Articular Cartilage Engineering Training School 2023

Program Handbook

İSÜ İSTİNYE ÜNİVERSİTESİ İSTANBUL

cost EUROPEAN COOPERATION IN SCIENCE & TECHNOLOGY

NetwOArk The European Network on OsteoArthritis

İstinye University Stem Cell and Tissue Engineering Research and Application Center

Funded by the European Union

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Welcome

dear participants and distinguished colleagues,

We are pleased to announce "Articular Cartilage Engineering Training School" event, which is scheduled to take place at Istinye University from December 1st to December 3rd, 2023.

At Istinye University, we hold a deep commitment to fostering innovation, promoting knowledge exchange, and advancing education across various fields. The "Articular Cartilage Engineering Training School" embodies these principles, and we are honored to endorse and host this event.

The "Articular Cartilage Engineering Training School" promises to be a remarkable platform for the dissemination of knowledge, the exploration of cutting-edge developments in the field of cartilage engineering, and the exchange of ideas among experts and participants. We encourage all interested participants to seize this opportunity to enhance their knowledge and skills. By engaging with the latest scientific advancements and innovative techniques shared at this event, participants can further advance their careers and make meaningful contributions to the ever-evolving field of cartilage engineering in healthcare.

We extend our sincere appreciation to all individuals and organizations who have contributed to the success of this event, as well as to the dedicated team behind the organization.

I take this opportunity to wish all participants, a productive training school and pleasant stay in Istanbul.

Prof. Dr. Erkan İbis
Rector of Istinye University

Welcome

As Chair of CA21110 NetwOArk I am very pleased that our Action will be organising a highly interesting Training School, hosted by Istinye University in Istanbul. The Articular Cartilage Engineering Training School fits very well with the objectives of our Action and offers great opportunity for our members to learn and meet one another.

Herewith I would like to invite young researchers within the Action to consider participating in this Training School and gain valuable insights, knowledge and hands on training. The programme promises to be highly informatve and relevant.

With kind regards, on behalf of the Action,

Corne BAATENBURG DE JONG
Action Chair
(Deputy Director of *ReumaNederland* – Dutch Arthritis Society)

Welcome

We are pleased to announce "Articular Cartilage Engineering Training School" event, which is scheduled to take place at Istinye University from December 1st to December 3rd, 2023.

Training Schools are integral educational activities of EU-COST-Actions. As with the first training school of the Building an open European Network on OsteoArthritis research" (NetwOArk) CA21110, we, organizers, are eagerly awaiting the opportunity to host this forum where enlightening experiences, exciting innovations and provocative ideas for new approaches will all be batted around with our osteoarthritis patients.

The training school (acets2023.istinye.edu.tr) will feature lecturers and panels as well as hands-on laboratory practice. The main aim of this training school is to share cutting-edge research with our patients and clinicians who are in the osteoarthritis field.

Along with the scientific aspect, it is our desire to take this opportunity to receive feed-back form the patient perspective. The intense program will introduce new research methods that influences osteoarthritis treatment.

With our best regards,

Prof. Dr. Feza KORKUSUZ
Hacettepe University

Assoc. Prof. Dr. Ayça BAL ÖZTÜRK
Istinye University

Detailed Course Program

Time	Activity	Location
01.12.2023 Friday		
09:00 – 09:20	**Registration**	Congress center
09:20 – 09:40	Disease and Illness in OA: netwOArk and building the European Society of OA (EUSOA) (Prof. Christoph Ladel, PhD)	Congress center
09:40 – 10:00	Welcome Erkan İbiş (Rector) & Hatice Gülen (Vice Rector) Ayça Bal Öztürk & Feza Korkusuz (MC) Çağatay Öztürk (Head of Department)	Congress center
10:00 – 10:20	Synthetic mRNA-based approaches for tissue regeneration – Application potential for the treatment of osteoarthritis (Meltem Avci-Adali, PhD, EKU Tübingen)	Congress center
10:20 – 11:00	**Break**	
11:00 – 11:30	Articular Cartilage Tissue Engineering – From Bench to Bedside (Prof. Feng Huei-Lin PhD, NHRI Taiwan)	Congress center
11:30 – 11:50	Bone and cartilage histology and stem cells in osteoarthritis. (Assist. Prof. Hakan Darıcı, PhD)	Congress center
11:50 – 12:10	Obtaining stromal cells from adipose tissue (TOST) with the ultra-sharp blade system and long-term results of orthopedic applications. (Prof. Eray Copçu, MD)	Congress center
12:10 – 12-30	3D bioprinting for tissue/organ engineering (Prof. Dr. Bahattin Koç, PhD)	Congress center
12:30 – 13:30	**Lunch Break**	Congress center
13:30 – 17:30	**Hands-on Laboratory Experiments**	Istinye University 4th floor

Time	Activity	Location
02.12.2023 Saturday		
09:00 – 09:20	The role of tissue engineering in osteoarthritis (Prof. Dr. Seda Vatansever, MD, PhD)	Congress center
09:20 – 09:40	Can microfluidics-enabled platforms expedite the assessment of immune response towards implantable biomaterials for the knee? (Prof. Dr. Özlem Yeşil Çeliktaş, PhD)	Congress center
09:40 – 10:00	Nanoparticular delivery systems for osteoarthritis therapy (Assoc. Prof. Pınar Çakır Hatır, PhD)	Congress center
10:00 – 10:20	Prospective in regenerative medicine and biosensor: when biology meets technology (Valentina Basoli, PhD)	Congress center
10:20 – 11:00	**Break**	
11:00 – 11:30	Current Situation of Adipose-Derived Stem Cells Treatments for Knee Osteoarthritis (Massimiliano Rucci, MD)	Congress center
11:30 – 11:50	Efficacy of stem cell-rich SVF - stromal vascular fraction fluid in treating osteoarthritis (Aşkın Nasırcılar, MD)	Congress center
11:50 – 12:10	Structure and Function of Articular Cartilage (Dr. Girish Pattappa)	Congress center
12:10 – 12-30	Stem cells in orthopedics. (Luminita Labusca, MD PhD, Romania)	Congress center
12:30 – 13:30	**Lunch Break**	-2nd Floor
13:30 – 17:30	**Hands-on Laboratory Experiments**	4th floor

Time	Activity	Location
03.12.2023 Sunday		
09:00 – 09:20	Cartilage-on-a-chip: Opportunities and challenges (Assoc. Prof. Hüseyin Avcı, PhD)	Congress center
09:20 – 09:40	Mesenchymal stem cell treatment for osteoarthritis (Assoc. Prof. Onur Uysal, PhD)	Congress center
09:40 – 10:00	Exosomes for Articular Cartilage Regeneration (Olcay Eren MD)	Congress center
10:00 – 10:20	Fresh Osteochondral Allograft Transplantation for Cartilage Defects (Prof. Gökhan Meriç)	Congress center
10:20 – 11:00	**Break**	
11:00 – 11:30	Safety of drugs used in osteoarthritis (Prof. Semra Şardaş, PhD)	Congress center
11:30 – 12:30	**Panel:** Knee Osteoarthritis Treatment Algorithms: Perspectives of patients, clinicians and researchers. (Prof. Dr. Çağatay Öztürk, Dr. Gürkan Gümüşsuyu & Prof. Dr. Feza Korkusuz)	Congress center
12:30 – 13:30	**Lunch Break**	Istinye University -2nd Floor
13:30 – 15:00	**Project Based Learning (project design - Mini Quiz)** (Assist. Prof. Hakan Darıcı, PhD, Assoc. Prof. Ayça Bal Öztürk, PhD, Assoc. Prof. Emine Alarçin, Gülşah Torkay & Remzi Okan Akar)	Health Sciences Classrooms (TK204)
15:00 – 15:30	**Break**	Health Sciences Classrooms (TK204)
15:30 – 17:30	**Feedback from participants** **Certificate Ceramony**	Health Sciences Classrooms (TK204)

Morning Lectures

01.12.2023 Friday

Prof. Christoph Ladel, PhD
CHL4special

Disease and Illness in OA: netwOArk and building the European Society of OA (EUSOA)

Abstract: Osteoarthritis is one of the most leading causes for disability. E.g. > 600 million people are suffering from knee OA. Despite this high incidence no effective disease-modifying treatment is available. One of the reasons is, that most of the investigated therapies are using a „one-fits-all“ approach, namely not looking in the individual patient pheno- and endotype. The disease AND illness should be taken into account when treating a patient. Already in 1978, Cassell described illness as: ‘what the patient feels when he goes to the doctor’, and disease as: ‘what he has on the way home from the doctor’s office’. In short, disease is something an organ has, as defined by modern medicine, and illness is something a person has. Although illness and disease usually coexist, disease may occur in the absence of illness and vice versa. A patient centricity leading to more personalized medicine is mostly missing and not always addressed, but would help to develop effective therapies. netwOArk was created due to a grant from COST to bring the OA patient in the center of research and investigations. With this approach disease and illness are addressed. The final result would be the creation of the European Society of OA (EUSOA) with large involvement of patients in research and therapeutic approaches. The current netwOArk spans all over Europe and has already created a lot of interactions. This will be further extended through business meetings, workshops and short term scientific mission funded by the COST action.

Prof. Meltem Avci-Adali, PhD
University Hospital Tübingen

Synthetic mRNA-based approaches for tissue regeneration – Application potential for the treatment of osteoarthritis

Abstract: In recent years, the prominence of synthetic messenger RNAs (mRNAs) has grown significantly, marking them as pivotal players in the field of innovative treatments for both disease prevention and therapeutic interventions. The unique ability of synthetic mRNAs to transiently express desired proteins, particularly those that are missing or defective, positions them at the forefront of cutting-edge medical advancements. Synthetic mRNAs can be applied to generate footprint-free induced pluripotent stem cells (iPSCs), which enable the generation of autologous cells for personalized cell therapies. In addition, synthetic mRNAs find application in the production of secretable proteins, the expression of receptors on cell surfaces, and the facilitation of gene correction processes. In the field of osteoarthritis, synthetic mRNAs have also been applied to prevent apoptosis, to stimulate proliferation and ECM homeostasis. This versatility of synthetic mRNAs underscores not only their importance in addressing diverse medical challenges but also their potential to revolutionize treatment approaches for a wide range of diseases and conditions, including osteoarthritis.

Prof. Feng Huei-Lin, PhD
National Taiwan University

Articular Cartilage Tissue Engineering – From Bench to Bedside

Abstract: Autologous fibrin glue has been demonstrated as a potential scaffold with very good biocompatibility for neocartilage formation. However, fibrin glue has been reported not to provide enough mechanical strength, but with many growth factors to interfere the tissue growth. Gelatin/hyaluronic acid/chondroitin-6-sulfate (GHC6S) tricopolymer sponge has been prepared as scaffold for cartilage tissue engineering and showed very good results, but problems of cell seeding and cell distribution troubled the researchers. In this study, GHC6S particles would be added into the fibrin glue to provide better mechanical strength, better cell distribution, and easier cell seeding, which would be expected to improve cartilage regeneration in vitro. Porcine cryo-precipitated fibrinogen and thrombin prepared from prothrombin activated by 10% CaCl2 solution were used in two groups. One is the fibrin glue group in which porcine chondrocytes were mixed with thrombin–fibrinogen solution, which was then converted into fibrin glue. The other is GHC6S-fibrin glue in which GHC6S particles were added into the thrombin–fibrinogen solution with porcine chondrocytes. After culturing for 1–2 weeks, the chondrocytes cultured in GHC6S-fibrin glue showed a round shape with distinct lacuna structure and showed positive in S-100 protein immunohistochemical stain. The related gene expressions of tissue inhibitor of metalloproteinases-1, matrix metalloproteinase-2, MT1-MMP, aggrecan, decorin, type I, II, X collagen, interleukin-1 b, transforming growth factor-b 1 (TGF-b1), and Fas-associating death domain were checked by real-time PCR. The results indicated that the chondrocytes cultured in GHC6S-fibrin glue would effectively promote extracellular matrix (ECM) secretion and inhibit ECM degradation. The evidence could support that GHC6S-fibrin glue would be a promising scaffold for articular cartilage tissue engineering.

Asst. Prof. Hakan Darıcı, PhD.
İstinye University

Histology and Stem Cells of Cartilage and Bone

Abstract: Cartilage and bone are two connective tissue subtypes with fibers, ground substance and cells. Cartilage is made of mostly type-II collagen while bone is abundant with type-I collagen and additional inorganic components such as hydroxyapatite crystals. Both tissues have active, extracellular matrix (ECM) producing cells blast cells as chondroblasts and osteoblasts, and their mature, metabolically less active forms as chondrocytes and osteocytes. Although both tissues originate from mesoderm and can regenerate from mesenchymal stem cells (MSCs) throughout the life, bone tissue have an additional progenitor cell between MSCs and osteoblast cells.

MSCs can differentiate both osteoprogenitor cells or chondrocytes easily during developmental period or during regeneration in adult life. Growth and regeneration of the cartilage happens from both inside and from periphery while bone can grow only from peripheral layers called periosteum or endosteum. Lengthening of the bone is more complicated and requires sequential growth differentiation and death of cartilage cells and replacement with bone progenitors in the areas of long bones called epiphyseal plates. Articular cartilage regeneration is different than regular cartilage growth and resembles the epiphyseal plate where new cartilage cells constantly produced and sent upwards to renew deformed articular surface. Diseases such as osteoarthritis develops when this regeneration slows down and the stem cell supply of the cartilage is not enough to counter the demand on the surface. Over time with the progress of the disease, eroding articular surface reaches the bottom stem cell layer and finally destroys the final defenses of the articles till no cartilage is left and only bone remains, which causes movement disabilities and too much pain in the patients.

Prof. Eray Copcu, MD
Adnan Menderes University

Obtaining stromal cells from adipose tissue (TOST) with the ultra-sharp blade system and long-term results of orthopedic applications.

Abstract: Regenerative medicine stands at the forefront of medical advancements, emerging as a promising branch with substantial developments, particularly after post-COVID-19 pandemic. This transformative period extends beyond healthcare, influencing sectors like energy and nutrition. Notably, the medical field has witnessed a paradigm shift from conventional pharmaceuticals to innovative cell-ceuticals, harnessing the inherent regenerative capabilities within an individual.

Our bodies comprise parenchymal and stromal cells, with stromal cells playing a pivotal role in regenerative practices. These cells, responsible for repairing damage caused by various factors, are integral to the evolving landscape of regenerative medicine and surgery. Among the various tissues housing stromal cells, adipose tissue boasts the highest concentration and diversity.

This abstract explores the methods employed to liberate and obtain stromal cells, focusing on the advantages of the ultra-sharp blade system. Direct methods involve enzymatic processes, demanding a controlled environment adhering to cGMP and cGLP standards, and costly equipment. Conversely, ultra-sharp blade systems offer a cost-effective, rapid, and straightforward approach, operating within minimal manipulation limits and complying with regulatory standards set by institutions such as the FDA and EMA.

The ultra-sharp blade system not only yields a higher quantity of cells but, crucially, preserves the extracellular matrix (ECM). The ECM serves as a conductor directing regenerative orchestration, proving essential in various regenerative applications. Its role in providing a cellular microenvironment for tissue engineering and disease treatment underscores its significance. The neutral, biocompatible, degradable, and controllable nature of ECM makes it an ideal biomaterial for mimicking cellular microenvironments.

In challenging clinical scenarios, such as cartilage healing, the ultra-sharp blade system becomes indispensable. This method ensures the protection of ECM and the maximum extraction of cells, making it particularly advantageous in orthopedic regenerative applications. By elucidating the merits of this approach, we aim to contribute to the optimization of regenerative procedures, fostering advancements in medical science and patient care.

Prof. Bahattin Koc, PhD.
Sabancı University

3D Bioprinting for Tissue/Organ Engineering

Abstract: Bioprinting is a relatively new tissue/organ engineering method that involves printing living cells, with or without biomaterials, layer by layer to create three-dimensional living structures. Unlike other tissue engineering approaches, this method fabricates complex biological structures using only live cells, biomolecules, and biomaterials. This presentation will discuss biomimetic 3D bioprinting, bioprinters, and bioinks for tissue and organ engineering. The bioprinting process, including how to digitally copy and design tissue and organs, how to prepare bioinks, bioprinting instructions, and how to print live cells, will be explained. The presentation will also cover several applications and challenges in organ printing.

