 days, a specific area of interest could be identified. Gradients from batch 2 (TGFβ-3, 30104, C and D, figure 22) resulted in an interesting area corresponding to a low particle density. Thus, surfaces with low particle density were produced. To be able to compare the aggrecan expression, surfaces with a high particle density was also produced.

Uniform surfaces, both with a low and a high particle concentration, showed no specific cell behavior such as observed for GDF5 and TGFβ-1. Cells were still evenly distributed on the surfaces, which was visualized using DAPI after 5 days of differentiation. In comparison to TGFβ-1, low concentrations of TGFβ-3 was not inferior by laminin and no cavities were formed. Using the IN CELL FITC filter for visualization of the aggrecan expression, a higher expression was observed on surfaces functionalized with TGFβ-3 than for surfaces with TGFβ-1 and GDF5. Comparing surfaces with low and high concentrations, there tends to be a quite small difference between the surfaces but a small favor of aggrecan expression at surfaces with high concentrations. TGFβ-3 is suggested by previous studies to induce aggrecan expressions [51] which is indicated in this project as well.

Removal of cells from gradient/surface for further cell culturing in a 3D structure

A possible method for culturing cells using Cline Scientific's nano gradients and surfaces have been demonstrated in this project. The gradient technology can be an important tool for determination of an optimal stimulus of proteins. The part of the project that describes how the nano surfaces are used for further high quality culturing was also confirming that it is possible to remove cells from the surface and form 3D structures for further culturing or differentiation. Two chondrocyte pellets were derived from two GDF5 gradients. One advantage of using gradients to form 3D structures is the possibility to mimic the natural development of e.g cartilage, joints and limbs since concentration gradients are present in the body and the gradient might function as an inducer to start a reaction. Two iPSc organoids were obtained from two uniform GDF5 surfaces and an advantage of using a uniform surface is the homogenous protein stimuli which will yield a homogeneous cell population, which is difficult to achieve in cell cultures.

Pellets

Pellets derived from a GDF5 gradient was observed in microscope indicated a combination of a blue color as well as a pink/red as can be seen in figure 28. The blue color is indicating production of GAGs and the pink/red color is indicating the formation of collagen. Aggrecan, which is a proteoglycan, are proteins that are heavily glycosylated with one or more covalently bound GAGs. The blue color observed in pellet might, therefore, indicate aggrecan production which was wanted within this project. On the other hand, in comparison to the pellet formed without GDF5 gradient, figure 29, the GAG production was significantly lower which also might indicate a less production of aggrecan compared to control. In comparison to control pellet (formed without GDF5 gradient) cultured in differentiation medium with 5% serum, figure 30, the appearance is more similar. That might indicate that the effect of the GDF5 gradient is more comparable to the effect of serum. Also, comparable to the serum-cultured pellet, the boundaries of the pellet are more distinct than in comparison to control pellet without serum. Chondrocyte pellet obtained from GDF5 gradient have not produced as many GAGs as pellet without gradient and without serum. Which pellet that is preferable is hard to say. Inducing a high expression of aggrecan was the aim with the project, but one the

other hand, pellet without gradient and serum did not indicate any significant production of collagen, which also is an important component in cartilage. The combination of TGFβ-1 and TGFβ-3 used in pellet medium seem to be a proper combination to induce a high aggrecan expression but the combination of GDF5, TGFβ-1, and TGFβ-3 are indicating a more cartilage-like structure due to indications of both collagen and GAGs.

From experiments on gradients and uniform surfaces, TGFβ-3 was indicated to be one of the three molecules that were most willing to induce aggrecan expression and this finding might also be partly supported by the results of the sectioned chondrocyte pellets without GDF5 gradient cultured in differentiation medium, seen in figure 29, (where TGFβ-1 and TGFβ-3 is added) since it resulted in a significant blue color which is indicating a significant GAG production. The surfaces functionalized with TGFβ-3 had TGFβ-1 added in the differentiation medium but since the aggrecan expression was not as significant on surfaces functionalized with TGFβ-1, indications are that the combination of TGFβ-1 and TGFβ-3 induces a high aggrecan expression but TGFβ-3 is the most important component for the induction.

Organoids

Organoids from iPS cells were differentiated in differentiation medium for six weeks. Every week after week 2, organoids were visualized using IN CELL to determine if aggrecan expression had increased from week 2 to 6. After week two it can be observed in figure 31 that the aggrecan expression was elevated. After week 3 the expression does not elevate significantly. This observation is indicating a peak in the expression of aggrecan after 3 weeks. Comparing the results from the organoids from week 2 to week 6, the indications are that 5 days of differentiation is too short to yield a high aggrecan expression.

