Tissue engineering at MHH – challenges and success in frame of the NanoMed TWIN project

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General activities for all visiting students

- Hannover Biomedical Research school (HBRS)
  - Good scientific practice course
  - Lectures in frame of the PhD program “Regenerative sciences”

- Rebirth lectures (scientist from MHH report about their research, open to all MHH staff)

- Monthly/bimonthly journal club in LEBAO (screening of publications)
- Weekly progress report of one of the members of LEBAO
- Mandatory weekly staff meeting

- Weekly journal club in working group Hilfiker
  - Each student presented at least one publication relevant to her/his field of research

4 Students visits

1. Olga Ignatov (October 2019 - March 2020)
   Biomechanical and biocompatibility tests of tissue strips made out of placental membrane loaded w/o nanoparticles (GaN or ZnO)

2. Tatiana Malcova (September 2020 - February 2021)
   Evaluation of blood vessel decellularization using a combination of physical and chemical methods

3. Alina Stoian (October 2020 - March 2021)
   Decellularization of composite bone graft vascularized by vascular pedicle

4. Vitalie Cobzac (October 2021 - March 2022)
   Storage of articular cartilaginous tissue for transplantation. In vitro cartilage growing on a 3D scaffold combined with nanoparticles with piezoelectric potential.

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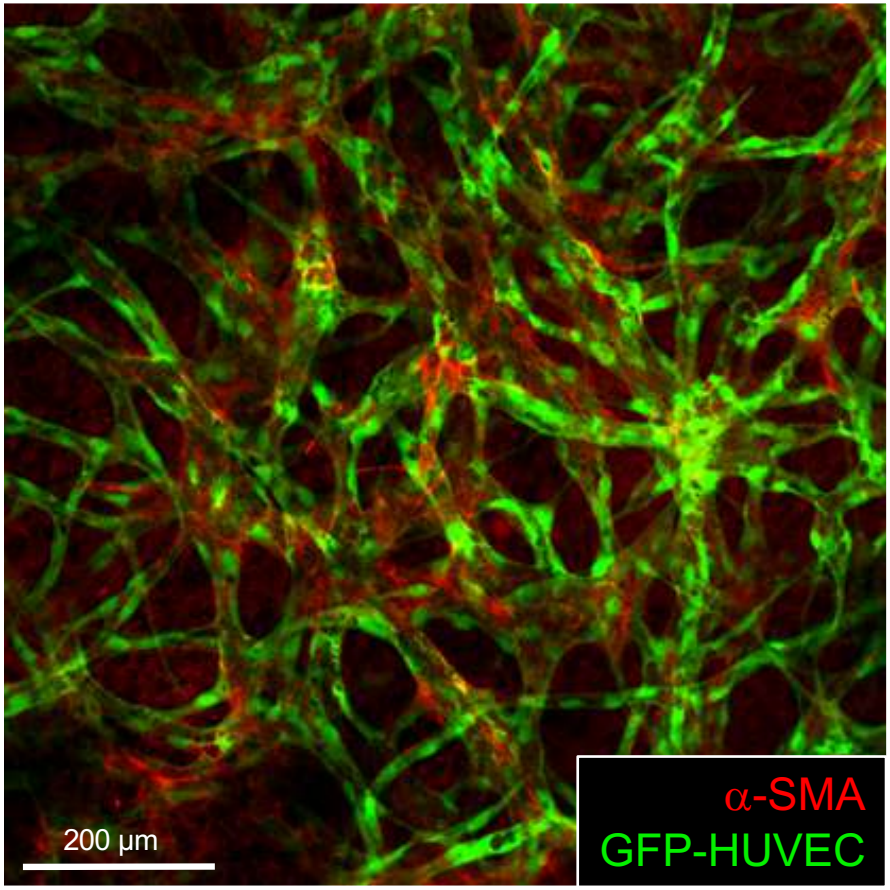
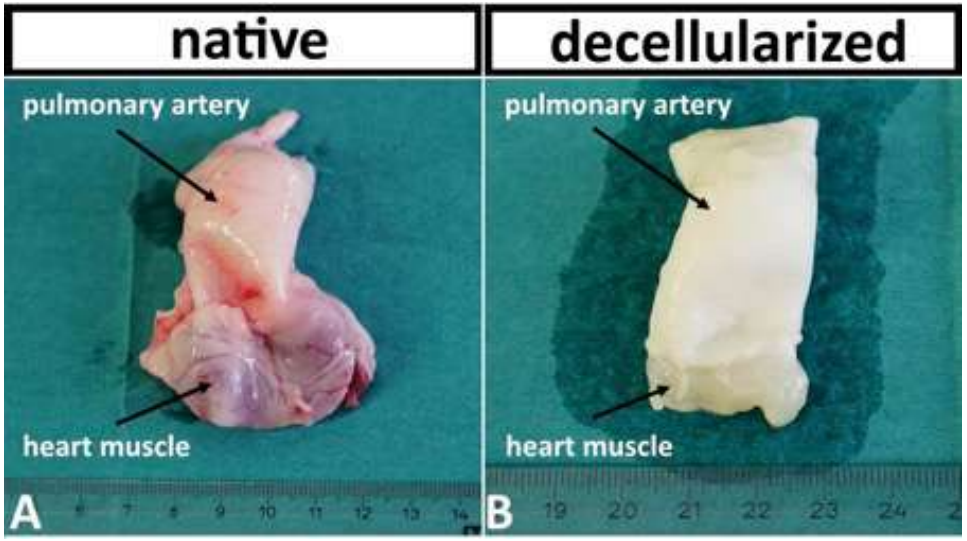
Tissue engineering