2.12.2023 Saturday

Prof. Seda Vatansever, MD
Celal Bayar University

The Role of Tissue Engineering in Osteoarthritis

Abstract: Osteoarthritis (OA) is defined as a whole joint disease and is characterized by cartilage destruction, subchondral bone change, osteophyte formation, and alterations of ligaments and meniscuses affects the quality of life of more than 300 million people worldwide. While significant progress has been made in defining the pathological mechanism of OA, current therapeutic approaches, including surgical interventions and pharmaceutical treatments, are palliative but not curative. Non-pharmacological therapies are used for patients in the early stages of OA to delay its development. However, the effects of these approaches on early symptoms are limited. Thus, it is necessary to develop new therapeutic strategies using tissue engineering techniques with or without cells. Both biomaterials and stem cells including mesenchymal and induced pluripotent stem cells can provide a good source for tissue engineering. These two type of stem cells can be obtain as autologous which is important advantages for cellular therapy. Mesenchymal stem cells can be differentiating under suitable culture condition to other type of cells such as osteoblast, chondroblast etc. The mechanism by which adipose tissue mesenchymal stem cells induce cartilage regeneration remains unclear. Secreted proteins, microvesicules or exsomes form stem cells are also detected. Therefore, rather than stem cells, the products secreted by stem cells may be alternate direction for OA treatment. Because of regulating of local microenvironment through paracrine nutritional factors and control of immune regulation during OA, it should be main aim for regeneration and repair and subsequently delaying cartilage degradation and improving joint function. Therefore, future studies on stem cells or their secreted products aim for therapeutic mechanisms in OA.

Prof. Özlem Yeşil Çeliktaş, PhD
Ege University

Can microfluidics-enabled platforms expedite the assessment of immune response towards implantable biomaterials for the knee?

Abstract: The immune response to the biomaterial in the patient's body after implantation may shorten the functional life of the implantable biomaterials depending on the degree of the response and adversely affect the quality of life. Therefore, understanding the immune response and designing strategies to modulate such a response represent a grand challenge for implantable devices and biomaterials. By developing 3D in vitro models, the M1/M2 macrophage ratios, pro-inflammatory and anti-inflammatory cytokine amounts can be determined to evaluate the immune response to the implantable biomaterials before clinical use. Here, the development of a microfluidic platform is reported for modeling the cascade of events during immune cell response to implants. The platform models the native implant microenvironment where the implants are interfaced directly with surrounding tissues, as well as vasculature with circulating immune cells. The materials representing the implant is located at the bottom layer. The responses towards titanium beads and various hydrogels that we have previously studied such as polyacrylamide-alginate (PAAm-Alg) hydrogel, which has been previously characterized as a biocompatible material and shown to enhance regeneration of cartilage in vivo, along with a graphite enhanced hydrogel (PAAm-Alg-G) as a non-biocompatible counterpart, to evaluate macrophage attachment and polarization to pro- or anti-inflammatory phenotypes are discussed. The study demonstrates that the release of cytokines such as monocyte chemoattractant protein 1 from the extracellular matrix-like hydrogels in the bottom tissue chamber induces trans-endothelial migration of circulating monocytes in the vascular channel toward the hydrogels, thus mimicking implant-induced inflammation. Predicting immune responses by developing an alternative 3D in vitro model provides various advantages such as increasing the success of treatment, thereby well-being of the patient and reducing the associated costs burdening the healthcare system.

Assoc. Prof. Pınar Çakır Hatır, PhD
İstinye University

Nanoparticular Delivery Systems for Osteoarthritis Therapy

Abstract: This abstract provides a comprehensive overview of the evolving landscape of nanoparticle-based drug delivery systems for osteoarthritis therapy. Osteoarthritis, the most common degenerative disorder of the whole joint, affects more than half of people over 65, which is associated with significant inflammation, cartilage degeneration, and joint pain. Changes in chondrocyte structure and an irreversible loss of extracellular matrix are characteristics of osteoarthritis, which frequently results in biomechanical failure. Drug therapy for disorders involving orthopedic tissues is often complicated by the anatomic structure of these tissues. For instance, drugs injected directly into intra-articular joint space clear out rapidly providing only short-term benefit. Therefore, the need for targeted drug delivery to the affected joints is arisen. Nanoparticles offer a promising avenue for overcoming these challenges, providing precise control over drug release, improved bioavailability, and enhanced therapeutic efficacy.

The talk will cover drug delivery systems with various nanoparticle formulations, including liposomes, polymeric nanoparticles, and micelles, highlighting their unique attributes for encapsulating diverse therapeutic agents for the treatment of osteoarthritis and the translational potential of these delivery systems.

Valentina Basoli, PhD
University of Basel

Prospective in regenerative medicine and biosensor: when biology meets technology

Abstract: Regenerative medicine aims to restore damaged or diseased tissues through tissue engineering strategies. These strategies involve the creation of tissue constructs that mimic the native tissue architecture and possess the ability to integrate seamlessly with the host tissue. However, ensuring the quality and functionality of these tissue engineering constructs remains a critical challenge.

Monitoring the physiological conditions within tissue engineering constructs is essential for successful tissue regeneration. Biosensors can detect deviations from optimal conditions, such as inadequate oxygen supply or accumulation of harmful metabolites. This real-time feedback allows researchers and clinicians to make timely interventions and adjustments to optimize construct development and improve tissue regeneration outcomes.

During the talk will be discussed the state the art in regenerative medicine, cartilage biology and osteoarthric model development and the future integration of biosensors for monitoring analytes, focusing on the best biological model for the test of nanoparticles for new therapies, scaffold development and use of emerging technology for real-time monitoring of physiological cell behavior.

The integration of biosensors in regenerative medicine has the potential to revolutionize the field of orthopedics. Indeed, sensor technology can offer a means to bridge the gap between conventional tissue engineering techniques and clinical success by providing continuous monitoring and feedback on the quality and performance of tissue constructs. The seminar will discuss applying the best sensing solutions, such as electrochemical biosensors or optical biosensors based on single-wall carbon nanotubes directly in the engineered construct and possible patient application.

Massimiliano Rucci, MD
University of Genoa

Current situation of adipose-derived stem cells treatments for knee osteoarthritis

Abstract: In a treatment panorama for Knee OA dominated, for the last 40 years, by TKA and other prosthetic joint replacements, the possibilities offered by Regenerative Medicine and MSCs have shifted the Surgeons' mindset to a more conservative one. My presentation will explore the possibilities offered in scientific literature by MSCs and ADSCs in OA and Knee OA, specifically. It will also cover several aspects of the treatment protocol we follow as a team in Turin, Italy.

Aşkın Nasırcılar, MD

Efficacy of stem cell-rich SVF - stromal vascular fraction fluid in treating osteoarthritis

Abstract: Osteoarthritis (OA) is the most prevalent joint disease in the world. There is no effective or curative treatment of disease progression. Stem cell-based therapies have emerged as possible disease-modifying treatments. Recently, mesenchymal stem cell-rich stromal vascular fraction (SVF) has gained popularity due to its easy availability from subcutaneous fat tissue and fewer legal restrictions. There are either mechanic or enzymatic ways to obtain SVF fluid. Mechanically obtained SVF fluid may called as Micronized / Microfragmanted adipose tissue - SVFs (MAT-SVFs) in the literature. Multiple studies have supported intraarticular injections of both mechanically and enzymatically obtained SVF for OA. This presentation will cover impacts of SVFs on OA as well as limitations and regulatory challenges.

Dr. Girish Pattappa
University Hospital Regensburg

Structure and Function of Articular Cartilage

Abstract: Articular cartilage is a tissue that lines the bones of synovial joints to help reduce contact stresses on the underlying bone and enabling smooth joint motion. Injury to the articular cartilage due to trauma or sports activities can result in cartilage lesions that lead to the onset of early osteoarthritis (OA). Due to the fact that it is an avascular and aneural tissue, natural repair mechanisms creates fibrocartilage that is structurally different to articular cartilage. Thus, with time, the tissue further degrades and this leads to exposure of the underlying subchondral bone that causes pain for the patient. Patients at late stage OA can have total knee replacement (TKR) to restore joint motion. However, interventions at the earliest stages of OA development, particularly treatment of focal cartilage lesion, could provide long-term benefits for the patient and ease healthcare burdens.

Thus, to create solutions for regenerating articular cartilage, an understanding of the development, structure and function of the tissue is required. This presentation will outline the embryology of articular cartilage, the structure and matrix components within the tissue and then how post-injury and with time, this develops into OA. The seminar will then describe how cellular-based therapies (e.g. chondrocytes or mesenchymal stromal cells) can prevent OA at early stages and potentially regenerate articular cartilage utilising environmental stimuli to optimise their performance and how potential drug therapies can be developed in the future.

Luminita Labusca, MD, PhD.
University Hospital Saint Spiridon Iasi Romania

Stem Cells in (regenerative) Orthopedics - Between Basic Science and Clinical Practice in Orthopedic Surgey

Abstract: The talk will provide basic notion regarding stem cell definition, function, currently accepted clasification, stem cell types and their importance for the rising field of regenerative medicine. The use of stem cell in regenerative medicine will be briefly introduced. The two major modalities of accesing stem cell use in clinical practice- third generation orthobiologics and stem cell based advanced medicinal products will beintroduced.. The principal currently used tissue sources of stem cells in orthopedic and access modalities s will be presented together with their particularities and modalities of use. Several general considerations and reccomandation for the use of orthobiologics will be provided.

03.12.2023 Sunday

Assoc. Prof. Hüseyin Avcı, PhD
Eskisehir Osmangazi University

Cartilage-on-a-chip: Opportunities and Challenges

Abstract: Cartilage-on-a-chip (CoC) platforms are one of the most promising recently emerged technologies to recapitulate their in vivo counterparts. Thanks to the lab-on-a chip system, there are great advantages to mimicking the dynamic and microenvironment of multifaceted joint tissue, i.e., integration with mechanical stimulation; multi-tissue interaction, and incorporating with immune cells; reducing human errors for long-term monitoring in addition to cost savings and faster analyses. On the other hand, the requirement for animal testing before clinical trials in the United States since the 1930s has been eliminated with the recent adoption of the FDA Modernization Act 2.0. This situation is expected to further accelerate organ-on-a-chip and CoC studies. However, despite all these achievements, the development of this technology and the establishment of health policies with standardization criteria are of great importance.

Specifically, for the CoC systems, there are many other areas that need improvement, such as designing complex microsystems; adding cell diversity with including nerves, ligaments, menisci and biochemical factors with ECM parameters; extending running time with cyclic stretch to mimic daily and sport activities; improving the materials used or investigate new substances. However, with the advantages of computational modeling, bioinformatics, artificial intelligence, and deep learning, it is expected that this technology will bring new perspectives and eliminate some of its drawbacks.

Assoc. Prof. Onur Uysal, PhD.
Eskisehir Osmangazi University

Mesenchymal Stem Cell Treatment for Osteoarthritis

Abstract: Osteoarthritis (OA) is a degenerative joint disease that causes destruction of the chondrocytes through destructive inflammatory cytokines, metalloproteinases and mechanical stress which subsequently leads to joint space narrowing, inflammation of the synovial membrane, degeneration of ligaments, enlargement of the joint capsule, formation of osteophytes and subchondral cysts. OA is not an inactive degenerative disease; on the contrary, it is a dynamic disease caused by the imbalance between restoration and destruction of joints. Previously described as a consequence of aging, it is now established that many factors can induce OA, such as genetic predisposition, abnormal joint biomechanics, traumatic injury, load excess, repetitive joint use, overweight or abnormal bone density.

Because cartilage has a unique physiological structure of lacking neural and vascular tissue, its ability of self-repair is extremely limited. The primary treatment goals for OA are to reduce pain and slow or halt the disease's progression. Numerous non-pharmacological and pharmacological treatments are currently utilized for the treatment of OA. Commonly used pharmacotherapies are acetaminophen, non-steroidal anti-inflammatory drugs, and opioid analgesics. Intra-articular injections of corticosteroids are also applied. Exercise, dietary advice, and weight loss are non-pharmacological treatments that can improve physical function and reduce pain as well as the progression of cartilage degeneration. Intra-articular injections of corticosteroids and hyaluronic acid in are commonly recommended for OA. However, these treatments can only transiently relieve OA symptoms and do not delay the progression of OA, and patients eventually must receive joint replacement. Moreover, joint arthroplasty carries its own risks: substantially more invasive, infection, persistent pain, leg length discrepancy, and elevated costs. It also requires long post-operative rehabilitation, and may require revision if the surgery fails, or if the components wear out over time.

Recent advancements in cell-based therapy offer new hope for the treatment of OA patients. Currently, autologous chondrocyte implantation (ACI) is the only cellular based therapy approved by Food and Drug Administration (FDA). However, currently, no therapy has been shown to protect articular cartilage, nor approved to prevent the progression of OA. Due to the complex pathology and its slow progression, and the lack of biomarkers for early diagnosis of OA, a challenge remains for researchers to develop efficient therapies to treat and modify OA.

Stem cell (SC) therapy is a turning point in for the treatment of OA in regenerative medicine. The principal objective of regenerative medicine is to repair defectives or aged tissues by preserving their morphology and native function.

Mesenchymal stem cells (MSCs) are non-hematopoietic, multipotent, and adult stem cells that can be obtained from various tissues, such as bone marrow, adipose tissue, umbilical cord tissue, synovial membrane, and synovial fluid. Many studies have shown that MSCs have the biological characteristics of multi-directional differentiation, diverse plasticity, and low immunogenicity.

MSCs can modulate local inflammation, cell apoptosis and proliferation, and participate in tissue repair, and can achieve certain effect of delaying degenerative changes when used in the treatment of OA. MSCs interact with immune cells and are responsible for the modulation of a number of effector functions, immunomodulatory properties, migratory abilities, the induction of peripheral tolerance, inhibiting the release of pro-inflammatory cytokines, and the promotion of tissue repair.

The advantage of using MSCs for treating OA include their capacity to differentiate into chondrocytes and their potential to prevent chondrocyte apoptosis and to prevent the overall process of degeneration (through a paracrine effect). The paracrine effect of MSCs on the surrounding tissue reduces inflammatory responses and helps repair damaged cartilage.

In conclusion, untreated OA will not heal spontaneously, and current standard treatments are very limited due to the lack of vascularization in the cartilage tissue. Due to their capacity for differentiation into chondrocytes and due to their immunomodulatory properties, MSCs have been the most extensively explored as new therapeutic agents in the cell-based therapy of OA. MSCs are a good candidate to meet the challenge in treating OA. They can repair the damaged tissues or provide immunomodulatory function to reduce inflammation in OA. Since OA is a degenerative joint disease likely involving the depletion of endogenous MSCs, and adult MSCs have the potential to differentiate into cells of chondrogenic lineage, investigation into MSC-based therapy should be supported for potential articular cartilage repair and regeneration.

Although the application of MSCs in joint repair is well established, it is particularly exciting about MSCs being used for OA treatment. Future challenges may include the efficient isolation and culture of MSCs from defined and reliable sources. MSCs made through good manufacturing practices must be carefully evaluated through a combination of means including biochemical, genetic, and epigenetic markers, as well as bioactive assays to establish the efficacy of cells, their proliferative activity, and reparative potentials before they can be used in humans. Furthermore, delivery systems for MSCs and evaluation of their safety and effectiveness also need to be investigated. It is hopeful that these studies can be accomplished in the near future and OA patients may receive much needed help soon.

The mechanism of action of the stem cell, as well as its chondrogenic potential and immunomodulatory effects in the OA model, must be thoroughly investigated before a new treatment strategy can be implemented. Proper isolation, delivery and management of the stem cells isolated from the patient must be carefully evaluated to avoid immunological rejection and to make sure optimal number of the MSC is obtained. Furthermore, innovative methods using MSC and biomaterial construct for cartilage tissue engineering must be investigated to improve the chondrogenic potential and immunomodulatory properties of the system for OA treatment.

Olcay Eren, MD
Istanbul Fatih Sultan Mehmet Training and Research Hospital

Exosomes for Articular Cartilage Regeneration

Abstract: Exosomes are small, membrane-bound vesicles that are released by cells into the extracellular space. They are a type of extracellular vesicle, ranging in size from about 30 to 150 nanometers in diameter. Exosomes contain various bioactive molecules, including proteins, lipids, and nucleic acids (such as RNA and DNA). These vesicles play a crucial role in intercellular communication, as they can be taken up by other cells, influencing their function and behavior. Exosomes are involved in various physiological processes and are implicated in disease progression, including cancer, neurodegenerative disorders, and inflammatory conditions.

Many recent studies have confirmed that, in both physiological and pathological states, various cell types, including synovial fibroblasts and chondrocytes, secrete exosomes in the internal environment of the joint. This secretion reflects the pathological changes associated with joint diseases, offering a potential avenue for early diagnosis. Additionally, exosomes, rich in cargo components such as RNAs and proteins, play a crucial role in mediating intercellular communication and enhancing cellular function. Owing to their nanocharacteristics, low immunogenicity, and excellent biocompatibility, exosomes hold the potential to serve as carriers for targeted delivery of bioactive molecules or drugs.

As a signaling molecule involved in cellular interaction, exosomes can accelerate the progression of OA or promote articular cartilage repair. Specifically, exosomes secreted by therapeutic cells, especially MSCs, have been used to treat OA by transferring their bioactive substances to regulate the damaged tissue environment and coordinate the subsequent regenerative process. The mechanisms of exosomes for cartilage repair include mechanisms that regulate the immune response, inhibit chondrocyte apoptosis and matrix degradation, promote proliferation and chondrogenesis of stem cells in situ, as well as promote directional migration to a site of injury.

