R.4.WP3.1 REPORT ON HCA FOR 2D CULTURE OF HETEROGENEOUS CULTURE

- R.4.WP3.1. Report sulle letture adatte per l'analisi HCA in condizioni 2D di co-colture eterogenee, quali infarto del miocardio, angiogenesi, modello di sensibilizzazione neuronale e modello neuro-infiammatorio/ Poroč ilo o od čitkih, primernih za HCA v 2D pogojih heterogenih sokultur, npr. model miokardnega infarkta, angiogeneze, nevronske senzitizacije in nevro-inflamacije

Responsible Partner: ICGEB
Contributing partners: Josef Stefan Institute and Experteam

High content analysis (HCA) set-up and software:

- ImageXpress Microscope from Molecular Devices, using MetaXpress software
- Operetta High-Content Imaging System from Perkin Elmer, using Harmony and Columbus software

The following 2D co-culture disease models have been set up by the ICGEB for HCA:

1. Model of myocardial infarction:
    - co-culture of cardiomyocytes, cardiac fibroblasts and cardiac endothelial cells in normoxia and hypoxia
    - HCA to evaluate cell composition, proliferation, apoptosis, viability, angiogenesis, fibrosis through myofibroblast activation and collagen secretion (GFP RFP mice), cardiomyocyte hypertrophy
2. Model of critical wounds:
    - culture of skin endothelial cells and fibroblasts on various extra cellular matrixes substrates
    - HCA to evaluate cell composition, proliferation, apoptosis, viability, migration, angiogenesis in vitro, fibrosis through myofibroblast activation and collagen secretion (GFP RFP mice)
3. Model of pain and neuroinflammation:
    - culture of dorsal root ganglion neurons with fibroblasts and immune cells
    - HCA to evaluate cell composition, mitochondrial density and distribution, and oxidative stress in response to pain killing approach
4. Model of mesenchymal stromal cell differentiation for the regeneration of osteo-articular pathologies:
    - mesenchymal stromal cell differentiated into chondro, adipose and osteocytes
    - HCA to evaluate cell composition (chondro, adipose or osteo), apoptosis, cell viability

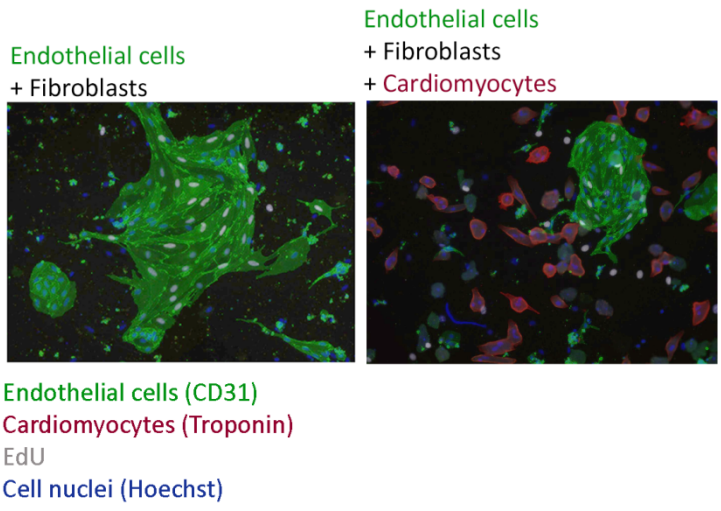
On the following pages each model is described in detail.