- Perfusable, Vascularized Matrices



- Allogenic & Xenogenic Heart Valves

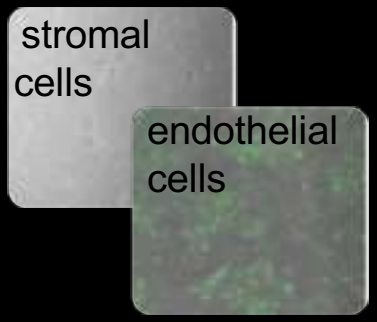


Vascularized Constructs

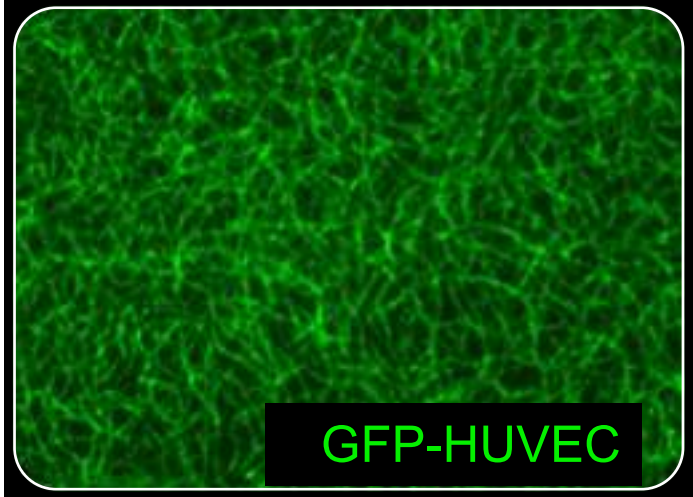
decellularized small intestine (SIS)