In addition, gene modification and tissue engineering can be used to modify exosomal secretion and contents and obtain more ideal therapeutic exosomes to increase their therapeutic efficacy. Tissue repair and regeneration has an exciting future in the treatment of osteoarthritis. The combination of gene therapy, stem cell therapy, and tissue engineering, as well as interdisciplinary collaboration between orthopedic surgeons, materials scientists, biomechanical engineers, and molecular biologists, is crucial to the future success of these technologies. The challenge will be to develop a reliable technology validated to treat the varying complexities of joint injuries and early degenerative lesions.

Prof. Gokhan Meric, MD
Yeditepe University Medical School

Fresh Osteochondral Allograft Transplantation for Cartilage Defects

Abstract: Large and complex chondral lesions of the joints are common, and their treatments are challenging. Several surgical treatment options are described in the literature for chondral lesions. In case of failure of other surgical methods or patients with the massive osteochondral defect, fresh osteochondral allograft (OCA) has been used as a biological salvage treatment option for the joints. The principle of fresh OCA is the replacement of cartilage defects and subchondral bone with an osteochondral allograft transplant. This allogeneic osteochondral unit consists of mature hyaline cartilage with viable chondrocytes along with an underlying subchondral bone. In the literature, fresh OCA transplantation is described as a reliable treatment option for cartilage defects.

Prof. Semra Sardas, PhD.
İstinye University

Safety of Drugs Used in Osteoarthritis

Abstract: Osteoarthritis (OA) is the most common musculoskeletal chronic progressive disorder of the synovial joints that is often age related and/or trauma induced. Existing treatments aim to reduce pain and symptoms, as well as improve joint functional capacity. Current pharmaceutical treatment for OA is largely restricted to analgesics including nonsteroidal anti-inflammatory drugs (NSAIDs), which are both palliative in nature and accompanied by adverse effects. The traditional medications can be categorized as; acetaminophen, non-steroidal anti-inflammatory drugs (NSAIDs), opioid analgesics, serotonin-norepinephrine reuptake inhibitors (SNRIs), intra-articular (IA) injections of corticosteroids, and dietary supplements. Although there are generally accepted guidelines for these traditional drugs, they need to be used with caution due to the growing concerns with adverse effects such as hepatotoxicity, gastrointestinal complications, septic arthritis, and cardiotoxicity. Currently, there are no US FDA- or EMA approved emerging disease-modifying OA drugs (DMOADs). However, most of the emerging drugs are in the clinical trial phase. Various OA phenotype-guided approaches and endotypes such as synovial inflammatory phenotype, osteoporotic phenotype, articular cartilage degradation phenotype, metabolic phenotype seems promising. Compared with traditional OA drugs, DMOADs which act by reducing inflammation, enhancing cartilage repair, and inhibiting OA degeneration, are associated with more pronounced effects and fewer adverse effects than traditional medications. However, we are still in need of large-scale randomized controlled trials for safety and efficacy for these candidate drugs.

PANEL: Knee Osteoarthritis Treatment Algorithms: Perspectives of Patients, Clinicians and Researchers

Prof. Dr. Cagatay Ozturk
İstinye University

Dr. Gurkan Gümüşsuyu
Liv Hospital Ulus

Prof. Dr. Feza Korkusuz
Hacettepe University

Abstract: Osteoarthritis is a degenerative joint disease characterized by the breakdown of cartilage in joints, leading to joint pain, stiffness, swelling, and reduced range of motion. Age, genetics, joint injury, obesity, and joint overuse are among the factors that can contribute to the development of osteoarthritis. Symptoms can range from mild to severe and tend to worsen over time. Orthopedic surgeons take a comprehensive approach to osteoarthritis management, aiming to alleviate symptoms, improve joint function, and enhance the patient's quality of life. Medical therapy involves nonsteroidal anti-inflammatory drugs, glucosamine and chondroitin sulfate. Corticosteroid injections provide pain relief and reduce inflammation. Hyaluronic acid injections aim to supplement the joints natural synovial fluid and provide lubrication. Platelet-rich plasma Therapy promotes healing and reduces inflammation. Stem cell therapy regenerates cartilage. Also using assistive devices like braces, splints can reduce stress on affected joints. As surgeons also we need to consider surgical options like total joint replacement, partial joint replacement, joint preservation surgery, osteotomy, cartilage repair and transplantation.

HANDS-ON LABORATORY EXPERIMENTS

Lab coats required!

Lab experiment 1:
Hydrogel preparation techniques for 3D cell culture, bioink preparation and 3D bioprinting with live cells
Location: 4th floor. **401/A** (Cell Loading)

Lab experiment 2:
Spheroid - Organoid preparation and culture
Location: 4th floor. **401/C** (3D Lab)

Lab experiment 3:
Imaging 3D cell culture systems
Location: 4th floor. **401/D** (Microscopy Lab)

Lab experiment 4:
Angiogenic potentials of 3D scaffolds *in ovo* technique
Location: 4th floor. Lab **401/I** (In ovo Lab)

Circulation

	Group A	**Group B**	**Group C**	**Group D**
Friday 13:30- 15:10	4th floor 401/A	4th floor 401/I	4th floor 401/D	4th floor 401/C
Friday 15:30- 17:30	4th floor 401/C	4th floor 401/A	4th floor 401/I	4th floor 401/D
Saturday 13:30- 15:10	4th floor 401/D	4th floor 401/C	4th floor 401/A	4th floor 401/I
Saturday 15:30- 17:30	4th floor 401/I	4th floor 401/D	4th floor 401/C	4th floor 401/A
Sunday 13:30- 15:10	Trainees will be divided into 6 roundtable groups.			

Trainees will spend 2 hours in each course and change laboratories during coffee breaks.

Hands-on Lab Experiment 1

Hydrogel Preparation Techniques for 3D Cell Culture, Bioink Preparation and 3D Bioprinting with Live Cells

Trainers: Ayça Bal Öztürk, PhD & Emine Alarçin, PhD

Emine Alarçin is an assistant professor of pharmacy at Marmara University, Turkey. She received her Ph.D. from Marmara University, Turkey from the Faculty of Pharmacy, and Department of Pharmaceutical Technology in 2011. Her Ph.D. research was mainly focused on fabricating polymeric microspheres for VEGF delivery in nerve graft prefabrication. She carried postdoctoral research fellow at Harvard Medical School in 2016–2017. Her current research focuses on developing multifunctional biomaterials, nanocarriers, and hydrogels for drug delivery applications and regenerative medicine.

Ayça Bal Öztürk is an Associate Professor at Istinye University, Faculty of Pharmacy. She also holds a faculty appointment at Istinye University Research and Application Center for Stem Cell and Tissue Engineering (ISUKOK). She received his B.Sc., M.Sc., and Ph.D. degrees in Chemical Engineering from Istanbul University. Awarded with The Scientific and Technological Research Council of Turkey (TUBITAK) in 2016, she joined Harvard Medical School as a postdoctoral research fellow. She has received an outstanding faculty award in 2019, 2020, 2021 and 2022 for her research, which has been given to only a few academic members at ISU. Ayca Bal Ozturk received the Young Scientist Award (BAGEP 2023) from Turkish Science Academy in 2023. Ayca Bal Ozturk, who is also a member of Biomaterials and Tissue Engineering Society (BTES). She has published over 60 papers with over 1100 citations (H-index 19). She has also presented her work to present at over 50 international/national conferences until today. She has hosted 12 projects as PI funded by TUBITAK and her university and participated as co-PI in many projects from TUBITAK or other universities. Her research interests include mainly smart biomaterials (i.e., tissue adhesives), design and synthesis of innovative micro/nanoscale biomaterials as well as regulating stem-cell differentiation with the conclusive purpose of generating tissue-engineered organs, designing the next generation bio-nanoparticles for efficient targeted cancer therapy. She is the co-founder of AdBioink Biosystem Technology Inc., established in TUBITAK Marmara Technopark. AdBioInk focuses on developing photo-polymerizable biomaterials and bioinks to speed up tissue engineering studies.

3D Cell Culture

Three-dimensional (3D) cell culture offers a promising avenue for research as an emerging technology. Cultivating cells in 3D models enhances their resemblance to the intricate *in vivo* environments. This approach has demonstrated increased realism in translating research findings to *in vivo* applications. Although cell lines offer homogeneous study material, adopting 3D models encourages cells to exhibit behaviors that closely mirror natural conditions (see Figure 1.1). Scaffold-based techniques, particularly those employing hydrogels, offer distinct advantages and diverse applications in the field.

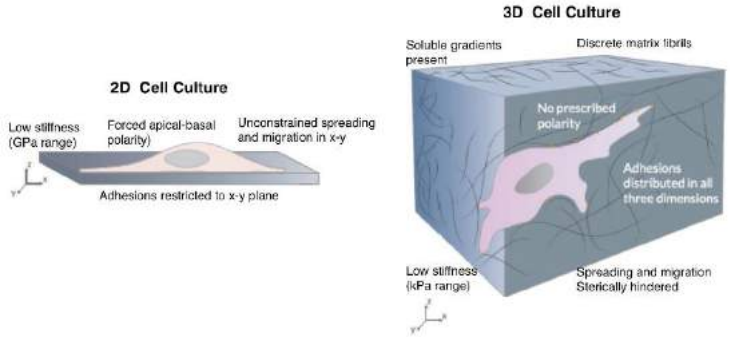

Figure 1.1. Cells in 2D and 3D microenvironments (Merino-Casallo et al. 2022).

The formation of hydrogels involves transforming liquid precursor solutions into solid materials, a process achievable through either physical (noncovalent) or chemical (covalent) crosslinking techniques. When working with cells, it is crucial to minimize polymerization times and employ non-toxic initiators, such as I2959 or lithium acylphosphonate salt for photopolymerization, to mitigate cell death and preserve overall cellular function. Rapid gelation is essential to prevent cell settling during the encapsulation process. The generation of free radicals in photopolymerization has been associated with cell damage, particularly in sensitive primary cell types. Therefore, it is imperative to assess the compatibility of crosslinking procedures with specific cell types of interest. Careful consideration must be given to designing polymerization times and

reagents to ensure that cell encapsulation occurs in a cytocompatible manner (Caliari et al. 2016).

Preparation of GelMA Hydrogel for 3D Cell Culture

This section of the course will cover the preparing a GelMA based hydrogel.

Materials and reagents

- Sterile gelatin methacrylate (GelMA; degree of methacrylation: >80%)
- Sterile alginate
- Sterile lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP)
- Cells
- Mem Alpha Medium
- Light curing platform

Protocols

1. Determine the mass of GelMA, Alginate and LAP required for your application based on the desired final concentration.
2. Prepare fresh photo-initiator solution by weighing LAP in a 15 ml centrifuge tube and add culture media. Cover the LAP solution with foil, and place in a 50°C water bath. Vortex after 20 minutes and ensure that the LAP is dissolved through the absence of undissolved solids. Sterilize it using 0.2-µm syringe filter units.
3. Weigh in the required amount of GelMA and Alginate. After the sterilization, dissolve the GelMA and Alginate in medium containing LAP solution. Place it in a 50°C water bath and vortex or use a shaker/rocker to accelerate the dissolution of GelMA. Make sure the complete mixture is protected from light.
4. The mixed solution can be stored at 4 °C for future use.
5. Warm the hydrogel solution to 50°C and vortex to liquefy before use if stored at 4°C. Mix with cell pellets and pipette it very gently.
6. Pour the cell-containing solution into the designated mold or well plate and crosslink it under the desired duration of light exposure.
7. Remove the cell-laden hydrogel from the mold and culture under standard conditions.

3D Bioprinting

3D-bioprinting is a type of additive manufacturing which can create 3D tissue constructs resembling *in vivo* tissues. Bioprinting enables the precise placement of cells, biomaterials, and bioactive molecules to construct intricate 3D tissue structures, catering to a range of biological and clinical applications.

In 3D bioprinting, a three-axis arm-mounted printer head dispenses bioink, depositing the material layer by layer with precision. During this dispensing process, the bioink is subjected to shear forces, and the resulting fluid-induced shear stress can negatively impact the cells. Optimizing dispensing parameters, including printing pressure, nozzle diameter, and bioink viscosity, is essential to strike the right balance between ensuring cell survival, achieving printing precision, and maintaining an optimal speed of deposition.

Throughout 3D-bioprinting process, a hydrogel-based solution containing biomaterials or a mixture of several biomaterials, typically encapsulating the desired cell types, is utilized. This specialized solution, known as bioink, is employed to fabricate tissue constructs. The bioink, housing the desired cells, can undergo cross-linking or stabilization either during or immediately after bioprinting, thereby defining the final shape, structure, and architecture of the intended construct.

An optimal bioink should exhibit appropriate mechanical, rheological, and biological properties that mimic those of the target tissues. functionality of the bioprinted tissues and organs.

Bioink Preparation and 3D-bioprinting

This section of the course will cover the preparation of bioink, 3D-bioprinting and *in situ* bioprinting.

Materials and Reagents

- Cell containing precursor solution
- Sterile syringe

- Syringe plunger
- Female Luer Syringe to Syringe Transfer Joint Lock
- Sterile nozzle

Protocols

1. Set up the printhead.
    a. Place ‘piece 1’ (Figure A) into syringe B and use a thin spatula to push it to the bottom of the syringe (Figure B).
        - Piece 1 should be level inside the syringe and air should not be able to escape between the syringe and piece 1
    b. Attach syringe A to syringe B via piece 2 (Figure C).
    c. Pour the bioink into syringe A.
        - Avoid creating or adding bubbles into the syringe.
        - A small scraper can be used to remove the remaining bioink from the conical.
2. Use the syringe plunger to push the bioink through piece 2 and into syringe B.
3. Remove piece 2 and attach piece 3 to syringe B (Figure D).
4. Insert syringe B into the printhead, referred to as “Tool 1” on the 3D Bioprinter (Figure E).
    - Attach syringe B to the pressure hose via the pressure connector above the printhead mount. Twist the pressure connecter to attach to syringe B.
    - Insert syringe B into the printhead by pushing down until the syringe is fully inserted.
5. Choosing a design and printing parameters

Choose the desired model from the “Model” tab.

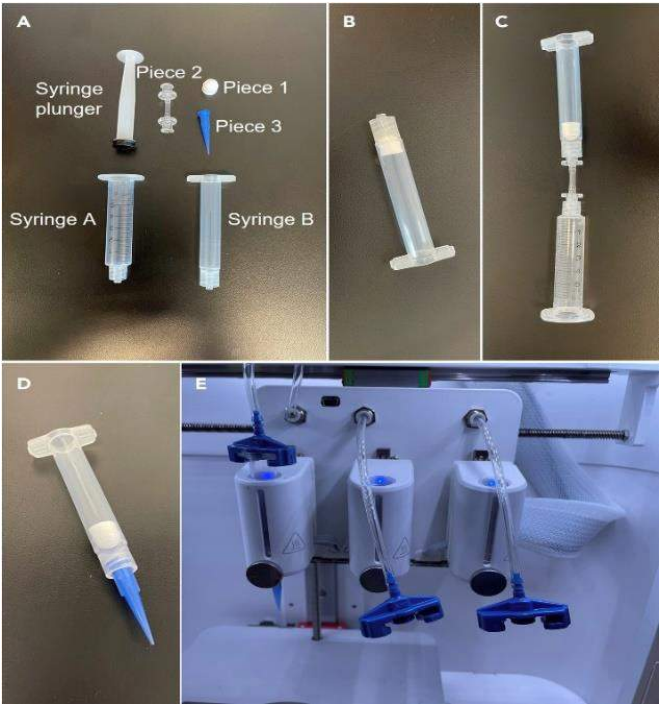

Figure 1.2. Syringe setup and 3D-bioprinting of cell-encapsulated bioink: (A) pieces required for printhead syringe setup, (B) syringe B with piece 1 (step 1a), (C) setup required to fill syringe (step 1b), (D) final set-up of syringe B (step 3) and (E) final set-up of syringe B and printhead (step 4).

In Situ Bioprinting: Handheld Bioprinter (BioPen)

Three-dimensional (3D) bioprinting technologies serve as a valuable tool for obtaining functional human tissues and organs, essential for applications in regenerative medicine. While traditional 3D bioprinters are typically employed to manufacture biological tissues externally, they may lack the versatility needed for *in vivo* transplantations and the creation of suitable models. Handheld bioprinters, address these limitations with its exceptional portability and user-friendly design, making it suitable for in situ tissue damage removal (Russell et

al. 2020). Notably, they allows direct work on damaged tissues and facilitates work on curved surfaces in the desired geometry.