1. Model of myocardial infarction

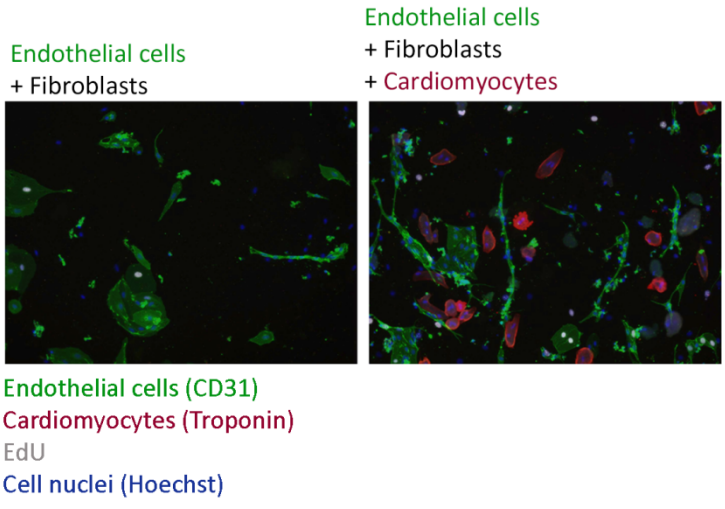
To set up an in vitro model of myocardial infarction we established a co-culture system, in which the three main cardiac cellular types (endothelial cells, cardiomyocytes and fibroblasts) coexist. Primary cells are isolated from various tissues using mechanical mincing followed by enzymatic digestion. Various cell types can then be selected using either MiltenyiBiotec magnetic beads or Dyna beads conjugated with antibodies targeting cell-specific surface antigens. Fibroblasts can be most easily selected through their propensity towards adherence on plastic; by plating enzymatically digested tissue on plastic plates, and allowing cells to attach for a short period of time, the attached cells will be largely fibroblasts. This model can be used to evaluate the intercellular cross-talk occurring in the normal heart and in pathological conditions (e.g. placing the system in a hypoxic chamber to mimic the lack of oxygen during myocardial infarction, adding a medium with high glucose to mimic diabetic conditions, etc.).

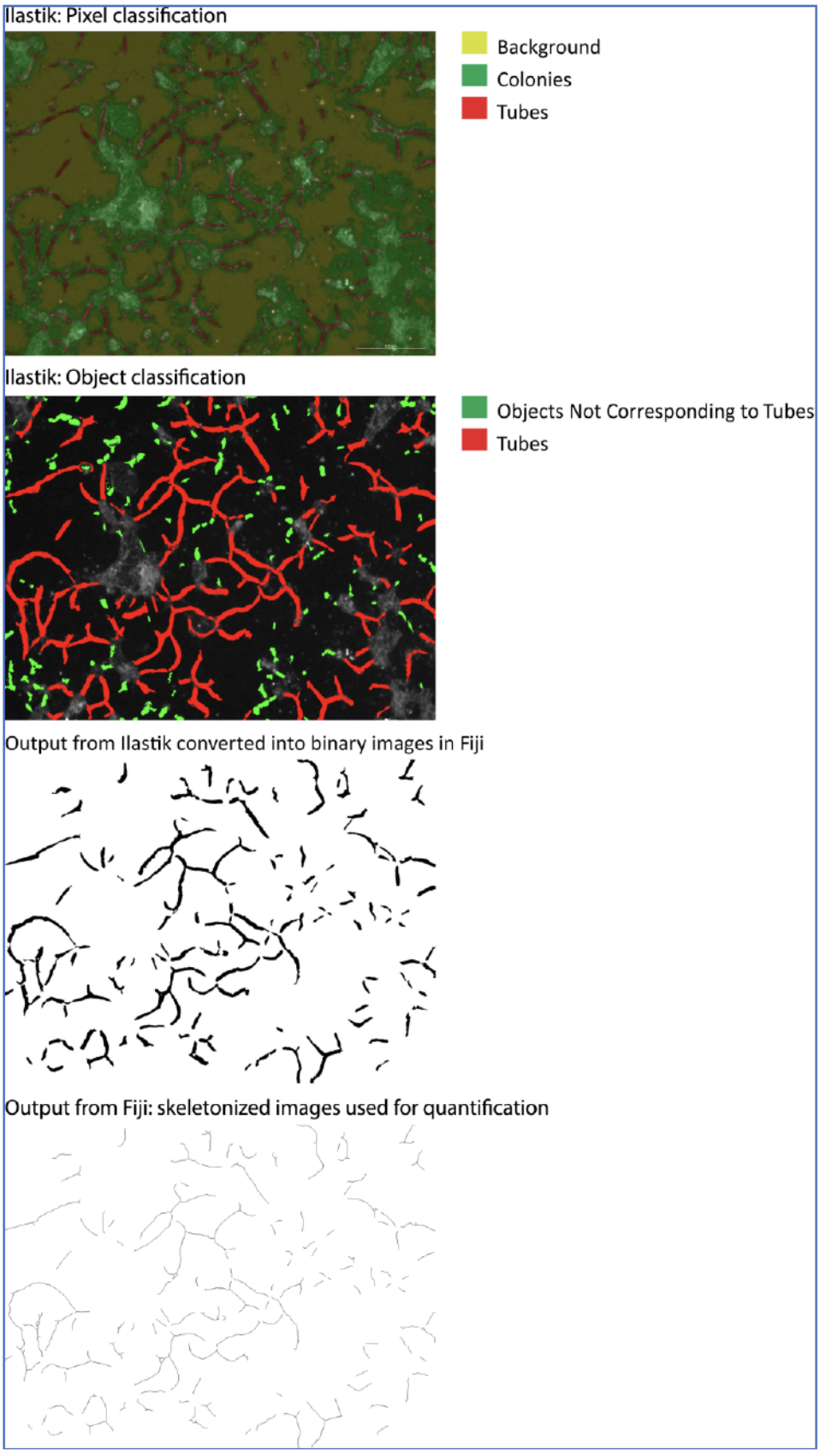
Through this model, various read-outs have been optimized which can be applied in an HCA setting. **Cell composition** can be evaluated using cell-specific antibodies (CD31 or ERG for endothelial cells, Vimentin for fibroblasts and Troponin for cardiomyocytes). Further, cells can be isolated from transgenic mice expressing cell type-specific fluorescent reporters. In this way cells can be identified by their expression of green or red fluorescent protein (GFP, RFP), for example under the control of a collagen promoter to identify fibroblasts, or under the control of a PDGF promoter to identify endothelial cells.