3D hydrogel

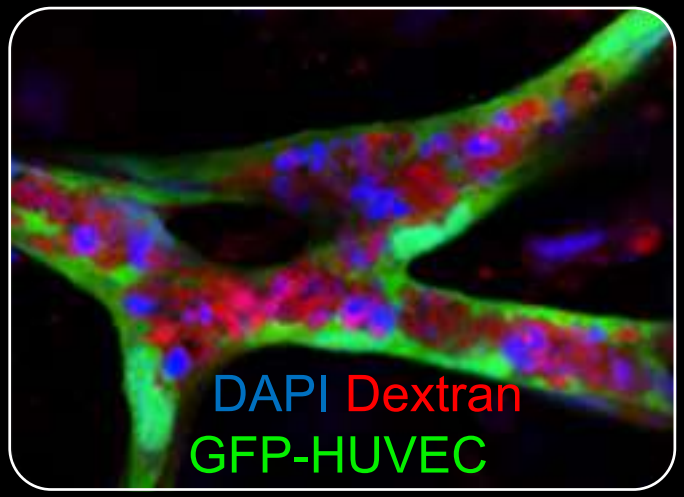
cells



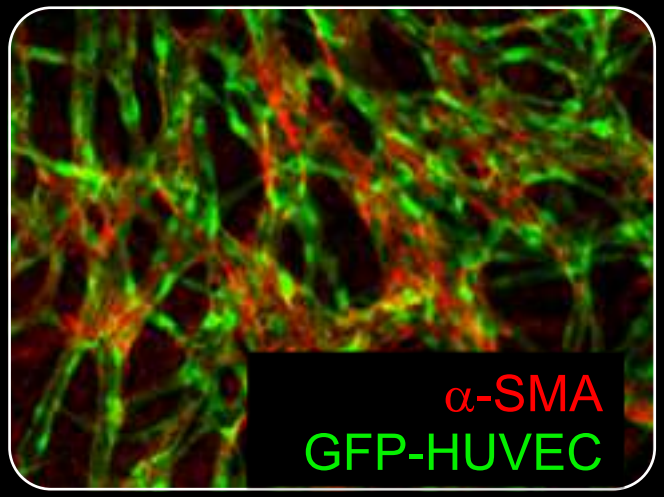
network formation



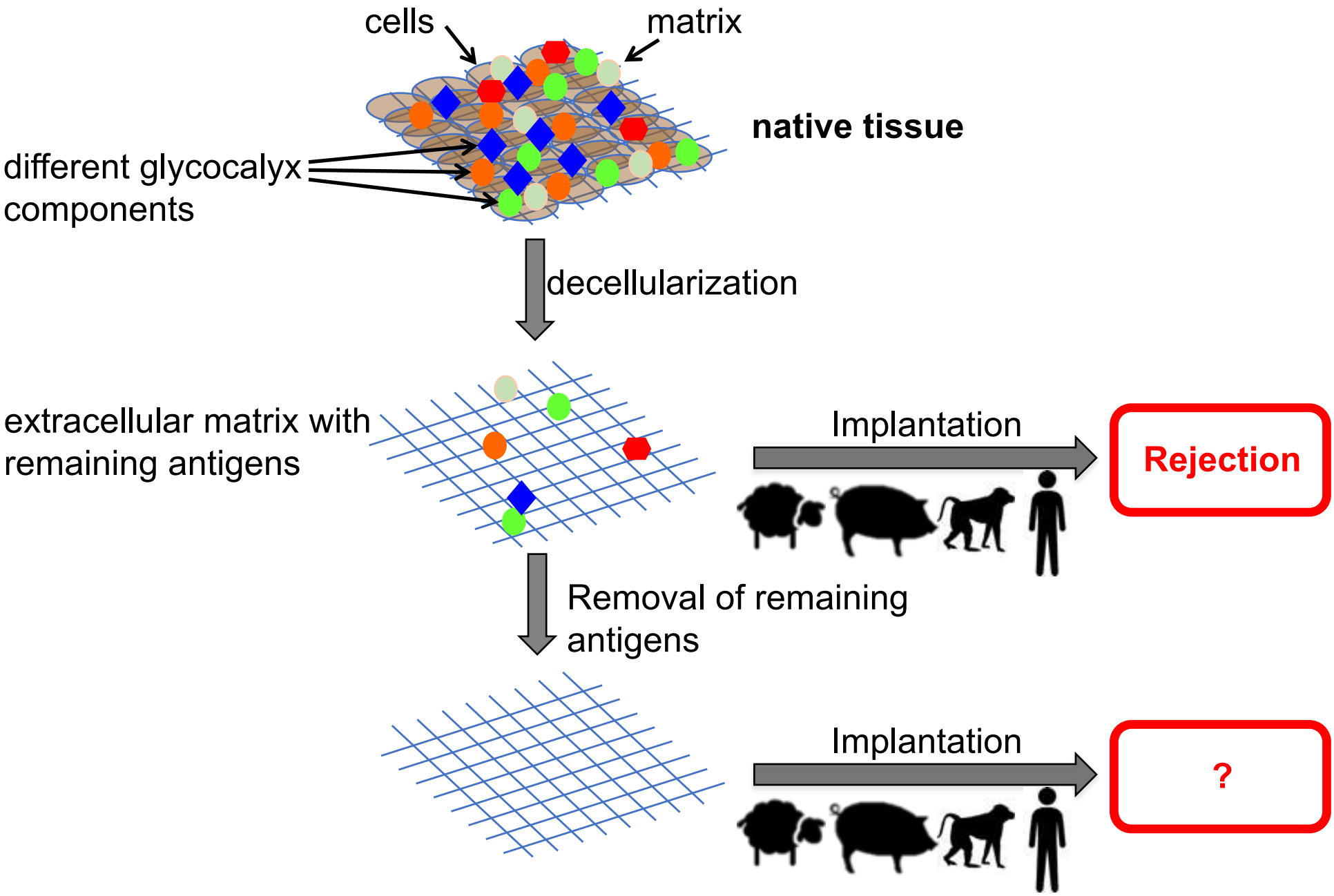
hollow structures



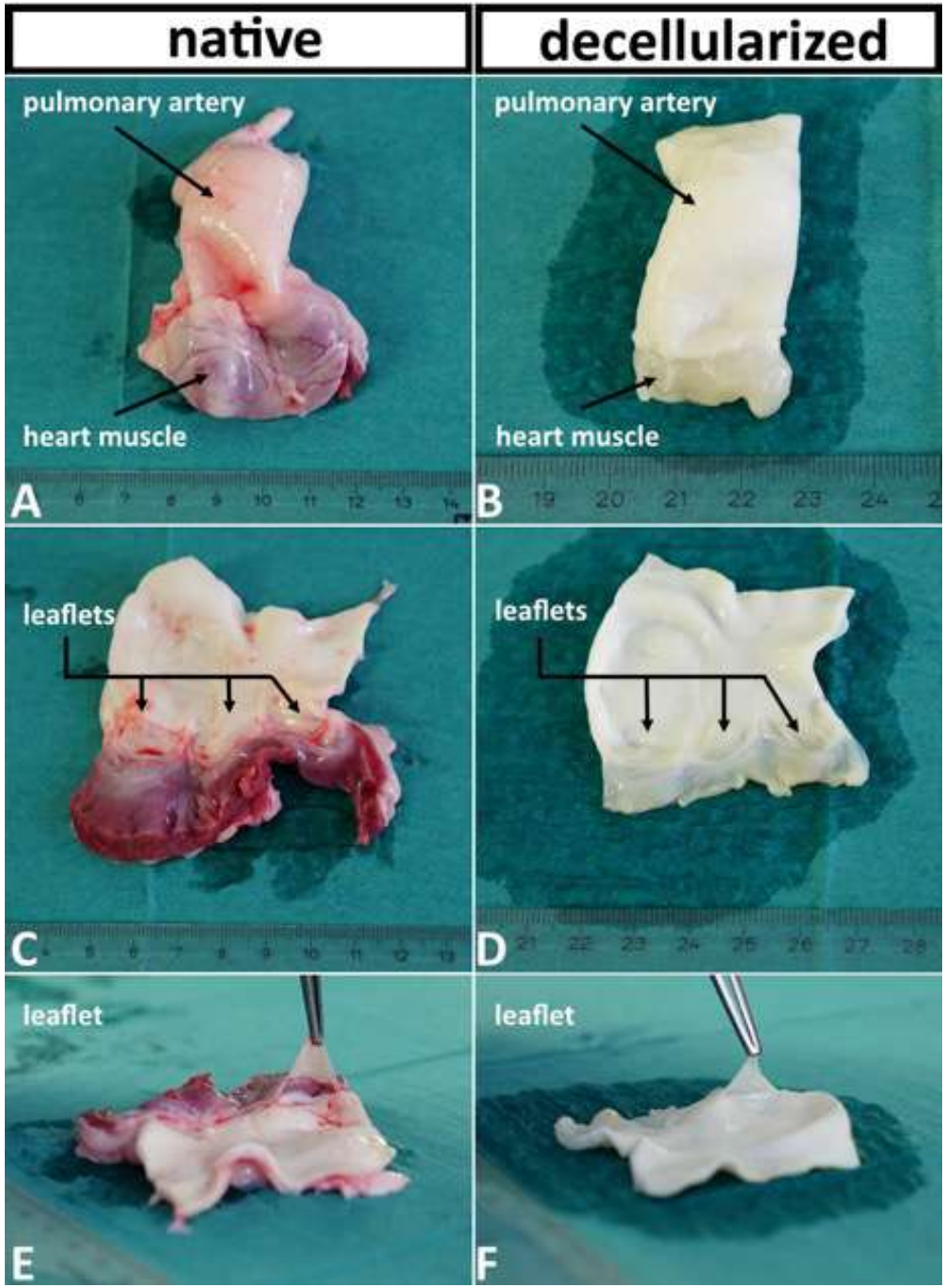
cell interaction



Allogenic & Xenogenic Heart Valves for Transplantation



Allogenic & Xenogenic Heart Valves



- Optimization of decellularization protocols
- Assessment of mechanical properties
- Analysis of composition by biochemical methods and histology