Proteins functionalized to nano gradient and surfaces

The proteins with a known concentration, 40 nM in suspension, was attached to functionalize the surfaces and gradient and after sufficient incubation, superfluous proteins were rinsed off with PBS. The binding of these molecules to each other is well known and used in many biochemical applications, e.g., ELISA and other such analytical kits. The protein linking mechanism used to attach the molecules to the particles, depending on the size and form of the biomolecule, is approximately 10-50 proteins attached per gold nanoparticle. Proteins are complicated. There is no universal rule for correlating size and molecular weight, since different proteins, and even the same protein in different environments, have different conformations. With that said, the size of the particles is approximately the same as many proteins used. That is one main reason for using 10 nm particles on the gradients and surfaces.

By rinsing away superfluous proteins, the exact concentration of protein will be unknown, though the linkers will be saturated. Studies have been performed to measure the exact amount of proteins attached to the surface, but the concentrations have been too small to be detected using both spectrophotometry and fluorometry. Mass spectrometry via Proteomics core facility, at Gothenburg University, have also been used to try to determine concentrations

and the results showed that the analysis was at the limit of detection and a specific concentration could not be determined. With certain optimizations though, there are high probabilities that mass spectrometry can be used in the future to determine concentrations of the proteins attached to surfaces.

Protein amounts on gold surfaces and gradients have been measured using a technique called iSPR (imaging Surface Plasmon Resonance) by Lundgren et al., showing that iSPR works well for detecting the number of proteins that bind to and around the gold particles on a gold substrate [52]. The method is not applicable to glass substrates and cannot be used to verify the number of proteins on Clines nano gradients/surfaces. Measurements with QCM-D silicon crystals have also been done to support above reasoning. Since Cline Scientific is using glass substrates and above validations are done on gold substrates the QCM-D measurements have been made to validate data on glass/silicon substrates. QCM-D is not quantitatively the same as iSPR, however, there is not anything observed to suggest that the silicon-gold nanoparticle surface would differ significantly from gold-gold nanoparticle surfaces. Regarding the particle distribution, the latter is confirmed using SEM measurements.

Lundgren et al. have by using time-of-flight secondary ion mass spectrometry (TOF-SIMS) shown the pattern of the gradient and the pattern of the space between particles which proves that molecules attached to particles are distributed in a defined pattern over the surface, see figure 34. They used RGD-peptides to investigate the distribution of the space between the particle gradient. PEG-5000 and Heparin were used to investigate the particle distribution of the gradient since they adhere to the particles [53].

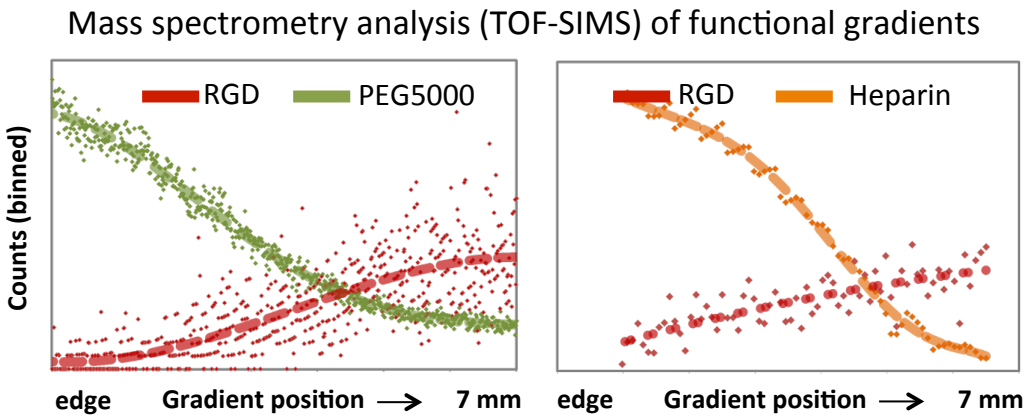


Figure 34. *Gradients with peptides RGD/PEG-5000 and RGD/Heparin were characterized by mass spectroscopy (TOF- SIMS) by Lundgren et al to determine the pattern of the gradient. RGD-peptides binds to the surface between particles and PEG/Heparin bind to particles [53].*

However, despite these results showing that the protein distribution is as claimed, one can also argue that the importance to determine the exact concentration of the proteins attached to the surface is not of total relevance. When using Clines nano gradients and surfaces there are

always possibilities to determine the exact number of particles on the gradient and to produce new surfaces with the desired particle density. It is also a bit problematic to compare proteins in solution and proteins bound to a surface. On a surface, the proteins will be distributed over the surface with a homogeneity and a defined distance to each other which is impossible to control in a solution where molecules constantly are moving. The stimuli from proteins attached to a surface will, therefore, be more concentrated than the stimuli from free proteins in a suspension which also makes it possible to use concentrations that is hard to achieve in a suspension.