Cell **proliferation** in this system can be easily identified by adding the nucleotide analogue 2'-Deoxy-5-ethynyluridine (EdU), followed by its detection by a chemical reaction that leads to a fluorescence signal. Alternatively, an antibody staining for the proliferation marker Ki-67 can be used. When coupled with the above mentioned cell specific antibodies, this can be used to understand which cell types proliferate in response to a specific stimulus. **Apoptosis** (programmed cell death) can be analyzed, again in a cell type specific manner, through terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, or by staining for cleaved caspase 3. When combining staining for apoptosis with a count of total cell number, the **viability** of various cell types can be evaluated.

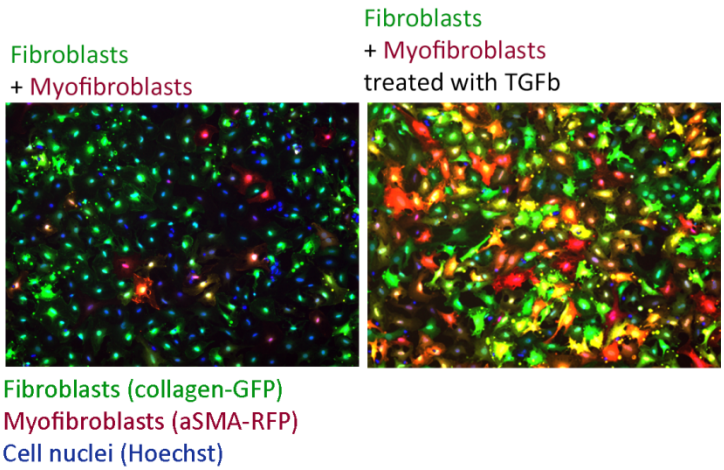


Within the model of myocardial infarction it is further possible to analyze **angiogenesis**, by plating cells on a layer of matrigel matrix, which together with a stimulation with Vascular endothelial growth factor A (VEGF-A), induces tube formation by endothelial cells. This assay in a plastic dish models the complex process of blood vessel formation in the heart and can provide quantitative angiogenic scores in terms of number of branch points, size of tube network and length of branches.