- Assessment of immunological properties *in vitro*
- *In vivo* testing

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Our expertise

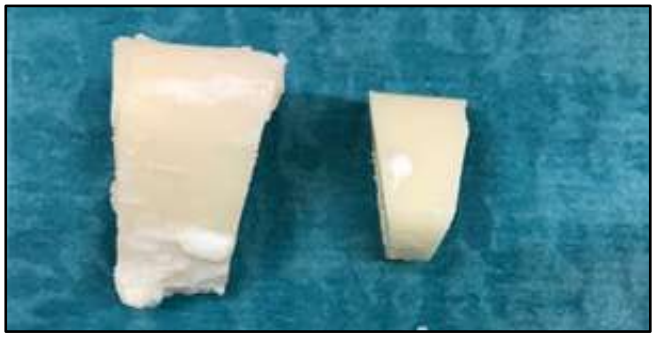
- Decellularization (SIS, heart valves, small vessels)
- Cell culture (diverse cell types)
- Cytotoxicity assays
- Analysis:
  - Histology
  - Immunofluorescence
  - Mechanical testing
  - SEM (facility on campus)

Decellulariaztion

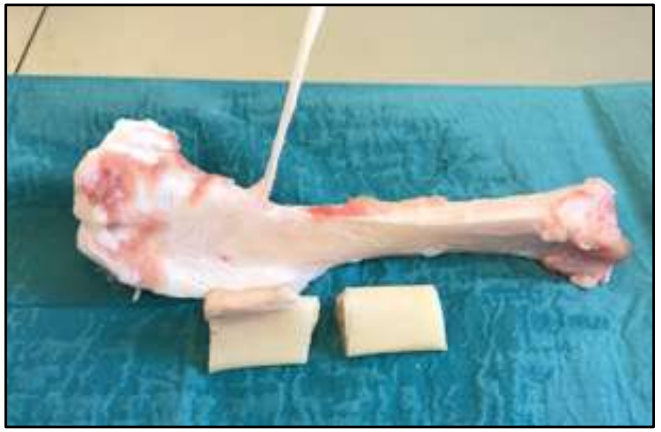