Handheld bioprinters, BioPen systems, comprise a motorized extrusion system, a bioink cartridge, a nozzle, and a photocuring unit. Its replaceable visible light unit facilitates the crosslinking of hydrogels using various photoinitiators. The adjustable extrusion system enables the creation of cell-friendly bioprints, allowing the printing of cell-loaded hydrogels at desired speeds within the cartridge. The hydrogel can be cross-linked during or after the printing process.

Protocols

1. Grow cells on the culture dish until the desired cell densities are achieved. Remove cells from the culture dish with trypsin and add them to the prepolymer at a concentration of $3x10^6$ cells per mL.
2. Prepare the cell-laden hydrogel for 3D bioprinting, ensuring that the cartridge is filled without any air bubbles.
3. Place the cartridge in the printhead.
4. Set the speed controller to its maximum setting and then press the button to advance the piston. Continue advancing the piston until the bioink starts to emerge from the tip of the needle.

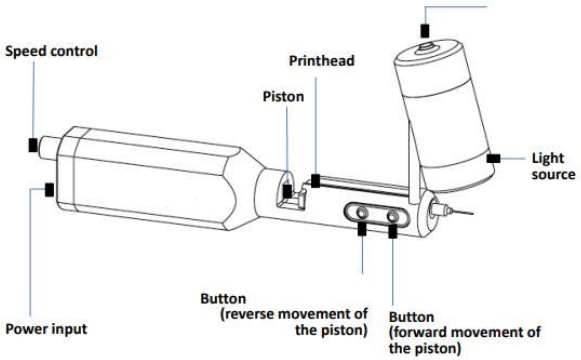

Figure 1.3. Handheld Bioprinter (BioPen).

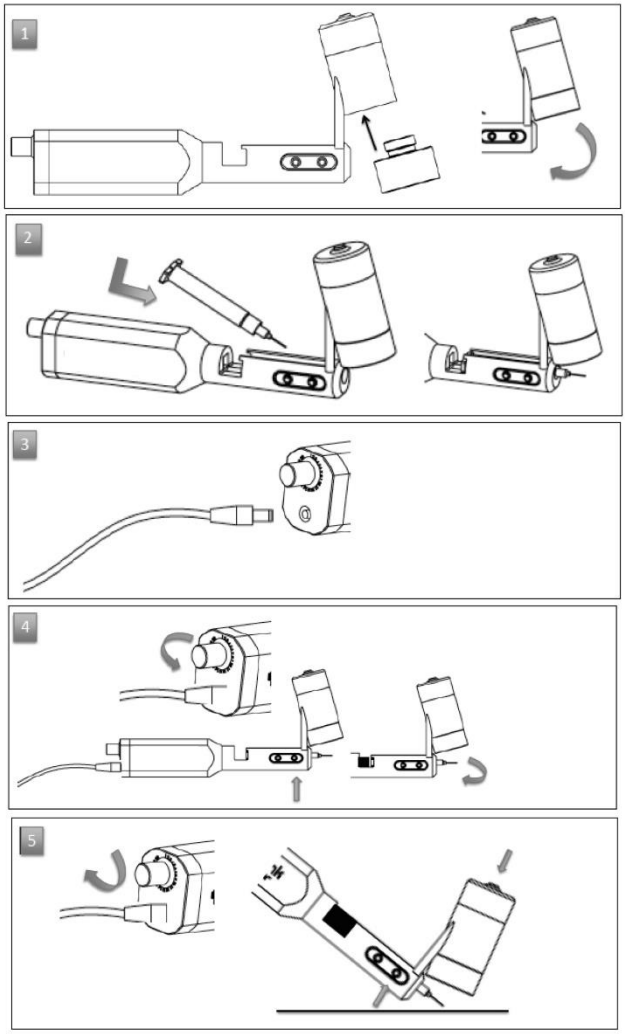

Figure 1.4. Schematic representation of the use of a handheld bioprinter.

References

Caliari, S. R., & Burdick, J. A. (2016). A practical guide to hydrogels for cell culture. *Nature methods*, *13*(5), 405-414.

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Russell, C. S., Mostafavi, A., Quint, J. P., Panayi, A. C., Baldino, K., Williams, T. J., ... & Tamayol, A. (2020). In situ printing of adhesive hydrogel scaffolds for the treatment of skeletal muscle injuries. ACS Applied Bio Materials, 3(3), 1568-1579.

Hands-on Lab Experiment 2

Spheroid - Organoid Preparation and Culture

Trainers: Hakan Darıcı, PhD, Elif Çıtak, MSc., Yağmur Ekin Boyoğlu, BS.

Hakan DARICI started his undergraduate education in Biology Department at Süleyman Demirel University in 2000. In 2004, he started his master's degree in the Department of Histology and Embryology, Faculty of Medicine, becaming the youngest research assistant of the university. During his Master's Degree, he conducted research on germ cells. In his PhD education his research focused on pluripotent stem cells. He received a scholarship from Singapore Stem Cell Association for his achievements. Immediately after completing his doctoral education, he worked as an assistant professor at the Department of Histology and Embryology at SANKO University as one of the 12 founding faculty members. In 2016, he participated the foundation of Istinye University, and establishment of the university's student and research laboratories. In addition to the courses in the Histology and Embryology department, he has been teaching Future Medicine, 3D Tissue Engineering and artificial intelligence, in Turkish and English.

Hakan Darıcı, is a member of the various foundations such as Stem Cell and Cellular Therapies Association, Turkish Histology and Embryology Association, Singapore Stem Cell Society, European Association of Cancer Research. Dr. Darıcı has worked in the establishment of Stem Cell and Tissue Engineering Research and Application Center (İSÜKÖK) at Istinye University and still conducts many stem cell associated projects in this center and provides training for both national and international students. Hakan Darıcı has also been the founder and executive of the Istinye University 3D Design and Prototyping Research and Application Center (İSÜ3D) since 2017. The studies carried out at the center are focused on 3D printers, 3D bioprinters, biocompatible polymers, tissue scaffolds, and tissues such as artificial skin, bone, cartilage, artificial organs and biomechanical human limbs.

He is also the founder and the CEO of HD Bioink Biotech. Corp since 2018, which is focused on 3D organ development, organoids, exosomes, CRISPR-based treatment methods and AI systems. Hakan Darıcı is currently working as an executive, researcher or consultant in many national and international projects and attends more than ten congresses and symposiums as invited speaker each year.

Research Areas: Stem Cells, Cellular Therapies Tissue Engineering, 3D Bioprinting, Transhumanism, Artificial Intelligence in Medicine.

Advantages and Disadvantages of 3D Cell Culture

Although conventional 2D cell culture is being used for more than 100 years, it does not resemble real 3D environment of the in vivo cells. To overcome this problem researchers developing 3D cell cultures to improve their experimental designs and to create 3D living tissues.

Within 3D cultures physiological conditions of tissues and organs in the human body are more realistic than 2D cultures. This better represents the native cellular microenvironment where cells have enhanced cell communication. Cells in 3D cultures have the ability to interact with neighboring cells and extracellular matrix therefore allowing for better representation of complex cell signaling and communication.

3D cultures can be used in drug testing and toxicity studies to have more accurate responses predictive drug responses. This allows researchers to study diseases such as cancer, neurodegenerative disorders, and infectious diseases in a more physiologically relevant context.

In stem cell research, 3D environment can mimic adult or embryonic microenvironment to promote self-renewal or cell differentiation. Therefore, it allows creation of tissue-like structures. They allow for the development of functional tissues and organs in vitro, with potential applications in regenerative medicine.

On the other hand, disadvantages of 3D culture are the requirement of somewhat more complex and expensive tissue culture methods or scaffolds and inter-laboratory variations of the results.

3D Culture Methods

There are several methods for 3D cell culture, each with its own advantages and applications. Here are some common 3D culture methods:

1. Scaffold-Based Methods:

Hydrogels are three-dimensional networks of hydrophilic polymers that provide a supportive environment for cells. They can mimic the extracellular matrix and are suitable for cell encapsulation. Scaffolds are often made of biodegradable materials like collagen, fibrin, or synthetic polymers. Cells can attach to and grow within these scaffolds, creating a three-dimensional structure.

2. Scaffold-Free Methods:

Cells are allowed to aggregate and form three-dimensional structures. These structures are usually creates a sphere like shape therefore called spheroids. This can be achieved using hanging drop methods, non-adherent surfaces, various devices, or specialized microplates.

- Hanging Drop Methods: Cells are suspended as hanging drops from the lid of a culture plate. Gravity causes the cells to aggregate and form spheroids.
- Magnetic 3D Bioprinting: Magnetic forces are used to levitate cells in a 3D space. This method allows for the assembly of three-dimensional structures without the need for scaffolds.
- Rotary Cell Culture Systems: These systems use rotating vessels to simulate microgravity conditions, allowing cells to aggregate and form three-dimensional structures.
- Microcarriers: Cells are cultured on small beads or microcarriers suspended in a liquid medium. This method is often used for large-scale production of cells for various applications, such as vaccine production or regenerative medicine.
- Cell Sheet Engineering: When cells are cultured on multiple layers they can form a thick extracellular matrix called cell sheets. These sheets can be layered to create three-dimensional structures. This method is particularly useful for tissue engineering applications.

3. 3D Bioprinting:

This technology allows for the precise deposition of cells and biomaterials in a layer-by-layer fashion to create complex three-dimensional structures. It is particularly useful for tissue engineering and organ printing.

4. Organ-on-a-Chip Systems:

- Microfluidic Devices: These devices recreate the microenvironment of tissues or organs by allowing the flow of fluids and nutrients. They enable the study of cell behavior in a more controlled and physiologically relevant environment.

Spheroids and Organoids

Spheroids and organoids are both three-dimensional cell culture models, but they have distinct characteristics and applications.

Spheroids are typically formed by allowing cells to aggregate spontaneously or through controlled methods, such as the hanging drop technique or non-adherent culture surfaces. They are simple, often spherical, cell aggregates and they lack the organized structures and differentiation seen in tissues.

Spheroids can be composed of a single cell type or multiple cell types. They are often used to study basic cell behavior, cell-cell interactions, and responses to stimuli in a more physiologically relevant environment than traditional 2D cultures. Spheroids are used in drug testing, toxicity studies, and to investigate basic cellular processes. They are a valuable intermediate between 2D cultures and more complex tissue or organ models.

Organoids however are more complex, often self-organizing structures that closely mimic the architecture and function of specific organs. Organoids have organized structures and can contain multiple cell types that are spatially arranged to replicate the architecture of the target organ. They may have functional characteristics similar to the original tissue.

Organoids are composed of multiple cell types that are specific to the organ they are intended to model. They can include differentiated cells, stem cells, and supporting cells. An organoid can be formed from pluripotent or multipotent stem cells following differentiation pathways of the target organ.

Organoids are used in disease modeling, drug screening, and personalized medicine. They offer a more advanced and organ-specific model compared to spheroids, making them particularly valuable for studying diseases and testing therapeutic interventions in a more relevant context.

In summary, while both spheroids and organoids are three-dimensional cell culture models, spheroids are simpler aggregates of cells used for basic studies of cell behavior, while organoids are more complex structures that aim to replicate the architecture and function of specific organs for applications in disease modeling and drug testing.

In this part of the training, we will demonstrate easy spheroid formation methods and we will use them to form organoids.

Part I: Spheroid formation using Spheromake™

Materials

- Standard cell culture laboratory devices (incubators, laminar hood, microscope, centrifuge etc.)
- Adherent cell line (MSCs, iPSCs, cancer cells)
- Cell specific culture media (DMEM/F12, mTeSR-1, etc.)
- Culture supplements (FBS, PS, PBS, etc.)
- Spheromake™ hydrogel*
- 6 well culture plates or petri dishes
- Autoclave or microwave oven

Low attachment plates, petri dishes (for hanging drop method) or agar agar for coating can be used alternatively. Protocols for these techniques are available on internet. This protocol is specific to Spheromake and allows you to produce mass number of spheroids at a significantly shorter period.

Sferoid protocol

This step requires 50% to 90% confluent cell lines. Cell number must be around 2-3 million per well of a 6 well plate.

We recommend using 5-6 ml Spheromake per 6 well plate (800-1000µl per well).

Hydrogel preparation

1. If the Spheromake is in gel form you can use it after heating to 37°C
2. If the Spheromake is in lyophilized form, appropriate amount can be weighed. Here we will calculate over 5 ml kits.
3. Transfer bottle contents into a 50 ml conical tube. Ad 5 ml of culture media or PBS (with Ca and Mg).
4. Do not close the lid of the tube tightly. Close loosely to allow water evaporation.
5. Place the conical tube into a bigger beaker. Make sure the conical tube is placed vertically or close to a vertical position.
6. a) Cover the top with aluminum foil if autoclave will be used. Put the beaker into autoclave and use water sterilization protocol (121 °C, 20 minutes).

   b) We recommend autoclaving but if autoclave is not available, you can use the microwave (takes less time but might be dangerous)

   Mix the lyophilized contents and media with a sterile glass rod or pipette. You can also use vortex for mixing.

   Adjust microwave to maximum setting and put the beaker inside. Watch the contents closely while heating. When the gel starts to boil shut down the microwave immediately and wait 10-20 seconds for contents to cool down. Repeat the boiling process for at least 2 more times.

   Make sure the lid of the conical tube is loose, otherwise the tube might explode! Also be careful during the boiling part. If the boiling continues for more than a few seconds, the contents can be spoiled into the microwave. Close the lid before taking out of microwave to avoid contamination.
7. The gel is now ready for coating. We recommend using gel over 37°C, preferably around 50-60 °C where it is less viscous. If you are using cold gel, warm it before use. Continue experiments in a sterile environment (in a laminar hood).
8. Use the conical tube and pour the gel into the wells of a six well plate, directly. Gel contents might stick to the walls of a serological pipette so we do not recommend using pipets, which can cause the loss of gel.

   Make sure all the area at the bottom is covered with the hydrogel and no non-covered surface remained.

800 µl Spheromake is ideal for a each well of a 6 well plate. However, you can also use 1 ml per well.
A single well might be left blank (uncoated) alternatively, to evaluate cell density and viability during the spheroid formation.

9. Place the plate into the incubator and wait around 20 minutes to acquire a flat surface on the gel.
10. Take the plate into the laminar hood and spray the wells with HD Bioink Crosslinker. Spraying allows micro pits formation on the surface of the gel while crosslinking. Make sure each well had enough crosslinker (around 1 ml) on top to cover the surface. Tilt the plate to check the crosslinking. Alternatively, you can use pipettes to transfer crosslinker, but this may cause formation of big pits therefore not recommended.
11. After crosslinking, aspirate and discard the extra crosslinker on top with gentle pipetting.
12. If the plate will be used immediately, wash the surface with culture media (preferably containing antibiotics) and discard the extra media. Do not use PBS-EDTA solution for washing.

Hydrogel preparation must be carried out separately, but preferably on the experiment day. However, sterile prepared Spheromake hydrogels can stored in conical tubes at +4°C up to a month or for a week when plates are coated. Cover the sides with parafilm to prevent evaporation or contamination before storage. Warm the hydrogel to 60°C for coating or warm the plates 37°C before washing with media. Culture plates can also be covered with hydrogels and stored at +4°C to be used in one week.

Cell preparation

1. Trypsinize, centrifuge and count the cells before use.
2. Adjust the numbers to 2-3 million cells per ml (or use a single T25 flask at 90% confluency) for a single well of a six well plate.
3. Put the cells on top of the hydrogel. Add appropriate amount of culture media to make total media amount is 3 ml per well.
4. You can use one uncoated well as 2D control. Use 1/8 of the cell amount (~$2{,}5*10^5$ cells) for control well.
5. Check the next day for spheroids. Measure and count if required.

Folow-up

- Spheroids start to form immediately and can be observed at the day 1.
- You can continue to culture for a few more days to get bigger spheroids or collect immediately.
- Since the number of cells is quite high, they will consume the nutrients faster and more frequent culture media changes will be required. Be careful not too collect spheroids during media changes.
- For differentiation studies, you can continue the experiment for up to 30 days. However, after 15 days, coated gel may start biodegradation, but cells keep their spheroid shapes.
- Make a thicker coat for long term culture. Make a thinner coat for better microscopic observation. Phenol red free culture media can be used for preparation for better microscopic transparency.

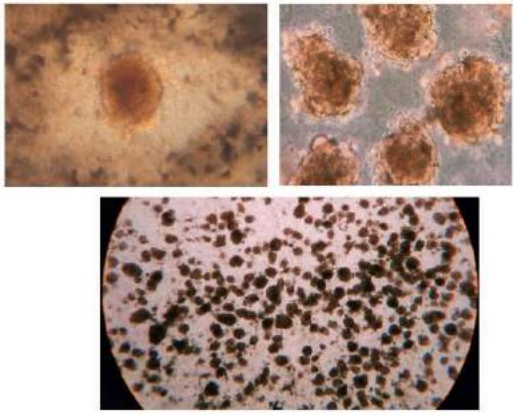
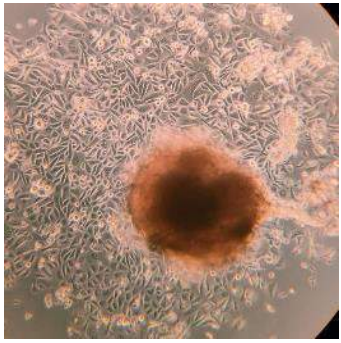


Figure 2.1. A single spheroid (top left) on the hydrogel, and multiple spheroids (top right and bottom).