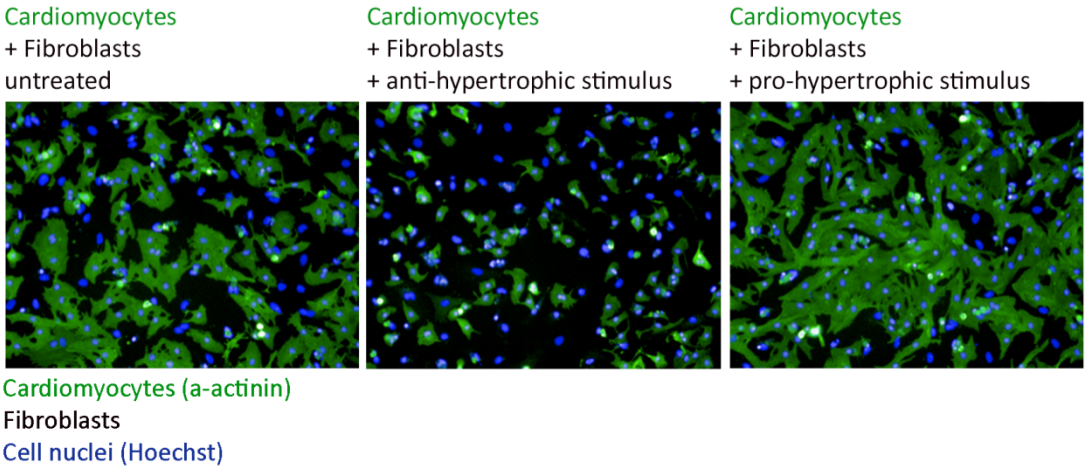


Quantification of angiogenesis is performed using a protocol developed in-house, where the number of tubes is normalized to the number of endothelial cells. Tube number is quantified using an interactive learning and segmentation software, Ilastik v. 1.3.0. Images are classified into three categories: endothelial tubes, endothelial colonies and background by setting specific size and intensity filters, and exported as binary images to the Fiji ImageJ software. Finally, tubes are analyzed using a Fiji plug-in for angiogenesis quantification, and nuclei are counted.



An additional typical feature of myocardial infarction is **fibrosis**, which results from myofibroblast activation and collagen secretion. Myofibroblasts represent an activated form of normal fibroblasts that in response to pro-inflammatory stimuli start expressing contractile proteins, such as alpha smooth muscle actin, and enhance the production of collagen. To reproduce this process in a dish, we isolated primary fibroblasts from GFP RFP mice, which express the green GFP protein under the control of the collagen promoter and the red RFP protein under the control of the alpha smooth muscle actin promoter. As green cardiac fibroblasts differentiate into myofibroblasts, as spontaneously occurs in culture or can be further induced using stimulation with transforming growth factor beta, they will start activating the alpha smooth muscle actin promoter, thereby expressing RFP and turning orange/red.

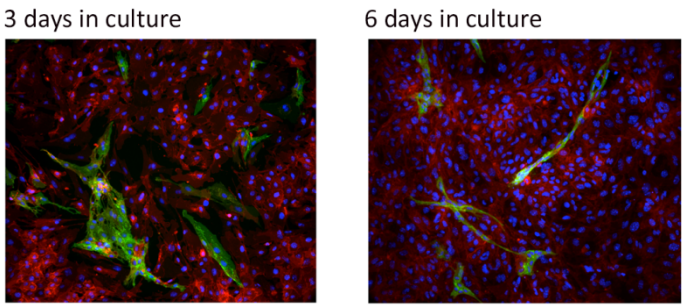
After myocardial infarction, the loss of contractile tissue induces the progressive **hypertrophy** of remaining cardiomyocytes. This effect can also be studied in a dish on isolated primary cardiomyocytes. Primary adult and neonatal cardiomyocytes are isolated and plated with other cardiac cell types to study the influence of various factors on cardiomyocyte hypertrophy. For this, cells are stained using antibodies that bind to the cellular skeleton (such as alpha-actinin), or else are stained with a cell mask, a lipophilic dye which allows for visualisation of the cell membrane. Using HCA, the size of the cells can thereby be measured, with any changes in hypertrophy easily quantifiable.



2. Model of critical wounds

To set up an in vitro model of wound healing we established a co-culture system, in which both fibroblasts and endothelial cells are cultured together. As above, primary cells are isolated from various tissues using mechanical mincing followed by enzymatic digestion. Endothelial cells can be selected using Miltenyi Biotec magnetic beads, or Dyna beads conjugated with an antibody targeting the endothelial cell surface specific antigen CD31. Fibroblasts are selected through adherence to plastic. This model can be used to evaluate the intercellular cross-talk occurring in wound healing, and the effect of various substances or other cell types on this process can be evaluated.