is a process to remove cellular material from a tissue leaving an extracellular matrix based scaffold of the original tissue behind, which can be used in artificial organ generation and tissue regeneration.

NanoMedTWIN:

- large and small blood vessels (aorta, carotid artery)
- Bone pieces



- Bone with vascular pedicle



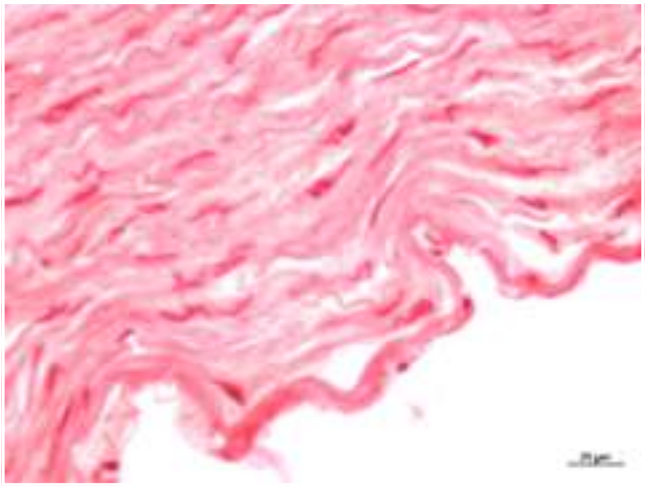
Evaluation of decellularization

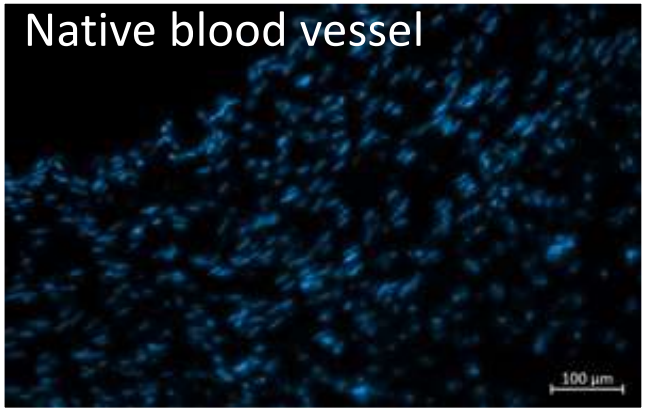
- Staining of sections from decellularized material in comparison to native material