Previous studies have been performed where the proteins have been attached successfully to gold nanoparticle surfaces and the concentration ranges of proteins used in these studies are around 5 nM up to 200 nM, which can argue for that proteins in a concentration of 40 nM is proper to add to gold nanoparticle surfaces [54] [55]. When the surfaces are functionalized with different proteins, the aim is to attach as few proteins per particle as possible but still cover all particles with protein. As long as the reaction goes to completion before rinsing off superfluous amounts of protein suspension, all binding sites will be covered and the reproducibility assured. A reason to keep the protein concentrations low are the high purchase costs of the proteins.

As an additional way to visualize protein binding, antibody labeling was performed in this project.

Immunoassay for visualization of proteins on a functionalized gradient

Two immunoassays were performed on GDF5 functionalized gradients. The immunoassays were performed on protein functionalized gradients to show the binding of proteins and no cells were seeded on the gradients. A GDF5 specific primary antibody was incubated directly to GDF5 on the surface.

Immunoassay using DAB for visualization of proteins on functionalized gradient

Precipitation of a brown color by DAB on the gradient labeled with a specific GDF5 primary antibody indicates a specific binding of the primary antibody to GDF5 attached to the nanoparticles. However, color development was also observed on the gradient with primary anti-GDF5 isotype control antibody which indicates a non-specific binding of the isotype control as well. The color intensity of the gradient coated with the isotype control antibody was not as strong as the color intensity of the gradient coated with GDF5 specific antibody. One reason could be that the isotype control used binds non-specifically to GDF5 or other surface structural proteins. Thus, being a less than optimal choice of isotype. Since the isotype gradient is, in comparison to the antibody gradient, less brown indicates that the isotype is not working as it should and it also indicates that it is not as specific as the primary-secondary antibody interaction. However, the gradient labeled with a specific GDF5 primary antibody had a higher color intensity, which may indicate a combined specific binding of the GDF5 specific antibody and non-specific binding. Without a fully functioning isotype, it is

hard to know for certain. When blocking procedure is totally completed there should not be any binding of the secondary antibody to thiolated streptavidin bound directly to particles, which might be a source of non-specific binding. Therefore, the observed non-specific binding can also be due to a not fully optimal blocking procedure. A non-specific binding of the secondary antibody to laminin is less likely since the laminin density increases inversely over the surface compared to the biomolecule density. But in order to make sure this is not the case the latter was also tested with the result that the colorimetric intensity of the non-specific binding did not correlate to the surface-bound laminin.

When primary antibody/isotype was removed, a light brownish precipitation was still observed in the microscope. The colorimetric intensity was lower compared to immunoassay using primary antibody and isotype control antibody. This might indicate a non-specific binding mediated by the secondary antibody. This also confirms a problem with the isotype or the blocking procedure as already discussed. Therefore, it is important to improve the method by e.g. optimizations of the blocking solutions and incubation time to achieve complete blocking and consequently to minimize the non-specific binding. Also, another compatible isotype antibody control is suggested for validations of the method.

Fluorescence immunoassay for visualization of proteins on a functionalized gradient

No fluorescence signal was observed on gradients when they were analyzed using fluorescence microscopy. One possible reason might be a partial dehydration of the primary antibody and the isotype when incubated overnight. However, the entire surface was not dehydrated (only the low-density part of the gradient) so if the protocol would work properly, there are strong reasons to believe a fluorescence signal would at least be observed on the non-dried half of the gradient.