Collecting the spheroids

1. Tilt the plate and carefully collect the spheroids via a 1 ml micropipette or a serological pipet.
2. Add additional media to collect remaining spheroids.

3. When spheroids are collected in a 15 ml conical tube, they will sink to the bottom by themselves over 10-15 minutes, so no centrifugation is required. However, you can used centrifuge at 300 g for 2 minutes for better isolation.
4. You can place the spheroids into new Spheromake coated or uncoated plates. If you are using normal culture plates, spheroids will start to attach the surface and bottom cells will migrate (Image on the right). In this form it is possible to fix and stain the spheroids.



- You can collect spheroid samples during long term culture to evaluate morphology, viability and differentiation.

Collecting DNA/RNA/protein from spheroids

It is possible to isolate DNA/RNA/protein from the spheroids at any point for further analyses.

You can collect the spheroids with the method described above or use the isolation protocol while on hydrogel. For hydrogel isolation

1. Aspirate the culture media as much as possible and wash the cells with sterile PBS solution. Discard the excess PBS.
2. Use HD Bioink LinkBreaker™ 1-2 ml per well. You will see the gel is disintegrating. You can stir with pipet tip to fasten the process.
3. Collect and centrifuge the contents.
4. Use your own DNA/RNA/Protein isolation kits.

Isolation with the hydrogel may cause extra protein and polymer accumulation and non-pure isolation, especially for RNA. Therefore, we recommend performing additional washing steps during the following centrifugations.

Video tutorial

You can visit the link in the QR code for video tutorial of spheroid formation in *JOVE* journal https://www.jove.com/v/63622/preparation-characterization-graphene-based-3d-biohybrid-hydrogel

Preparation and Characterization of Graphene-Based 3D Biohybrid Hydrogel Bioink for Peripheral Neuroengineering. 10.3791/63622-v • 10:17 min • May 16th, 2022.

Part II: Organoid formation

Since the organoids are relatively much bigger than the spheroids, bigger scaffolds are necessary to provide 3D environment. Matrigel is a common material for organoid development. Also our product OrganoMake™ can be used to form organoid scaffolds. Here we will describe both methods.

Materials

- Standard cell culture laboratory devices (incubators, laminar hood, microscope, centrifuge etc.)
- Orbital shaker
- Adherent cell line (MSCs, iPSCs, cancer cells) *or preferably spheroids*
- Cell specific culture media (DMEM/F12, mTeSR-1, etc.)
- Differentiation media or supplements (small molecules)
- Culture supplements (FBS, PS, PBS, etc.)
- Matrigel™ , parafilm *or* Organomake™ hydrogel, OrganoMake Crosslinker
- 6 well culture plates or petri dishes

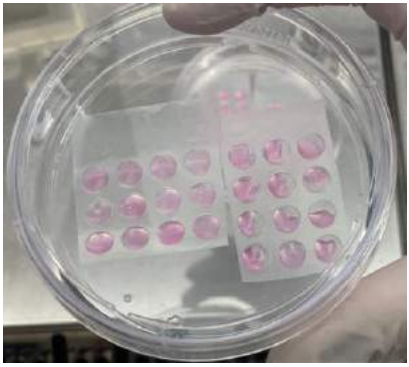
Matrigel™ Protocol

Tip: You can start organoid formation with small spheroids (E.g.: Embryonic bodies) instead of single cells.

Parafilm scaffolds will be used to shape the Matrigel droplets. After forming semi-solid Matrigel beads, in an 37°C incubator, stem cells must be inserted into the beads. Lastly Martigel beads must be transferred to the culture plates.
Parafilm scaffolds can be made prior to the experiment. However, cells and Matrigel must be prepared simultaneously.

Scaffold preparation

1. Cut appropriate amounts of parafilm. Wash with 70% ethanol.
2. Form pits with 6-7 mm by pressing parafilm into a plastic scaffold (E.g.: micropipette box holes) like shown in the figure.
3. Place parafilm on sterile petri dishes and wash with alcohol then PBS. Lastly sterilize via UV light.



4. Thaw Matrigel at 0-4°C in an ice box.
5. We recommend mixing Matrigel with culture media on 1:1 or 1:2 ratios. However, pure Matrigel can also be used.
6. Add Matrigel into the pits as droplets to make sphere-like shapes.
7. Place the parafilms into 37°C incubator for gelation. Wait no more than 10 minutes.
8. Scaffolds are ready for cells. But you must act quickly before Matrigel solidifies.
9. Trypsinize and centrifuge the cells simultaneously with the Matrigel steps.
10. Collect cells with 100µl pipet tips and transfer them into the center of the Matrigel beads. This part is tricky since too solid gels might split while cells tend to fall to the bottom in less viscous matrigels.
11. Place the parafilms with prepared beads into the incubator to finish gelation.

12. Take the parafilm and transfer the Matrigel beads into a petri dish. You can use culture media, ejected through pipet tip to force gels to leave the parafilm and slide down to the petri dish. You can also press the parafilm from behind to dislocate sticky beads.
13. Transfer the beads into the final culture plate. Adjust each well to have 3-4 beads.
14. Place the culture plate into an orbital shaker, which is placed in an incubator. Shaking must not be less than 50 RPM otherwise organoids may attach the surface.
15. Change media regularly and add differentiation factors, specific to the organ development.
16. Continue to culture for 2 to 4 months.
17. Collect and image the organoids.

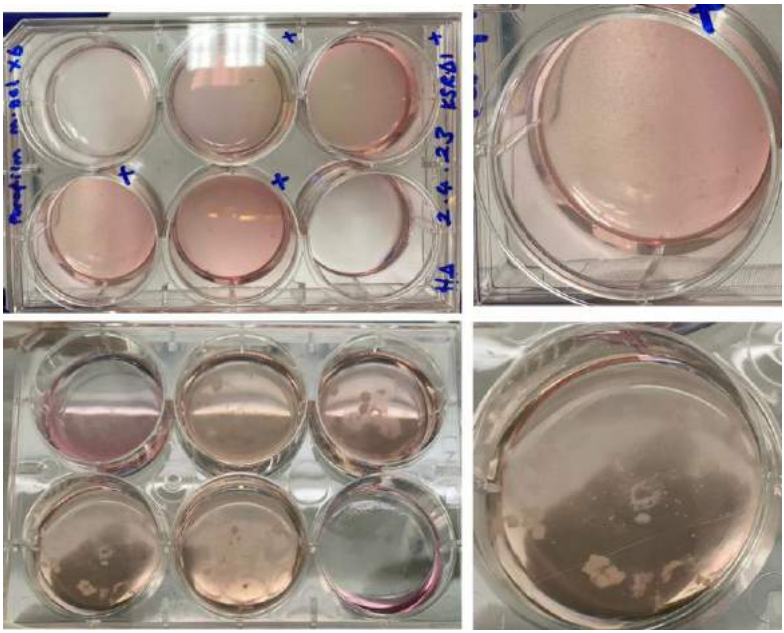


Figure 2.5. Organoids on the first day as small dots (top). Organoids after 12 days of culture measuring 2-4 mm (bottom).

OrganoMake™ Protocol

OrganoMake™ is a hydrogel which is relatively too easy to form organoid scaffolds and allows cells to grow into embryo like structures within the gels resulting in organoids.

Preparation of the OrganoMake is similar to SpheroMake and using is even easier. You can use autoclave or microwave to prepare and store at fridge till use. But we advise prepare fresh on the experiment day.

OrganoMake™ preparation

1. Transfer bottle contents in a 50 ml conical tube.
2. Dissolve the lyophilized powder with culture media. Stir with a sterile pipet if necessary.
3. Use autoclave or microwave to turn into gel form.
4. Store at 4°C or use fresh (recommended).

Organoid scaffold preparation

5. Seed cells on Spheromake™ and culture for 20 to 36 hours.
6. Collect spheroids into a 15 ml conical tube.
7. Add fresh culture media to the conical tube and mix gently.
8. Let the spheroids sink to the bottom. Discard excess media. Centrifuge at 300 g for 2 minutes if necessary. Spheroids must be floating only a few µl of culture media.
9. Add 1-2 ml OrganoMake™ on top of the spheroids. Mix gently.
10. Pour OrganoMake Crosslinker into 35 mm petri dish or a well of a six well plate.
11. Collect hydrogel-spheroid mixture using a 1 ml pipet. Let the pipet leave droplets into the crosslinker.
12. Droplets immediately form sphere beads. You can collect the beads with tweezers (image) or bigger pipettes.
13. Put the 2-4 beads into a well of a six well plate. Add specialized culture media.
14. Place into orbital shaker. Use minimal 50-60 RPM rotation.
15. Culture the organoids for 2-4 moths.
16. Collect and analyze the organoids.

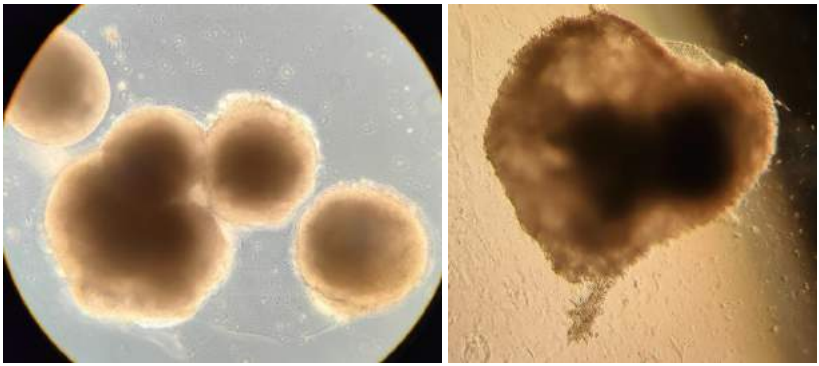


Figure 2.6. Organoids developing within Matrigel (left) and OrganoMake (right) around 2nd week.

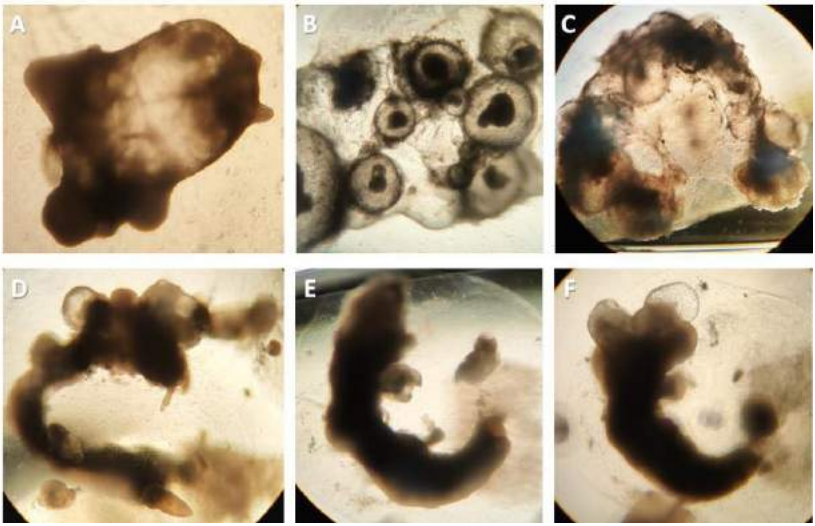


Figure 2.7. Organoids in the OrganoMake on the 4th week (A-C), 6th week (D-E) and 8th week (F).

Hands-on Lab experiment 3

Imaging 3D cell culture systems

Trainers: Hakan Darıcı, PhD & Ayça Bal Öztürk, PhD

Most microscopic imaging techniques depends on the transmission or emission of the light through or from the samples, respectively. This is quite easy on 2D cultures which have only few μm thickness. However, when the researcher needs to observer 3D structures and especially cells in it, more advanced techniques or devices are required.

Brightfield microscopy is a standard method for observing cellular morphology. It provides detailed images of spheroids and organoids without the need for special staining. However, simple to more complex staining can also be used to observe cells using visible light. When the samples are too thick for light to pass, histologic tissue preparation and sectioning methods must be used before staining.

Fluorescence microscopy allows the visualization of specific molecules within cells using fluorescent dyes or genetically encoded fluorescent proteins. It is used for studying specific cellular structures, protein expression, and dynamic processes within spheroids or organoids.

Confocal microscopes use a pinhole to eliminate out-of-focus light, providing higher resolution and optical sectioning compared to traditional fluorescence microscopy. They are ideal for imaging thicker samples, such as organoids, and obtaining detailed 3D reconstructions. However, these microscopes are comparatively more expensive than the fluorescent microscopes and requires better tissue preparation more user skills.

Both **electron microscopy** methods, Transmission Electron Microscopy (TEM) or Scanning Electron Microscopy (SEM) can be used to image 3D cultures with a high resolution. TEM provides high-resolution images of internal cellular structures by transmitting electrons through thin sections of the sample, offering ultrastructural details of cells within spheroids or organoids. SEM provides detailed surface topography images by scanning a focused electron beam across the sample surface which is useful for studying the surface morphology of spheroids or organoids.

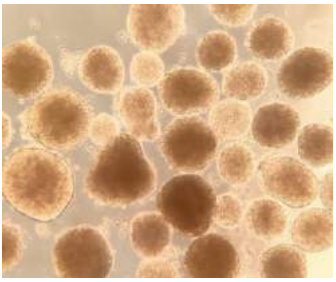
Live-Cell Imaging can be performed via recording cell responses in real time. However, since the cells move a little slow for human eye to catch movement, time-lapse imaging can be used to captures dynamic processes, allowing the observation of cell behavior and responses even within spheroids or organoids. This method is useful for studying cell migration, proliferation, and responses to external stimuli.
Artificial intelligence can also be applied to the imaging methods to optimize results, reduce human error, analyze large amounts of data or distinguish smallest differences between samples.

In this part of the training, we will demonstrate some of the imaging methods for spheroid or organoids along with AI based methods.

I. Brightfield imaging

a) Imaging of unstained samples

Imaging of 2D conventional culture is trivial since the light can pass through thin cells without hitting many obstacles. However, 3D cultures have a bigger thickness, from ten micrometers to millimeters. Therefore, observing them on visible light becomes tricky in since the light cannot pass those structures.
Two options remain for visible light imaging, observations on thinner objects till the transparency become low or preparing tissue sections through histological procedures.



Spheroids are on the first group, which are somewhat transparent until certain size. When the diameter gets bigger than roundly 100 μm, the cells in the middle part become to die. While live cells are more transparent, dead cells looks darker therefore creates a darker central zone within the spheroids. This model is similar to the growing tumors until they establish a circulation system into the tumor via signaling for new blood vessel formation. However, in 3D cultures, such a circulatory system has not yet been developed. This limits internal observation of visible light on spheroids on

bigger sizes. Nevertheless, spheroid diameter can still be measured and be used to evaluate the effects of the chemicals on 3D cultures.
When the diameter becomes too thick for light transmission, stereomicroscopes can be used the evaluate organoids from outside, which gives observer the 3D morphology. However, images taken from stereomicroscopes are still acquired through one objective and therefore 2D view of a 3D image.
Histology becomes the most accepted and easy solution to observe inner structures, developed during organoid formation. For histological procedures, samples from the developed 3D cultures must be collected on several time points, fixed, paraffin embedded, and tissue sections must be taken to obtain 4-5 µm thick slides.

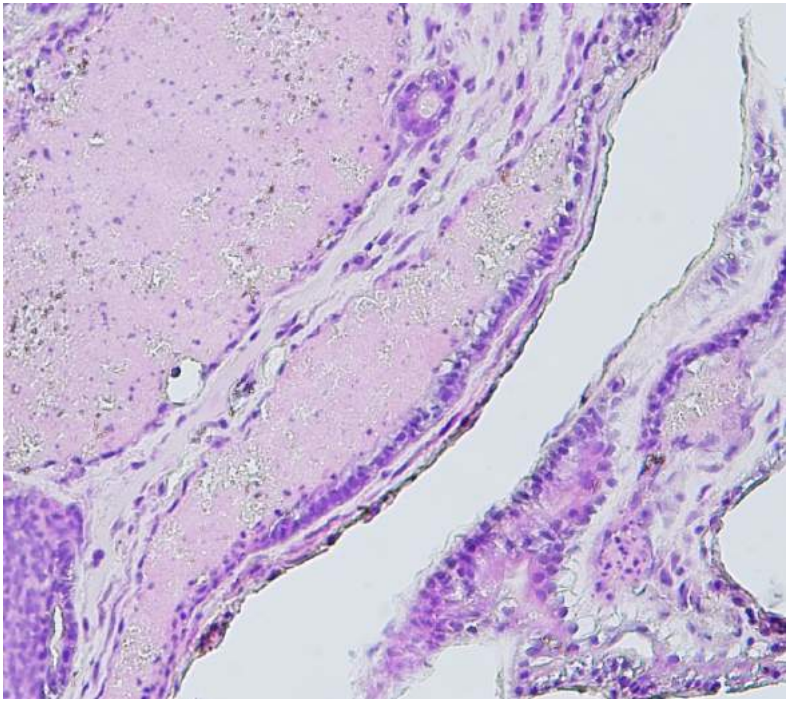


Figure 3.2. This section was taken from an organoid sample, developed in OrganoMake hydrogel. Epithelial structures, blood vessel like organizations, ducts and connective tissue fibers are visible.