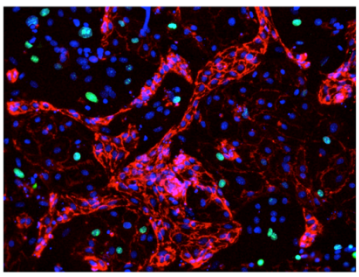
Various read-outs have been optimized which can be applied in a HCA setting. As above, **cell types** can be identified using cell-specific antibodies (CD31 or ERG for endothelial cells, and Vimentin for fibroblasts). As an alternative to staining, cells can be isolated from various transgenic mice to differentiate between cell types. For example, in some images below, endothelial cells were isolated from Rosa26mT/mG mice, in which all cells are constitutively expressing the red tomato gene, whereas fibroblasts were isolated from actin-GFP mice and are therefore labelled in green. Alternatively, as shown above, cells can be isolated from CDH5-CreER;Rosa26mT/mG mice after tamoxifen injection; in these conditions, endothelial cells express green fluorescent protein and are green, whereas other cells including fibroblasts are labelled red by tomato red. Alternatively, cells can be isolated from transgenic mice in which the majority of cells are not fluorescently labeled, and with cell type specific reporters with fluorescent markers. In this way cells can be identified by their expression of green or red fluorescent protein, for example under the control of a collagen promoter to identify fibroblasts, or under the control of a PDGF promoter to identify endothelial cells.



Endothelial cells (CDH5-CreER;Rosa26mT/mG mice)
Fibroblasts (Rosa26mT/mG mice)
Cell nuclei (Hoechst)

The use of primary cells isolated from transgenic mice and genetically labeled by fluorescent proteins offers numerous advantages. First, staining to identify the cell type is not required, thus allowing tracking of cell behavior over many days and by live imaging. Second, additional phenotypic markers can be analyzed using alternative colors, as one of the most common limiting factors in HCA analysis is that antibodies are generally produced in a few host species and thus are difficult to combine in the same reaction without cross-talk.

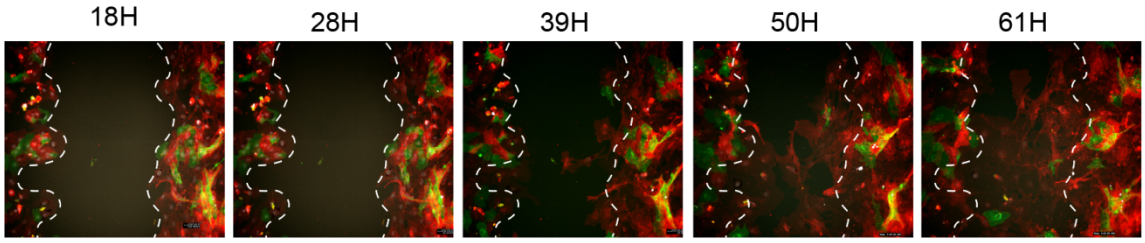
Endothelial cells
+ Fibroblasts



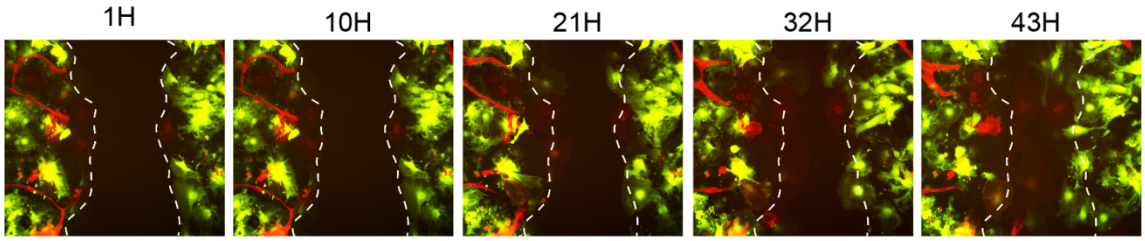
Proliferation (Ki67)
Endothelial cells (CD31)
Cell nuclei (Hoechst)

As above, using various markers it is possible to evaluate **proliferation**, **apoptosis**, and **cell viability**, all in a cell type specific manner. Also in this model, **fibrosis** can be studied, using cells isolated from GFP RFP mice to see differentiation into myofibroblasts.