The fluorophore attached to the secondary antibody was Alexa Fluor 546 which has an excitation maximum at 556 nm and an emission maximum at 573 nm. When a fluorophore is used in presence of a metallic nanoparticle, the fluorescence emission might be affected and altered. A quenching effect, directly correlated to the distance between particle and fluorophore has been proved in a study by Kang et.al. which describes how a fluorophore, directly connected to a 10 nm gold nanoparticle is affected by the gold particles strong metallic plasmon field and the electrons from the fluorophore will interact with the field. Kang et.al. describes how the fluorescence signal from the fluorophore will be quenched and how the quenching decreases when the distance between fluorophore and particle increases [56]. Gold nanoparticles, in the range of 10 nm, are exciting light around 520 nm, which is in the same range of excitation as Alexa Fluor 546 and the energy from the fluorophore will be absorbed by the gold nanoparticles resulting in no emitted fluorescence [56]. Quenching from the gold nanoparticles is highly suspected in this case since the fluorophore did not show any light at all. Perhaps, the fact that Alexa Fluor 546 has the same absorption range as the gold nanoparticles makes the fluorophore inappropriate for the study. When choosing the fluorophore, the quenching effect of the nanoparticles was overlooked. This effect is highly

relevant and cannot be excluded as the reason for the lack of signal. For optimizations of the protocol, preferably, a fluorophore in another excitation range might be evaluated before further optimizations can be made.

Conclusion

The aim of the project was to study the effect of the concentrations of proteins (TGFβ-1, TGFβ-3, and GDF5) on cells adhered to a nano gradient. The intended outcome was to generate important information of aggrecan expression dependent on different concentrations of proteins for future studies to try to reveal disease mechanisms for osteoarthritis. One first conclusion that can be drawn is that 5 days of differentiation was not long enough to turn on high aggrecan expressions as much as wanted. Comparing gradients/surfaces from 5 days of differentiation to organoids from 6 weeks of differentiation, the aggrecan expression was indicated to be higher in organoids that were further differentiated.

Even though aggrecan expression was not elevated as much as hoped for, other cell responses to growth factors could be observed. GDF5 tended to induce the formation of condensed cell structures, regardless of concentration. However, the condensation tended to appear faster in stimuli of a lower concentration of GDF5 than in higher concentrations.
Low concentrations of TGFβ-1 showed a similar behavior as control surfaces functionalized with only laminin which might be an indication that the cells lack sensitivity to TGFβ-1 in low concentrations, higher concentrations of TGFβ-1 is required for an effect. Since all surfaces with a low particle density were produced with the same density, TGFβ-1 tend to have less effect at low concentrations than both GDF5 and TGFβ-3.
Regarding TGFβ-3, based on visualizations of surfaces, the indications are that TGFβ-3 induces aggrecan expression more than GDF5 and TGFβ-1. Only a small difference is observed in terms of a concentration dependence, however, the results point towards higher concentrations of TGFβ-3 inducing the highest aggrecan expression. That high TGFβ-3 concentrations is important for inducing aggrecan expression is a very important finding since degradation of aggrecan has been suggested to be a part of the OA disease mechanism.

Attempts to visualize coupled proteins at the nanoparticle gradient surfaces with immunoassays were only partly successful. While GDF5 gradients could be visualized with a DAB based readout, there were indications of non-specific antibody binding to the gradient surface. Fluorescently labeled antibodies were suspected to be quenched by the gold nanoparticles and no signal was detected.

Future research

Since no significantly high aggrecan expression was induced after five days of differentiation, results that also was confirmed in the organoid study, there are strong recommendations to try to differentiate the cells longer on the surfaces, preferably as long as possible (regarding confluency and passaging of cells). Another suggestion if further differentiation on surfaces is not possible, further differentiation after removal from surfaces in a 3D structure is suggested for at least three weeks to induce the aggrecan expression. Also, a complementary analysis for determining if there is an elevated aggrecan expression between week 3 and week 6 in organoids is suggested to be performed since it is hard to distinguish the expression using only fluorescence imaging. A microarray assay is suggested to determine if the ACAN gene is upregulated or not.

Suggested optimizations regarding both immunoassays are also suggested to be performed. Immunoassay experiment using DAB for visualizations of the GDF5 gradient indicated a specific binding of a GDF5 specific primary antibody to GDF5 on the surface. Further optimizations are needed to ensure the statement since some results indicate that a non-specific binding may be suspected. Prolonged blocking procedures are suggested to ensure the completion of the blocking avoiding a non-specific binding to anything else than the protein of interest. Also, another isotype is suggested to be used since there were reasons to expect that the isotype was less than optimal.

Despite no detectable fluorescence signal on gradients labeled with a fluorescent antibody, there are reasons to suggest trying another fluorophore in another excitation range to avoid quenching of the signal. This will not be included in this work, but a suggested first step in optimizations of the method to visualize proteins on the surface.

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