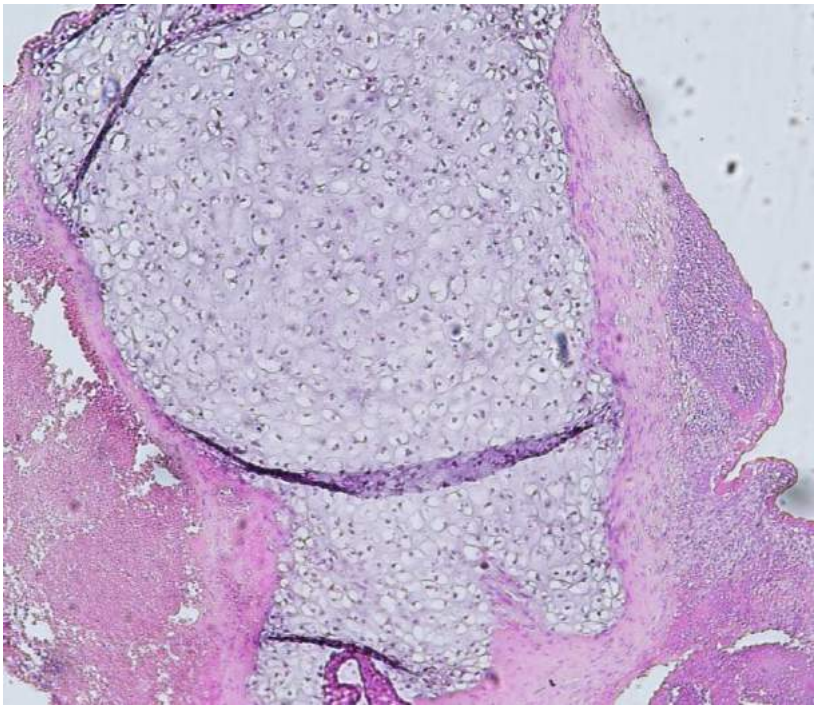


Figure 3.3. These sample is also taken from an organoid, developed in OrganoMake hydrogel. Fully grown cartilage is developed with perichondrium and external dense connective tissue.

II. Fluorescent staining methods

Although the transparency of the visible light can be drop to zero in bigger 3D structures, fluorescent imaging becomes advantageous since it uses reflected light instead of transmission. Therefore, superficial layers and cells embedded in the scaffolds can become visible among non-fluorescent environment.
Various methods are being used in the imaging of 3D structures. Simpler ones are viability assays and direct fluorescent stains. For more specific imaging, immunofluorescent (IF) or immunohistochemical (IHC) staining must be performed.

Live/Dead Staining

The LIVE/DEAD® Viability/Cytotoxicity Assay stands as a rapid and straightforward two-color methodology tailored for the realm of tissue engineering, offering a means to assess the viability of cellular populations through the simultaneous evaluation of plasma membrane integrity and esterase activity. Its efficacy lies in leveraging the distinctive characteristics of live cells, characterized by ubiquitous intracellular esterase activity and an intact plasma membrane. In the context of tissue engineering, the LIVE/DEAD® Viability/Cytotoxicity Assay expeditiously discriminates between live and dead cells.

The vitality of live cells is marked by their intrinsic intracellular esterase activity, discerned through the enzymatic conversion of the nearly non-fluorescent, cell-permeant calcein AM into the highly fluorescent calcein. Calcein's polyanionic nature ensures its substantial retention within viable cells, emitting a vivid and uniform green fluorescence (excitation/emission ~495 nm/~515 nm). In contrast, EthD-1 permeates cells with compromised membranes, undergoing a 40-fold increase in fluorescence upon binding to nucleic acids and emitting vibrant red fluorescence in non-viable cells (excitation/emission ~495 nm/~635 nm). The intact plasma membrane of live cells prevents the entry of EthD-1. The assessment of cellular viability in the context of tissue engineering relies on these inherent physical and biochemical attributes of cells, providing valuable insights into the health and functionality of engineered tissues.

Materials and Reagents

- Cell-laden hydrogel
- Calcein AM (Component A), 4 mM in anhydrous DMSO
- Ethidium homodimer-1 (Component B), 2 mM in DMSO/H_2O 1:4 (v/v)
- Sterile DPBS
- Well-plate
- Fluorescence Microscope

Protocol

1. Cell viability in cell-laden hydrogel can be determined by Live/Dead staining. First, the medium is withdrawn from the well-plate and samples washed with DPBS.
2. Allow the LIVE/DEAD® reagent stock solutions to reach room temperature after removing them from the freezer.
3. Prepare an approximately 4 µM EthD-1 solution by adding 20 µL from the supplied 2 mM EthD-1 stock solution (Component B) to 10 mL of sterile DPBS. Ensure thorough mixing by vortexing.
4. Combine the reagents by transferring 5 µL from the provided 4 mM calcein AM stock solution (Component A) into the 10 mL EthD-1 solution. Ensure thorough mixing of the resulting solution by vortexing.
5. Add 200 µL of the resulting approximately 2 µM calcein AM and 4 µM EthD-1 working solution directly to the cells.
6. Incubate the samples in the dark for 20 minutes in a 5% CO_2 incubator at 37 ^{o}C.
7. Wash the samples with PBS to remove excess staining solution.
8. Image the samples using appropriate fluorescence filters for Calcein AM (live cells, green) and Ethidium Homodimer-1 (dead cells, red).
9. Count the live and dead cells to calculate viability.

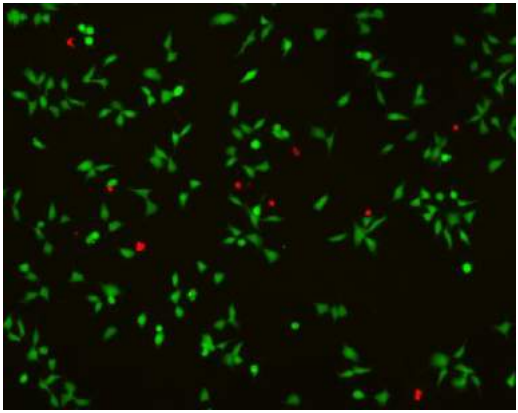


Figure 3.4. Representative image of Live/Dead staining in the sample, highlighting live cells (stained with calcein AM) in green and dead cells (stained with EthD-1) in red.

DAPI-Actin Staining

DAPI-Actin Staining plays a crucial role in the field of tissue engineering, offering a sophisticated methodology to explore and visualize the intricate structural arrangement of actin filaments within engineered tissues. Actin, a key component of the cytoskeleton, is instrumental in providing structural support to cells within these engineered constructs, actively participating in fundamental cellular processes critical for the success of tissue engineering, such as cellular motility, division, and maintaining the desired morphological characteristics. Phalloidin is the active component of the stain which directly attaches actin molecules and give fluorescent emission, rendering cell skeleton visible.

In the realm of molecular and cell biology, the DAPI staining technique holds particular significance in the context of tissue engineering. This powerful tool allows researchers to visualize and discern the nuclei of cells within engineered tissues. The acronym DAPI stands for 4',6-diamidino-2-phenylindole, a fluorescent dye specifically engineered to bind to DNA, which is essential for understanding the cellular composition of engineered tissues. Given the concentration of DNA within the nuclei of eukaryotic cells, DAPI staining facilitates precise visualization, aiding researchers in assessing the structural integrity and cellular organization of engineered tissues at the molecular level. This nuanced approach contributes valuable insights, guiding the refinement and optimization of tissue engineering strategies for enhanced regenerative outcomes.

Materials and Reagents

- Cell-laden hydrogel sample
- Paraformaldehyde (4% solution)
- PBS
- Triton X-100
- Bovine serum albumin
- Tween-20
- DAPI (4',6-diamidino-2-phenylindole)
- Phalloidin-488 or another fluorophore-conjugated actin stain
- Well-plate
- Sterile DPBS

Protocol

1. Fixation
- Gently aspirate the culture medium from the hydrogel samples.
- Rinse the samples with PBS to eliminate residual cell culture media.
- Fix the samples by applying 4% paraformaldehyde in PBS, ensuring the samples are fully covered, and incubate for 30 minutes at room temperature or following the recommended protocol for your specific hydrogel and cell type.

2. Permeabilization
- Treat the fixed samples with a permeabilization solution (PBS with 0.1% (w/v) Triton X-100) for 30 minutes.
- Follow with a thorough rinse using PBS.

3. Blocking
- Incubate the samples in a blocking solution (e.g., 1% BSA and 0.1% Tween20 in PBS) for 30 minutes to minimize non-specific binding.
- Follow with a thorough rinse using PBS.

4. Actin Staining
- Prepare the actin staining solution by diluting 1:60 in PBS with 200 units/mL of Phalloidin-488, resulting in a final concentration of approximately 6.6 μM.
- Add 200 μL of the staining solution per well and incubate for 1 hour in the dark.
- Wash the samples with PBS to remove excess actin staining solution

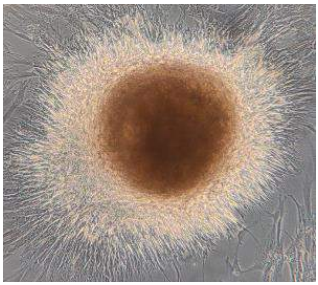
5. DAPI Staining
- Incubate the hydrogel samples with DAPI solution (0.1μg/mL-10μg/mL) in the dark for about 30 minutes.
- Wash the samples with PBS to remove excess DAPI solution.

6. Imaging and Analysis
- Capture images of the samples using suitable fluorescence filters for DAPI (blue) and the fluorophore-conjugated actin stain (green).
- Employ appropriate software for the analysis of the acquired images, allowing visualization and quantification of DAPI-stained nuclei and actin filaments.

You should adjust concentrations and incubation times according to your specific experimental conditions and the unique properties of the hydrogel and cells under investigation. Tailoring these parameters ensures optimal compatibility and accurate assessment within your specific research context.

Immunofluorescent Staining

Although Live/Dead and DAPI/Actin systems use fluorescent dyes, immunofluorescent staining is more complex and requires the use of specific antibodies.



Both IF and IHC staining requires multiple wash steps. If the sample is small like a spheroid, the first problem will be the fixation of the sample in a certain location for not to lose during these wash steps. Our technique is to collect spheroids, seed them onto normal tissue culture plates and let them to attach naturally, via migrating. Then samples can be fixed with paraformaldehyde and stained accordingly. The image on the right shows such a spheroid, attached to the culture plate and sending extensions.

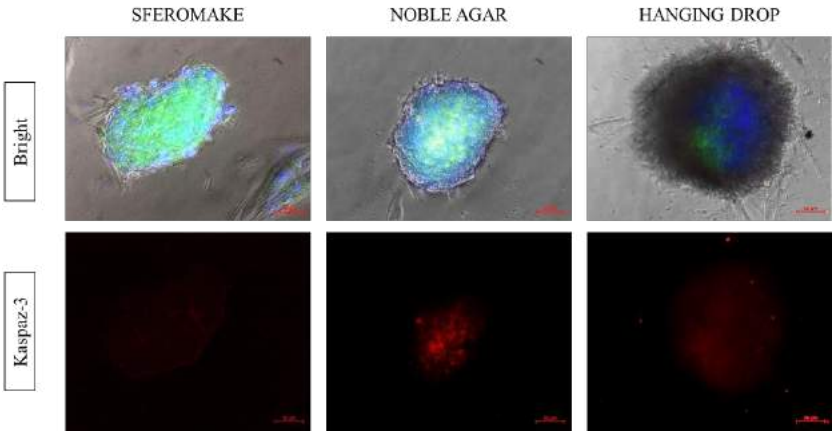


Figure 3.5. This image shows Caspase-3 staining of different spheroid models. More prominent staining is visible in the core of the spheroid formed on agar.

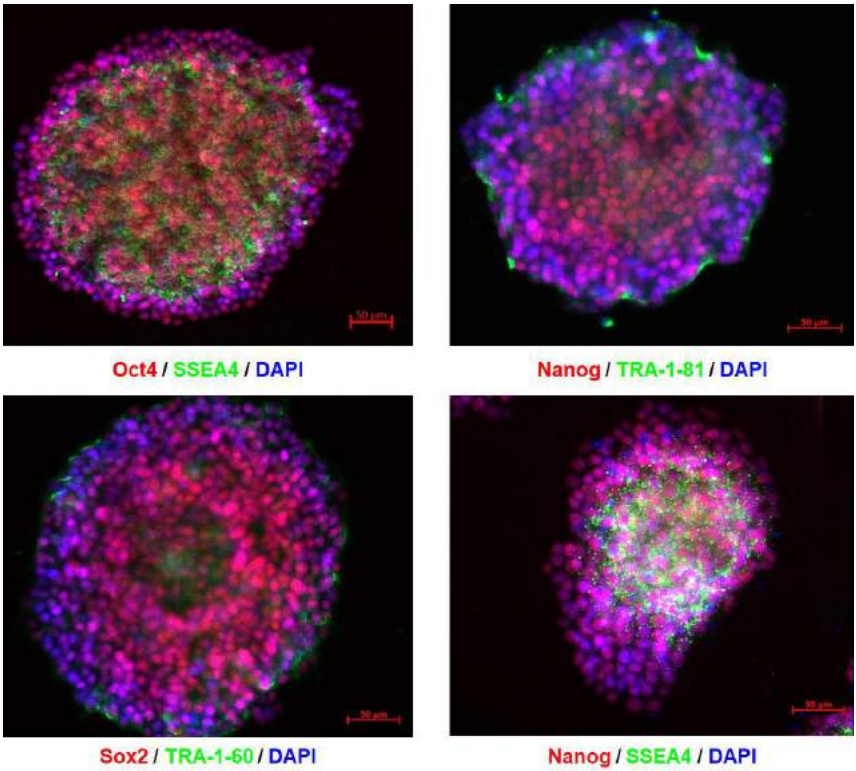


Figure 3.6. Various stains of iPSC colonies. Double staining was performed for the iPSC colonies for embryonic marker proteins. DAPI used as counter-stain.

Artificial Intelligence Analyses

Artificial intelligence (AI) systems integrating more and more in our lives as well as our laboratories. AI imaging systems have various advantages such as observing images without human error or inter-observer differences. AI also can be used to analyze high-throughput or bulk data and can finish tasks in minutes or hours which can take years for a human.

We use Intellesis system, a module integrated to the Zeiss microscopy software Zen. In order to use AI based systems, a teaching stage must be completed for

AI to learn distinguish various cell structures. Since the brightfield images are only gray-scaled images and color variations between a cell cytoplasm and culture plate plastic is not that different, learning of the AI system is harder. On the contrary, fluorescent images of the 2D and 3D samples can easily be distinguished from dark background therefore easy to learn and analyze for AI.
The training/learning process is simply showing which pixels of the sample is a certain object or stain. E.g., green pixels may represent live cells while red ones are dead cells, while blue ones are just all cells. You can also teach the AI to identify background from the cells.

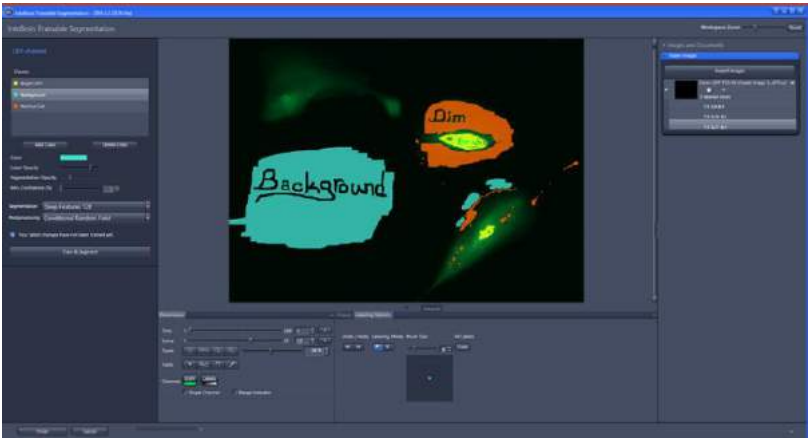


Figure 3.7. Training process of the GFP gene transfected MSCs where the dim or bright staining and background were thought to the AI software.