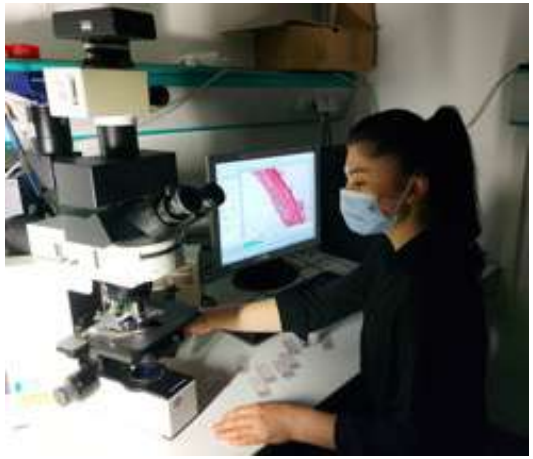
  - **Hematoxylin and eosin staining:** Hematoxylin stains nucleic acids deep purple Eosin stains proteins nonspecifically pink. In a typical tissue, nuclei are stained blue, whereas the cytoplasm and extracellular matrix have varying degrees of pink staining.

  - **DAPI** (4‘,6-diamidino-2-phenylindole): a blue-fluorescent DNA stain

Native blood vessel

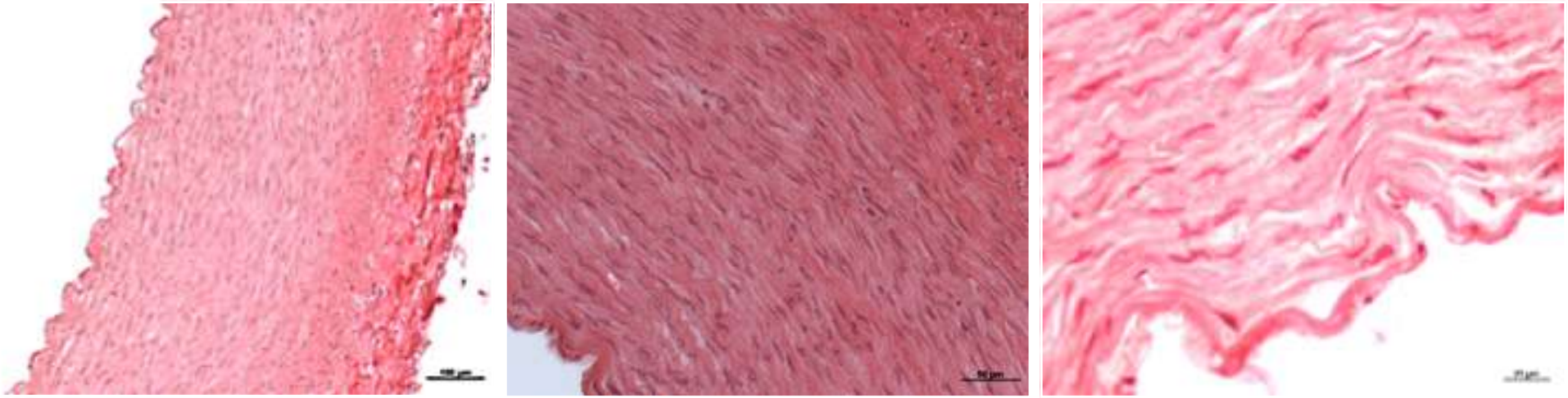




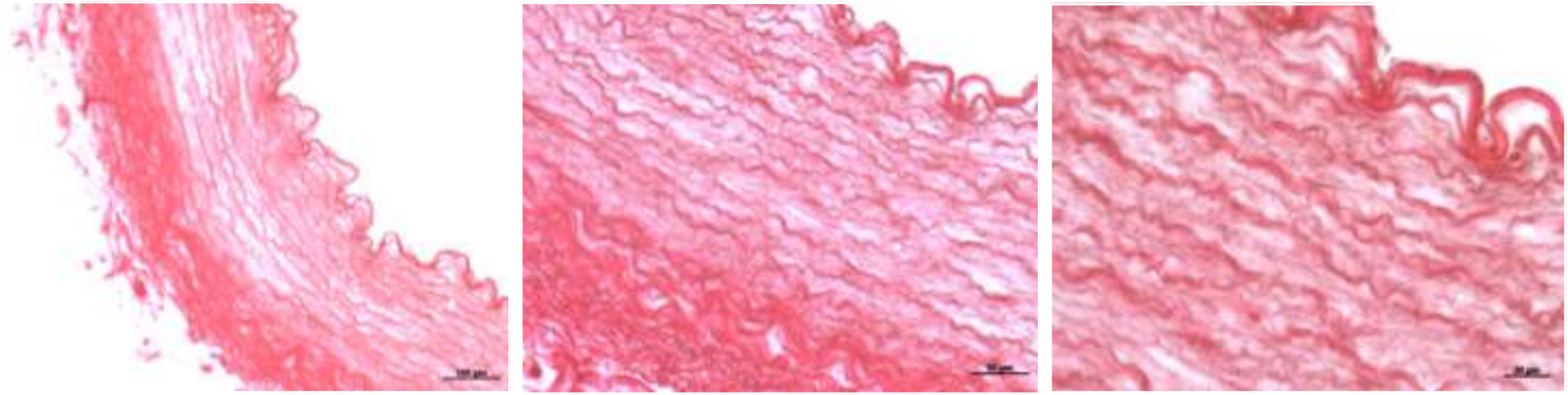


H&E staining of porcine carotid artery

Native

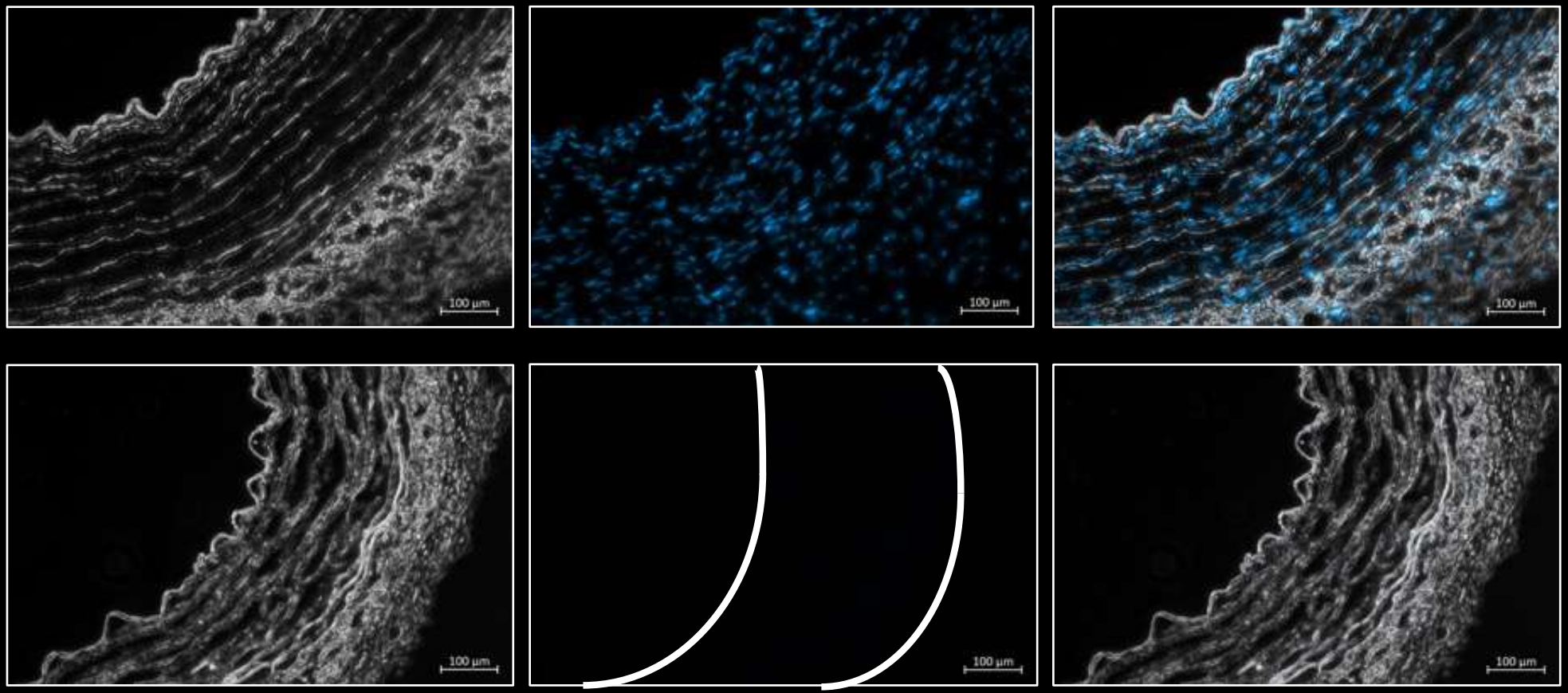


Treated



Nuclear material was successfully removed.
Matrix proteins and structure are preserved.

DAPI staining of porcine carotid artery



Nuclear material was successfully removed.