Extremely important to wound healing is the **migration** assay, in which a monolayer of fibroblasts and endothelial cells is disturbed by a scratch. This can be performed manually using a pipette tip, or in a standardized manner using specialized chambers in which cells are seeded and an insert is removed to create an homogenous gap in the monolayer. This allows for the quantification of the extent and speed of wound closure. Through live imaging the entire process of wound closure can be closely observed. For this, live imaging is initiated 1 to 18 h after disruption of the monolayer, and images are acquired every 10 min for two days.

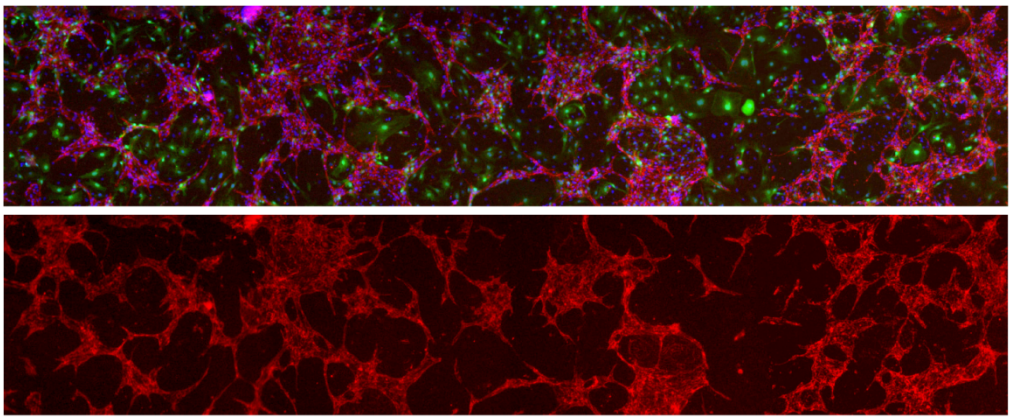


Endothelial cells (CDH5-CreER;Rosa26mT/mG mice)
Fibroblasts (Rosa26mT/mG mice)
Cell nuclei (Hoechst)



Fibroblasts (actin-GFP mice)
Endothelial cells (Rosa26mT/mG mice)
Cell nuclei (Hoechst)

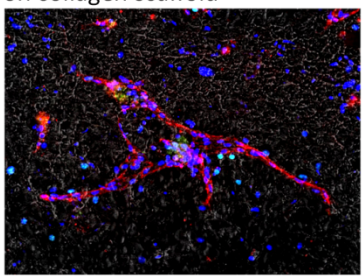
Also in the context of this model of wound healing it is possible to analyze **angiogenesis** in vitro, a process which plays a large role in wound healing in vivo. To analyze angiogenesis, fibroblasts and endothelial cells are co-cultured on matrigel, in the presence of VEGF-A to stimulate angiogenesis. Various factors can be added in order to evaluate their pro- or anti-angiogenic activity during wound healing.



Fibroblasts (actin-GFP mice)
Endothelial cells (Rosa26mT/mG mice)
Cell nuclei (Hoechst)

Further, cells can be grown on various **cellular matrix substrates**, to study the effect of these on wound healing. These include simple matrixes such as a coating of fibronectin and gelatin, matrigel or collagen, as well as more complex matrixes such as the clinically used Integra scaffold.

Endothelial cells
on collagen scaffold

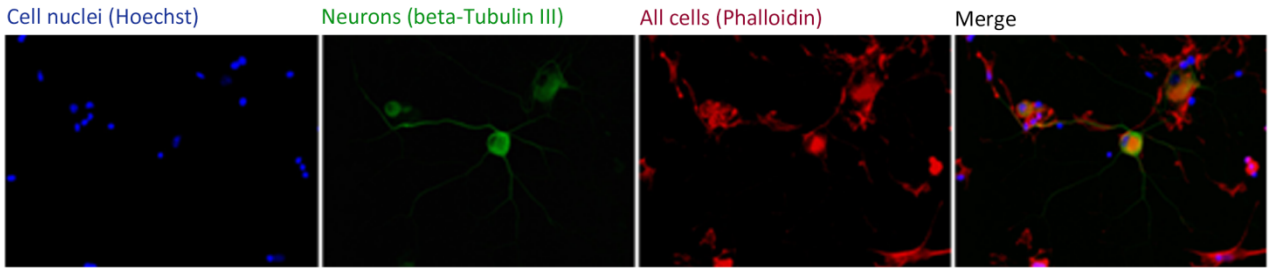


Proliferation (Ki67)
Endothelial cells (CD31)
Cell nuclei (Hoechst)