Time-lapse imaging

Some experiments require the demonstration of the change in the observed cells, such as wound healing experiments, where a scar imitating line is formed among confluent cells. Normally, images around every 6 to 24 hours were taken from the same position to demonstrate change, this imaging with long intervals does not give information between time periods and only focuses on the end results. However, time lapse systems capture images with short intervals and create a stop-motion video for the cells, allowing us to observe changes.
Here we have applied AI systems and time-lapse imaging into 3D cultures and observed how the spheroids are formed. AI allowed us to analyze hundreds of

gigabytes data, from hundreds of thousand images which are digitalized and quantified.

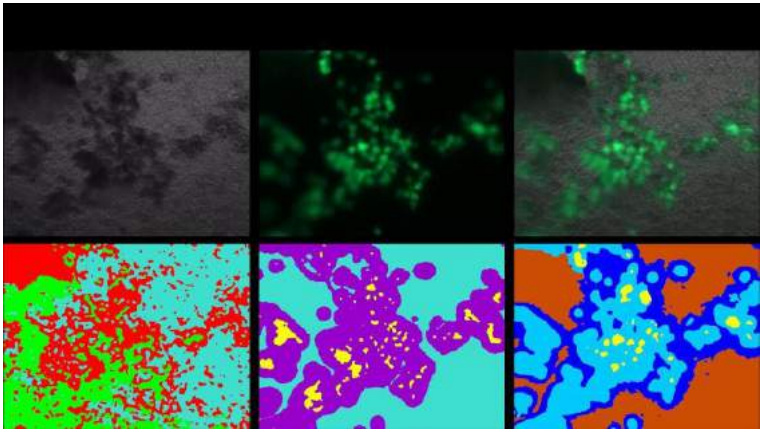


Figure 3.8. GFP tagged MSCs. Brightfield (left), fluorescent GFP 8middle) and merged (right) images were analyzed by AI and simplified images on the bottom were produced. These secondary images were analyzed and data like cell covered spaces, viability, spheroid formation rate were calculated.

Time-Lapse Protocol

- For the time-lapse imaging, Zeiss microscope incubation system were prewarmed, 30 minutes before analyses.
- CO_2 sonnection was established and andjusted.
- Cells were placed into the microscope 15 minutes before the analyses to equalize temperature differences.
- Places to be observed were marked on X,Y and Z axis. Ideally 12 different locations in a plate can be marked while up to 50 is possible. However, more image locations mean more data and requires more storage. If multiple channels will be used, each channel multiplies the data amount.
- The interval between each photo is adjusted. We use 4 or 5 minutes intervals between each photo, to produce a fluidly movement.

- Lamp brightness and exposure times were adjusted to a fixed point. Auto exposure may cause sharp changes therefore must be avoided.
- Sample locations were checked for a second time to be sure there is no focus loose. Warming or cooling plates can change their shape in micrometer level, which is not visible to human eye but may cause a loss of focus on images, especially on Z axis. Therefore focus must be check in the beginning of the adjustments and right before start.
- Experiment started and imaging observed for a few turns (around 15 minutes). If a problem or focus shift is observed, it is better to stop the experiment, adjust and restart.
- We found that experiments taking up to 16 hours are ideal for time-lapse imaging even though more is possible.
- At the end of the expertiment another one can be started, samples can be kept or discarded.
- Collected photos can be saved as a video file in AVI format.
- For AI analyses, videos must be saved in CZI format.

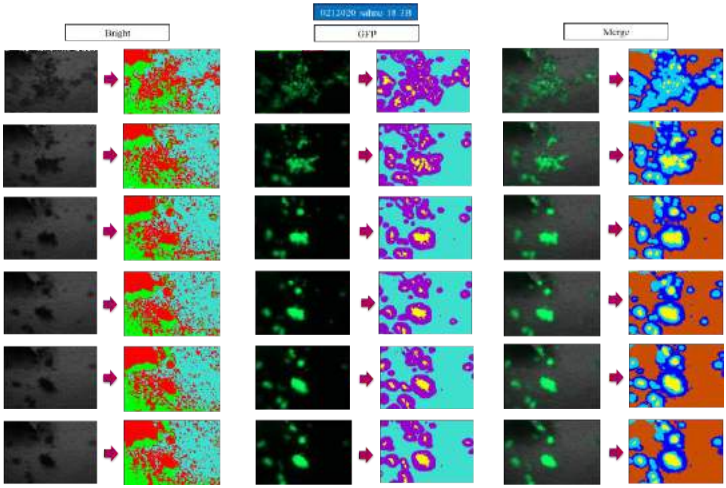


Figure 3.9. Formation of a spheroid. Brightfield (left), fluorescent GFP 8middle) and merged (right) images were analyzed by AI over time series and formation of the spheroid turned into data.

Hands-on Lab Experiment 4

Angiogenic potentials of 3D scaffolds in ovo technique

Trainers: Remzi Okan Akar PhD Cand & Gülşah Torkay MSc

Remzi Okan Akar completed his undergraduate studies in Molecular Biology and Genetics, and pursued his master's degree in Cancer Biology and Pharmacology. He is currently working towards his PhD in Molecular Oncology. His research focuses on molecular cancer pathways, anti-cancer drug discovery, cell death, and in ovo studies. He holds memberships in the Molecular Cancer Research Association (MOKAD) and the European Association for Cancer Research (EACR). He has published 10 articles in international journals and holds a PCT patent for a potential anti-cancer compound. In addition to being a research assistant at the Medical Biochemistry Department of Istinye University Faculty of Medicine, he also works at the Istinye University Molecular Cancer Research Center (İSÜMKAM). Alongside his academic endeavors, he is a co-founder of Ovoboard Biotechnology Inc. The aim of the company is drug candidate molecule screening in fertilized chicken eggs. Ovoboard Biotechnology is working on the investigation of the Angiogenic effects of various active substances on the chorioallantoic membranes (CAM) of fertilized chicken eggs. In addition, it is also aimed to provide personalized treatment approaches by developing patient derived-xenograts for patients and test anticancer drug candidate molecules on cell line-derived tumors for researchers. Ovoboard Biotechnology was established in September 2022 after more than 20 studies and 2 years of R&D started in 2020.

Gülşah Torkay graduated from the Biology Department of Istanbul University in 2020 as the top student, high honor (Magna Cum Laude) student, and third in the faculty. She studied in Poland as part of the Erasmus student exchange program during her undergraduate education and worked on a DNA barcoding project at the molecular genetics laboratory at Poznań Adam Mickiewicz University. She worked on drug resistance in cancer cells in an undergraduate student project funded by Istanbul University BAP unit. She received a travel scholarship at the 1st International Cancer Research Congress organized by the Molecular Cancer Research Association (MOKAD). During her undergraduate education, she took part in various projects and in the management team of a start-up that developed mobile health applications with artificial intelligence technologies. She obtained a domestic master's

scholarship from TÜBİTAK after being accepted to the İstinye University Graduate Education Institute Stem Cell and Tissue Engineering program with an academic merit scholarship. The TÜBİTAK 1002 project she wrote with her advisor Assoc. Dr. Ayça Bal ÖZTÜRK was accepted and she completed her thesis in the field of neural tissue engineering. At the MOKAD 2nd International Cancer Research Congress, she was awarded a young researcher scholarship. She is a co-founder of Ovoboard, a start-up backed by the TÜBİTAK Individual Young Initiative 1512 program that performs chorioallantoic membrane testing, which are highly significant in cancer drug development studies. The company was formed by Kaan ADACAN and Remzi Okan AKAR's studies and operates under the supervision of Prof. Dr. Engin ULUKAYA. The angiogenic behaviors of active substances and tissue scaffolds are explored within the scope of the company using artificial intelligence-mediated image analysis. Tumoroids produced from cell lines and patient xenographs are also being examined for drug efficacy. Gülşah TORKAY participates in numerous research projects in addition to her entrepreneurial endeavors and thesis studies. She was deemed worthy of the 2022 Academic Incentive Award by İstinye University. Within the scope of the 1004-Center of Excellence Support Program carried out within Sabancı University, she works on developing sustainable advanced nanotechnological materials. In 2023, she started her PhD studies at Yıldız Technical University Bioengineering program. Her thesis is on applications of electroconductive biomaterials in neural tissue engineering. Gülşah TORKAY has 7 international SCI indexed research articles, 1 ESCI indexed national review paper, 1 national book chapter, and 13 national and international posters and oral presentations.

Tissue engineering delivers oxygen and nutrients to growing tissues by stimulating the angiogenesis process, which promotes structural and functional repair of damaged tissues, in addition to many of the previously detailed purposes. Comprehensive *in vivo* models that include all stages of the angiogenic process are necessary to explore pro- or anti-angiogenic behaviors of tissue scaffolds or active components, as well as the corresponding tissue regeneration potential. In comparison to other *in vivo* models, the fertilized chicken egg's highly vascularized extraembryonic chorioallantoic membrane (CAM) provides a simple, accessible, and affordable angiogenic screening tool (Figure 1). Reducing animal experimentation, on the other hand, is a significant problem for pharmaceutical corporations as well as a much-discussed societal issue. Regulatory organizations such as the FDA, EMA, ICH, and the European Commission strongly support the 3Rs (Replacement, Reduce, Refine) aims and alternative ways to animal testing. Because the *in ovo* method is not deemed

animal experimentation by European authorities, it is not subject to ethics committee approval and documentation procedures (Fischer et al., 2022).

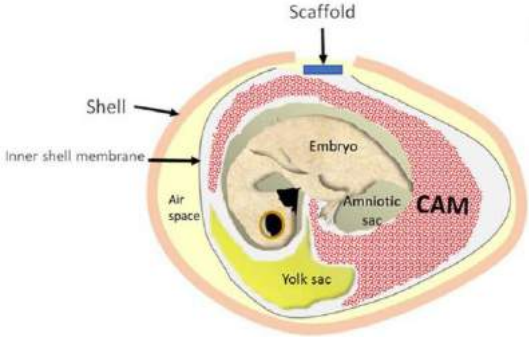


Figure 4.1. Schematic illustration of the in ovo CAM assay with a defect in the eggshell to allow application of the scaffold on the CAM (Reprinted with permission from Marshall et al., 2020).

Osteoarthritis, a degenerative joint condition, is the most frequent type of joint illness in adults over 50. The joint cartilage structure deteriorates with osteoarthritis. As a result, the bone tissue underlying the joint cartilage undergoes modifications. Bone growths and protrusions on the joints' margins alter the natural structure, producing mobility limits and discomfort (Loeser et al., 2012). Figure 2 both depicts intact cartilage with no fissures and no signs of synovial inflammation and osteoarthritis is characterized by soft tissue swelling, osteophyte formation, meniscus deterioration, and degeneration of cartilage (Zhang et al., 2022).

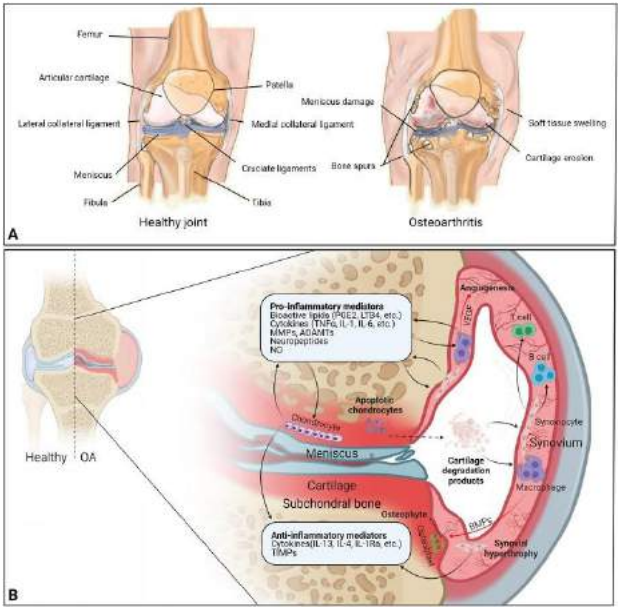


Figure 4.2. Overview of the healthy and osteoarthritic joint along with the pathophysiology of osteoarthritis (Reprinted with permission from Zhang et al., 2022).

Angiogenesis rises in the menisci, synovium, and osteophytes during osteoarthritis, resulting in the ossification of the deep layers of articular cartilage and osteophytes (Mapp & Walsh, 2012). In osteoarthritic joints, both angiogenic and antiangiogenic factors may be increased; nonetheless, vascular development is predominant, and the articular cartilage becomes less resistant to vascularization. Consequently, a few viewpoints can be used to assess the application of the *in ovo* approach in osteoarthritis. While angiogenesis is anticipated to proceed normally with evaluating the biocompatibility of tissue scaffolds to be utilized in therapy, antiangiogenic active chemicals alone or loaded tissue engineering systems may be expected to have the opposite effect.

This section of the course will cover subjects such as preparing fertilized chicken eggs for CAM testing, inserting tissue scaffolds on the layer, acquiring CAM photos, and artificial intelligence analysis.

Materials and reagents

- Fertilized chicken eggs
- Laboratory disinfectant, 70% ethanol, and towels
- Egg incubator with rotation and humidity (37°C)
- Distilled water for the incubator to maintain humidity (60-80%)
- Torch for candling eggs
- 3 mL Silicone Rubber Suction Bulb
- Class II laminar flow cabinet
- Powered engraving pen
- 5 mL sterile syringe with needle
- Powered circular saw blade
- Sterile forceps for opening shell membrane
- Flexible Medical Patch
- Materials to be implanted

Protocols

Egg preparation and scaffold implantation

1. When the eggs come from the manufacturer, they are checked for any breakage or damage, cleaned with a paper towel gently dampened with 70% ethanol, and placed in the incubator.

The incubator be kept on rotation until egg development day 3 (EDD3) with 1h scheduled rotation.

2. The eggs are candled with a torch at EDD0 and the air sacs are marked.
3. Using a powered engraving pen, a hole is drilled from the side of the egg and the part with the air sac to allow the needle to enter.

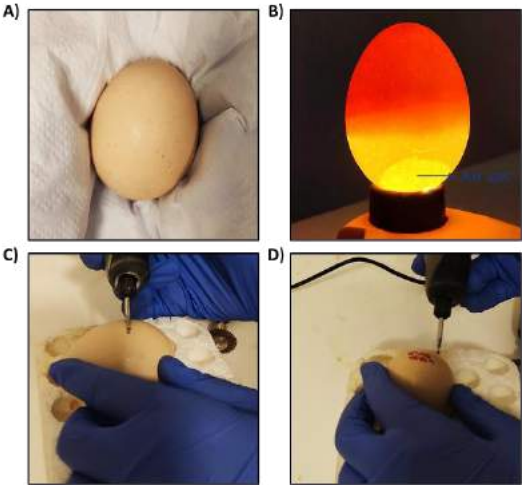


Figure 4.3. A) The eggs are cleaned with a paper towel gently dampened with 70% ethanol. B) The eggs are candled and the air sacs are marked. C) A hole is drilled from the side of the egg. D) A hole is drilled from the part with the air sac.

4. The inner membrane is pierced with the help of a sterile needle tip.
5. The egg is placed on its side, with the side hole facing the torch for candling. At this stage, both the viability (Figure 5) of the egg and the position of the air sac are checked.
6. While the air in the air sac is withdrawn with the help of a silicone rubber suction bulb, the air filling the upper hole is controlled with the help of the light.
7. The eggs are incubated upside down, with the hole where the old air sac was located downwards.
8. On EDD3, 3 mL of ovalbumin is withdrawn from the eggs.
9. On EDD5, the eggs are carefully cut from the upper part where the air sac is located, using a powered circular saw blade.

The cutted eggshell is taken from the egg with sterile forceps under the laminar flow cabinet and a piece of flexible medical patch is covered the hole. The following experimental processes are carried out in the cabinet.

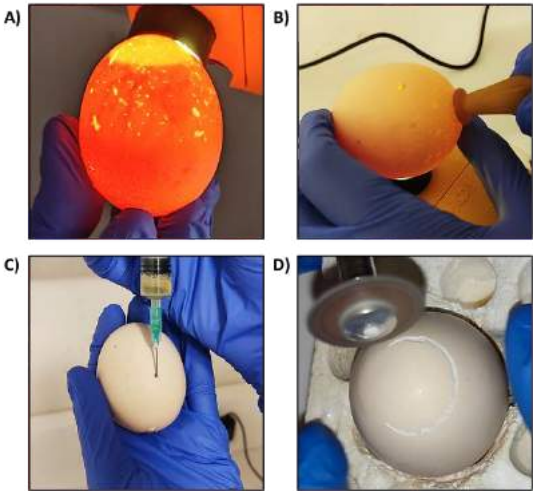


Figure 4.4. A) Viability and position of the air sac are controlled by light. B) If the air sac has not descended, the procedure is performed again with a suction bulb. C) Ovalbumin is withdrawn from the hole opened on the side of the egg. D) The eggs are cut from the upper part, using a powered circular saw blade.