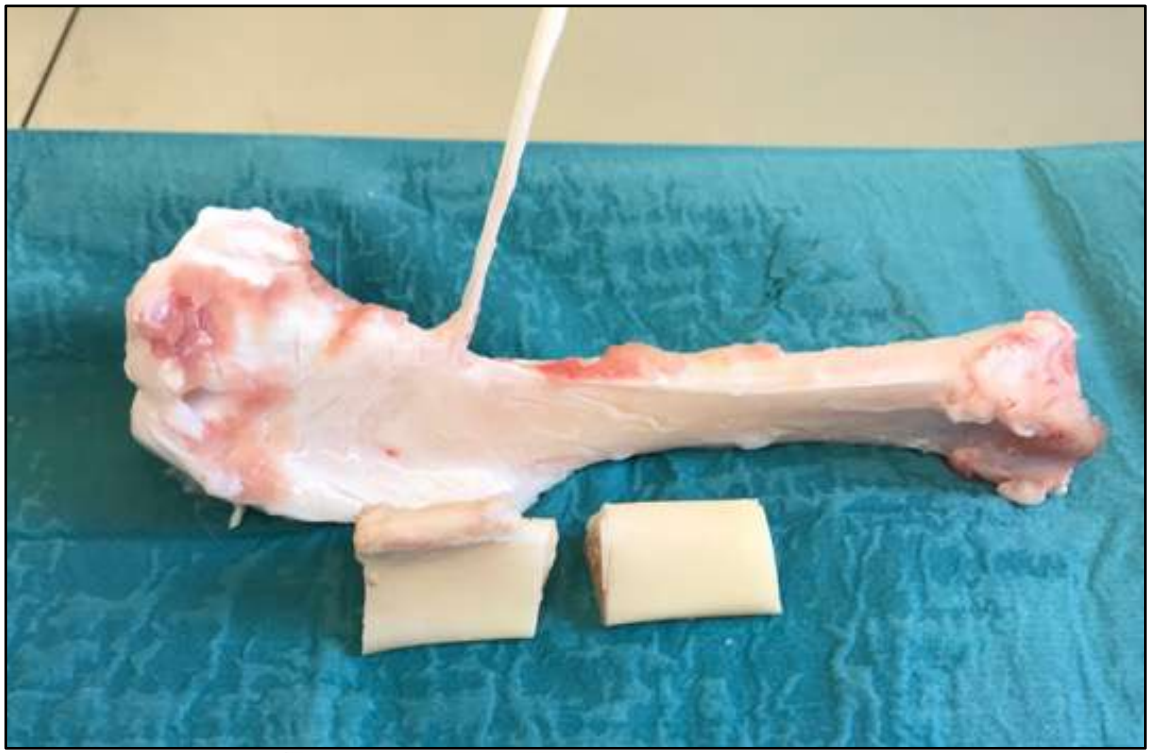
Decellularization of bone with vascular pedicle

Main challenge of such a complex material:
Finding a protocol that decellularizes both tissue – bone and vessel - without harming the extracellular matrix of both tissues

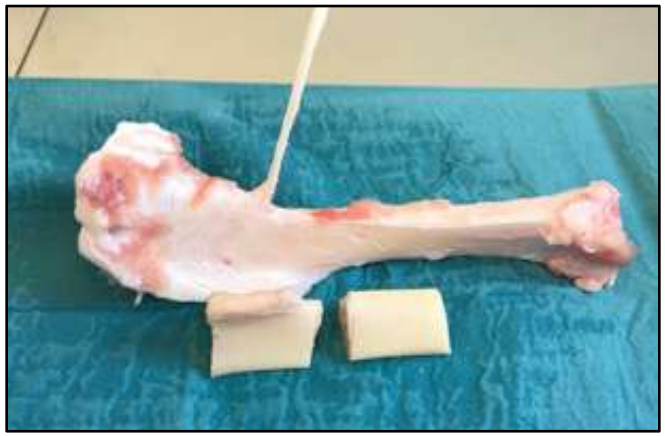
Challenge: Which material to use at MHH?

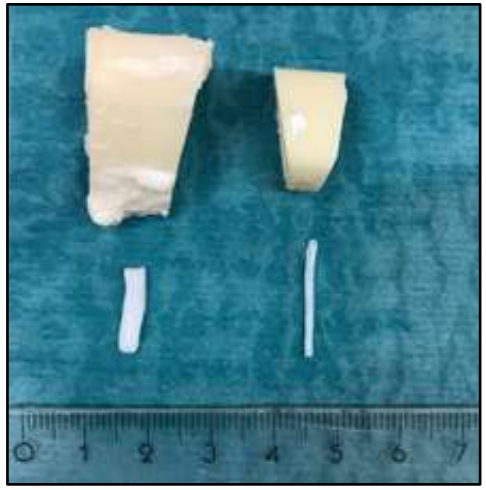
Moldowa

Germany



Decellularization of bone with vascular pedicle





Test protocol first independently on bone and blood vessels as the structure in the pig is quite large