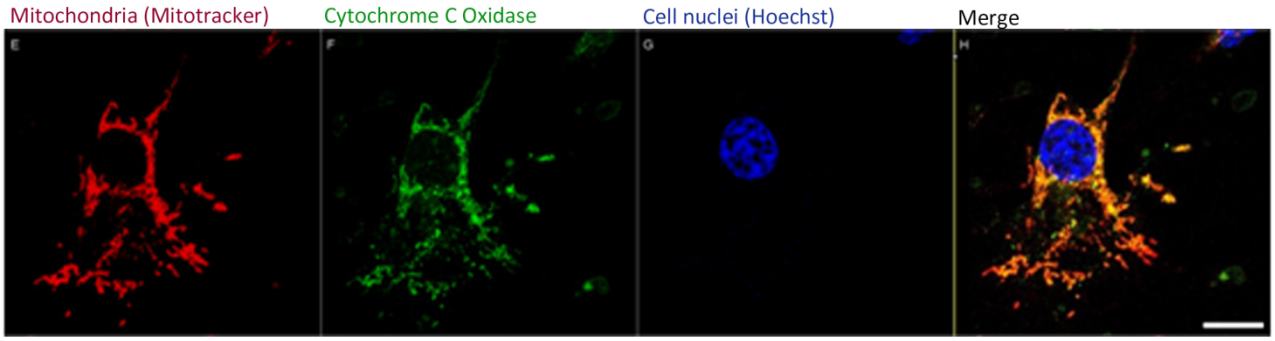
3. Model of pain and neuroinflammation

Pain and neuroinflammation can be studied in vitro through a co-culture of neurons, fibroblasts and immune cells. Dorsal root ganglion neurons are isolated using enzymatic digestion, resuspended to break up cell clumps, and plated on a commercial extracellular matrix made from mouse sarcoma cells. Cells are ready to be analyzed at 72 h after plating, when they present branches, thereby resembling mature neurons. When plated with fibroblasts and immune cells, these co-cultures can be used to study the effect of various pain and inflammation treatments.

Cell composition can be analyzed using cell specific antibodies, e.g. anti-β-Tubulin III to recognize neurons and Vimentin to stain fibroblasts. Phalloidin can be used to stain cytoskeleton F-actin, in order to stain all cells in the dish.



Several assays can be applied to this disease model, to be used in HCA. For instance, **mitochondrial density and distribution** can be measured using mitochondrial markers such as mitoRed and cytochrome C. **Oxidative stress** was measured using the cell-permeant 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) dye (Invitrogen), while mitochondrial superoxide was measured using MitoSOX (Invitrogen). This assay is particularly useful to study the mechanisms by which pain-killers or analgesic strategies impact on sensory neurons, and in particular on their mitochondrial activity, which is known to importantly affect neurotransimission of the pain stimulus.



4. Model of mesenchymal stromal cell (MSC) differentiation for the regeneration of osteo-articular pathologies

Mesenchymal stromal cells are the ideal cells to study osteo-articular pathologies, as they differentiate into several cell types in vivo, including chondrocytes, osteocytes and adipocytes. Primary cells are isolated from bone marrow and can then be differentiated through various mediums (such as ChondroMAX, Osteo-MAX-XF, and AdipoMAX differentiation medium from Sigma Aldrich) into these cell types. A co-culture of chondrocytes, osteocytes and adipocytes can act as a model of osteo-articular pathologies, and the effect of various treatments on chondro and osteogenesis can be studied in this model.

Cell composition can be analyzed by staining for specific cell types. Specifically, chondrocytes, which grow in structures which need to be fixed and sectioned, are stained with Alcian-Blue. Osteocytes are stained with Alizarin-Red and adipocytes are stained with Oil-Red-O. The latter is particularly adaptable to microscopy, as Oil-Red staining is visible by eye as well as being fluorescent in the red channel, and can therefore be acquired both in bright field and in red fluorescence. **Apoptosis** and **cell viability** can be evaluated using HCA as detailed above, by quantifying total cell number, as well as cleaved caspase 3 positive cells, or by using the TUNEL assay.