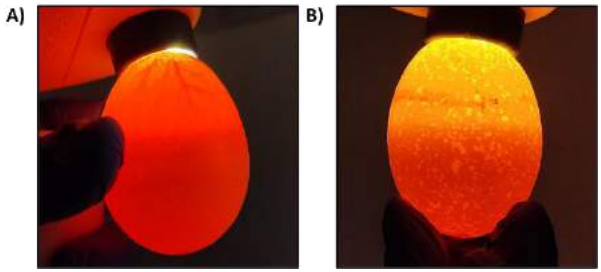


Figure 4.5. A) Live egg. B) Dead egg.

10. Each condition, including the non-treated control, requires at least six eggs in the experimental setup. During the experiment, uncontrolled losses may occur.
11. Tissue scaffolds are implanted onto the CAM surface rich from vessels and far from the embryo (Figure 6.A). The hydrogel-based scaffolds used in this experiment were lyophilized and UV sterilized. Tissue scaffolds in different forms can also be used.

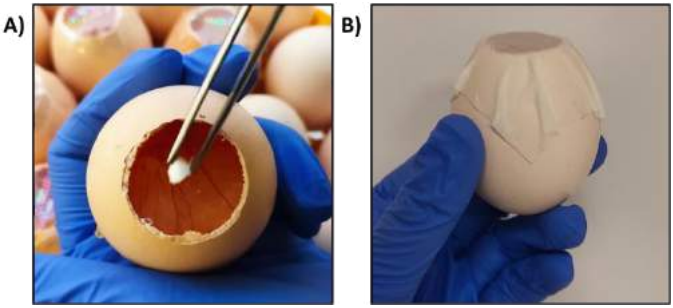


Figure 4.6. A) Tissue scaffolds are implanted onto the CAM surface rich from vessels and far from the embryo. B) After photography, the window opening to the eggs is closed appropriately and returned to the incubator.

12. The CAM is photographed by a proper camera and labeled as time zero and the eggs are put back to the incubator.
13. Photographs continue to be taken in the following days.

Angiogenesis Analysis by a Software

The quantification of angiogenesis studied by CAM varies considerably between studies in the literature. On the other hand, since the technique was first developed, the experimental approaches and analysis methods used have become increasingly effective due to the influence of technology. One of the observer-based manual counting techniques that was developed in the past and is still used today is the Chalkley scoring method (Figure 7).

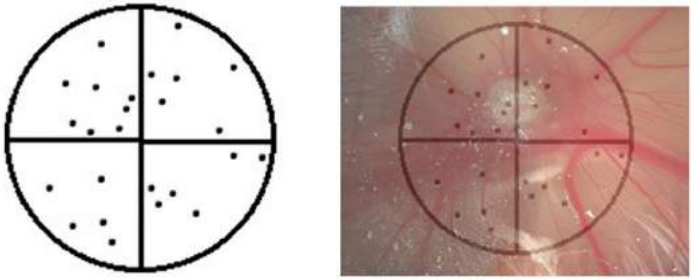


Figure 4.7. Example drawing of a Chalkley graticule eyepiece, showing the arrangement of points to align on blood vessels, allowing measurement of angiogenic response (Reprinted with permisson from Marshall et al., 2020).

Injecting a contrast material, such as hand lotion, under the CAM from a distance to the scaffold, is another alteration to help vessel numeration.

Computer models have been created in recent years to automate the process of measuring vascular parameters and speed up analysis, and they appear to be as reliable as manual counting (Faihs et al., 2022). Software such as Photoshop (Adobe Inc., US), Image J (National Institutes of Health, US), ImageScope (Leica Biosystems Nussloch GmbH, Germany), Angiotool (Zudaire et al., 2011), IKOSA (KML Vision GmbH, Austria), or AngioQuant (ELIXIR bio.tools) can be used to perform automatic counting.

IKOSA CAM analysis, an image processing software, is used for analysis in our laboratory (Figure 8). The analysis result gives us the vessel total area, vessel total length, vessel mean thickness, and vessel number of branching points from the CAM image (Annese et al., 2022). Therefore, different quantitative parameters can be obtained and compared (Table 1).

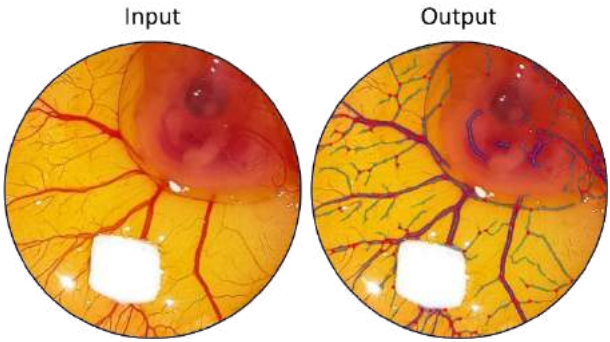


Figure 4.8. Input image and output result of IKOSA CAM Assay (v3.1.0) analysis of a tissue scaffold implanted on the CAM surface of the chicken egg.

Table 4.1. Quantitative results of IKOSA CAM Assay (v3.1.0) analysis of tissue scaffold implanted on the CAM surface of the chicken egg.

Parameter	**Results**
roi_size [Px^2]	1127844,00
vessels_total_area [Px^2]	56211,00
vessels_total_length [Px]	8799,39
vessels_mean_thickness [Px]	6,39
vessels_num_branching_points	127,00

Here, results for a single time point and one sample are given for illustrative purposes, but it is possible to evaluate different parameters in an experimental setup. There may be blank control groups with no treatment, negative groups with a treatment that suppresses angiogenesis, or vice versa, positive groups. As a result of the analysis, fold change rates of each egg to its own time zero results can be obtained. From the images taken on different days, it can be understood angiogenesis is increased or suppressed.

References

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Test Yourself

Please try to answer the following questions and self-evaluate yourself about the knowledge you have gained at the end of the course.

1. **In which layer the stem cells of articular cartilage reside?**

a) Superficial zone b) Transitional zone c) Radial zone d) Calcified zone

2. **How does the osteoarthritis progress?**

3. **What are the advantages of synthetic mRNAs?**

4. **For which applications can synthetic mRNAs be used?**

5. **What is the mean of Tri-copolymer?**

6. **Why is fibrin glue needed for to combine with tricopolymer?**

7. **Which of the following is not one of the advantages of obtaining stromal cells from fat tissue mechanically compared to enzymatic methods?**
   a) It is cheaper
   b) Requires shorter process
   c) More equipment is needed
   d) More cells are obtained to
   e) Extra-cellular matrix is preserved

8. **Which of the following is not a reason for choosing to use fat tissue, unlike all other tissues, as the source of stromal cells in regenerative medicine and surgery?**
   a) It is found in excessive amounts in the body
   b) The number and diversity of stromal cells is very high
   c) Allows repeated applications
   d) Simple and easy to obtain
   e) There is no contra-indication

9. **Do you prefer using stem cells or their product to treat OA as tissue engineering? Why?**

10. **Can you list the advantages and disadvantages of stem cells and their products?**

11. **What are the main cell types required in a microfluidic-enabled model to assess immune response?**

12. **What are the parameters to consider for determining the biocompatibility of a material?**

13. **If you are asked to develop a 3D in vitro co-culture model for evaluating biomaterial-mediated modulation of foreign-body responses, how would you approach?**

14. **Which of the following <u>is not</u> one of the limitations of traditional drug therapy for orthopedic disorders?**

a) Painful methods of administration b) Sustained drug release
c) Risk of systemic toxicity d) High rate of clearance

15. **Which of the following is not a correct statement?**

a) Drug accumulation in the liver and spleen is one of the problems with non-targeted drug delivery.
b) Physical and electrical barriers in the ECM reduce the bioavailability of the drugs in the cartilage.
c) Small drug molecules exhibit limited clearance from cartilage tissue, whereas drug molecules with high molecular weights readily leak out of the cartilage space.
d) Targeting of nanoparticles allows the attachment to specific cells such as chondrocytes as well as to inflammatory agents.

16. **Targeted drug delivery involves _____**

a) Delivering a drug from the factory to the patient.
b) Delivering a drug directly to the diseased part of the body.
c) Making more drug available to the affected population.
d) None of the above.

17. **The prefix ""nano"" comes from a _____**

a) French word meaning ""Billion""
b) Greek word meaning ""Dwarf""
c) Spanish word meaning ""Particle""
d) Latin word meaning ""Invisible""

18. What is the main aim in tissue engineering?

a) Printing donor specific cells using specific supports implantable into patients
b) Developing mature tissues that can be functional inside a body
c) Creating Frankenstein
d) I don't know

19. What is a biological sensor?

a) A biosensor is an analytical device composed by sensitive biological element and a transducer element able to detect undefined chemicals
b) A biosensor is an analytical device composed by some element able to detect something.
c) A biosensor is an analytical device made by a bio-receptor part (enzyme/antibody/cell/nucleic acid/aptamer), transducer component (semi-conducting material/nanomaterial), and electronic system which includes a signal amplifier able to transform the signal in useful information.
d) No clue, I was looking at my phone while someone explaining.

20. What is the oxygen tension of articular cartilage in vivo ?

a) 20% Oxygen
b) 5% oxygen
c) 2-5% oxygen
d) 0.5-2% oxygen

21. What are the predominant types of loading that articular cartilage subjected to ?

a) Compression
b) Compression, hydrostatic pressure
c) Shear and compression
d) Hydrostatic pressure, compression and shear

22. **What is the most appropriate cell type for the development of articular cartilage therapies?**
    a) Chondrocytes
    b) Articular cartilage progenitor cells
    c) Mesenchymal stromal cells (bone marrow, synovium, adipose)
    d) Co-culture of two cell types described above.

23. **What is regenerative medicine?**
    a) Another recently introduced medical field
    b) A multidisciplinary field that encompass regenerative approaches in many medical specialities
    c) A product

24. **Which types of stem cells are currently used in clinical practice?**
    a) Embryonic stem cells
    b) Induced pluripotent stem cells
    c) Freshly isolated mesenchymal stem cells

25. **Briefly describe the advantages of cartilage-on-a-chip when compared with their in vitro traditional counterparts.**

26. **What can be future perspectives on improving cartilage-on-a-chip systems to better recapitulate their in vivo counterparts?**

27. **What markers can generally be used for exosome characterization?**
    a) CD 9, CD 81, CD 63
    b) CD 145, CD 21
    c) CD 8, CD 17
    d) CD 21, CD 45
    e) CD 21, CD 38

28. Which of the following is incorrect about exosomes?

a) Exosomes are small vesicles, typically ranging from 30 to 150 nanometers, that are released by cells into the extracellular space.
b) The cargo carried by exosomes reflects the physiological or pathological state of the originating cells, making them valuable indicators of cellular health and disease
c) Due to their phospholipid layer, exosomes cannot pass many physiological barriers within the body, such as the blood-brain barrier.
d) These membrane-bound vesicles contain a variety of bioactive molecules, including proteins, lipids, and nucleic acids such as RNA.
e) The contents of exosomes, particularly RNA molecules, have been found to reflect the genetic information of the parent cells, serving as messengers of cellular status.

29. Which surgical treatment option is suggested for >4cm2 osteochondral defects of the knee?

a) Microfracture
b) Mosaicplasty
c) Debridement
d) Osteochondral Allograft Transplantation
e) Total Knee Arthroplasty

30. Describe the current pharmaceutical treatment for Osteoarthritis and concerns with their adverse effects.

31. Which of the following treatment method for cartilage defects can achieve hyaline cartilage?

a) Microfracture
b) Mosaicplasty
c) Debridement
d) Osteochondral Allograft Transplantation
e) Autologous Chondrocyte Implantation

32. **Write down what you know about the recent emerging drug developments for the treatment of Osteoarthritis?**

33. **The chorioallantoic membrane (CAM) is formed by the fusion of the ……. and the …… …**

34. **Which of the following is not an advantage of the in ovo technique performed as an alternative to in vivo?**
    a) The chicken embryo is naturally immunodeficient at early stages.
    b) It is possible to work with more individuals per experiment group.
    c) The cost of the basic equipment and the eggs themselves is low.
    d) The post-treatment observation period is short.
    e) Results can be obtained in a short time.

35. **What should be the correct sequence of the metastatic cascade, the stages of which are given below, in a CAM model where tumor studies are performed?**

    I. Survival in circulation
    II. Invasion
    III. Metastasis formation
    IV. Extravasation
    V. Primary tumor
    VI. Intravasation

    a) V -II -I -VI -IV -III
    b) V -II -VI -I -IV -III
    c) V -VI -II -I -III -IV
    d) I -V -II -VI -IV -III
    e) I -V -VI -II -IV -III

36. **Which 3R principle is fulfilled by the *in ovo* technique?**
    a) Replace b) Reduce c) Refine d) Recovery e) Recycle

37. **Which of the following software is not a computer-aided quantification used in angiogenesis calculations in the CAM test?**
    a) Chalkley Point Array b) IKOSA
    c) Image J d) Angiotool e)AngioQuant

38. **Are pharmacological and non-pharmacological methods used in the treatment of OA effective and why?**

39. **What MSC resources can we use in OA?**

40. **Which MSC source do you think will be most effective in the treatment of OA and why?**

41. **What criteria should be taken into consideration when treating a patient with MSC?**

42. **What is the current situation for adipose-derived stem cells treatments for knee osteoarthritis?**

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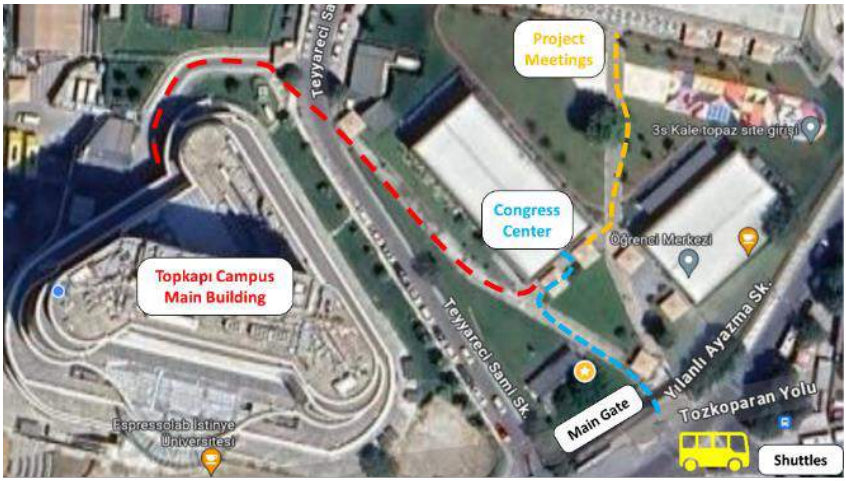
Navigation thorough ISU Topkapı Campus

Morning lectures will be given in Istinye University (ISU) Topkapı Campus Congress Center. You can follow the blue lines from the main gate.



Afternoon hands-on courses will be at the main building Laboratories. You can follow the red lines to reach main building. For the laboratories, take the elevator to 4th floor. Sunday afternoon project meetings will be on Health Science School Classrooms (orange line).

Lunch will be on the main building -2nd floor on Saturday and Sunday.

Coffee service will be at the 4th floor, Friday and Saturday afternoon.

University shuttles will be in front of the main gate. Use this link for shuttle times. https://www.istinye.edu.tr/tr/iletisim/servis-saatleri

General Course Program

Time	Activity	Location
01.12.2023 - Friday		
09:00 - 09:20	Registration	Congress center
09:20 – 12:30	Lectures	Congress center
12:30 – 13:30	Lunch	Congress center
13:30 – 17:30	Hands on laboratory courses	Main Building
18:00 – 21:00	Dinner (Optional)	Turkish Kebab Restaurant
02.12.2023 - Saturday		
09:00 – 12:30	Lectures	Congress center
12:30 – 13:30	Lunch	Main building -2nd floor
13:30 – 17:30	Hands on laboratory courses	Main Building 4th floor
19:00 – 24:00	Gala Dinner (Optional)	Kazancı Meyhane
03.12.2023 - Sunday		
09:00 – 11:30	Lectures	Congress center
11:30 – 12:30	Panel	Congress center
12:30 – 13:30	Lunch	Main building -2nd floor
13:30 – 15:30	Project based learning	Health Science Classrooms
15:30 – 16:30	Feedback and Certificates	Congress center

Laboratory Circulation

	Group A	Group B	Group C	Group D
Friday 13:30- 15:10	4th floor 401/A	4th floor 401/I	4th floor 401/D	4th floor 401/C
Friday 15:30- 17:30	4th floor 401/C	4th floor 401/A	4th floor 401/I	4th floor 401/D
Saturday 13:30- 15:10	4th floor 401/D	4th floor 401/C	4th floor 401/A	4th floor 401/I
Saturday 15:30- 17:30	4th floor 401/I	4th floor 401/D	4th floor 401/C	4th floor 401/A