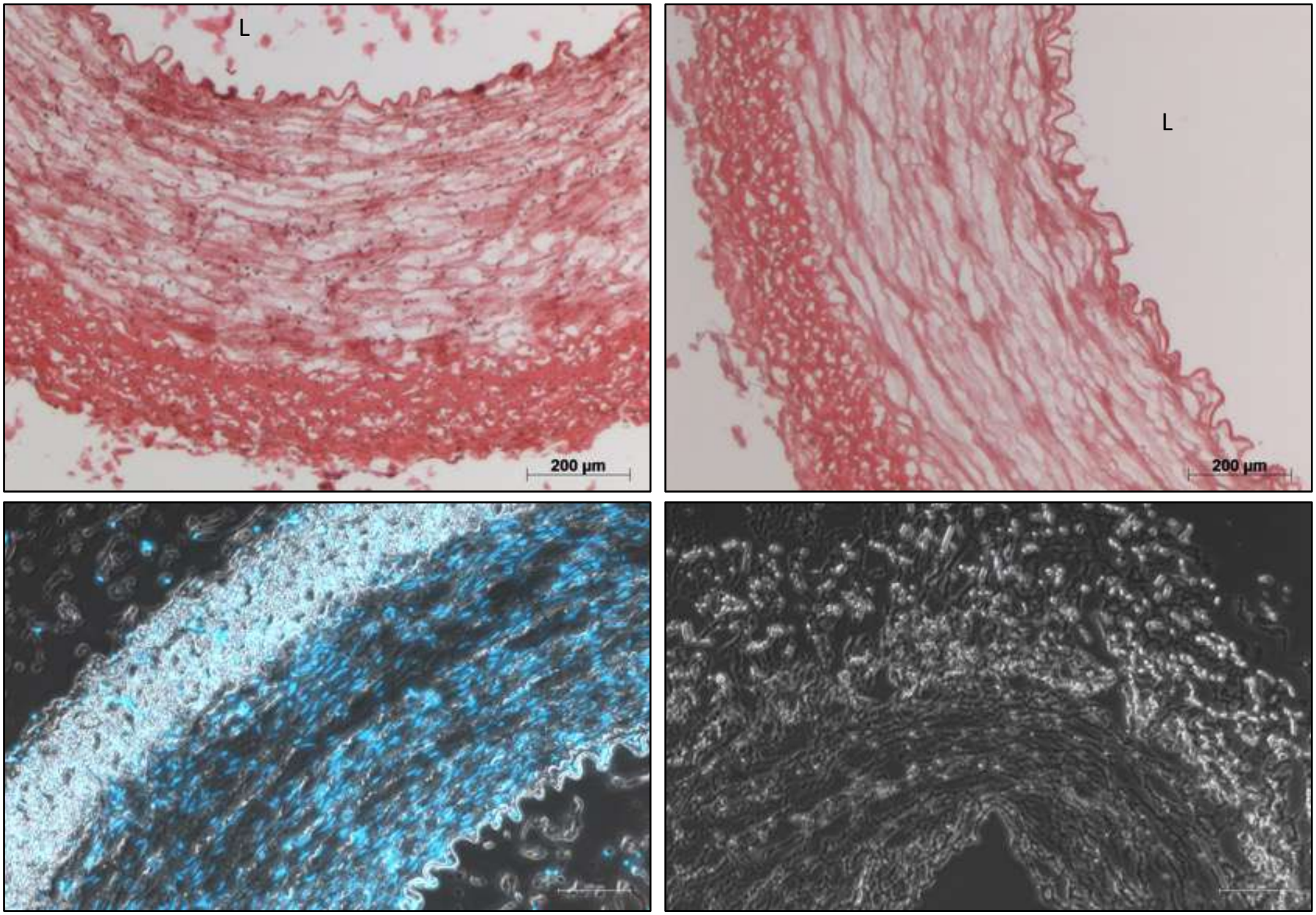
Decellularization of vessel

Native vessel

After processing

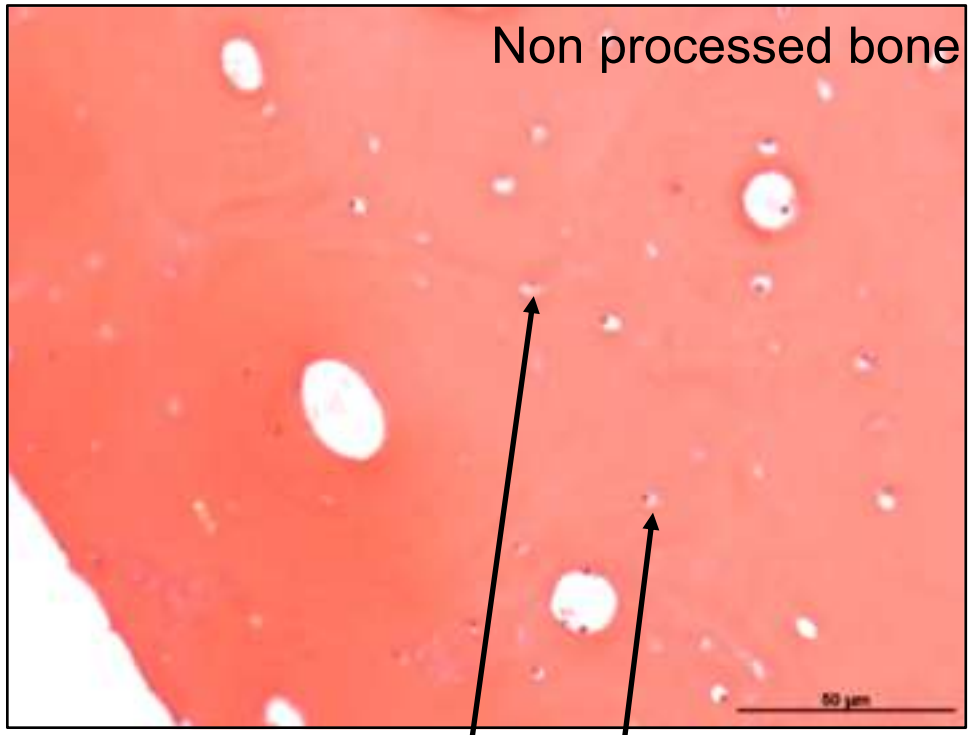
H&E staining

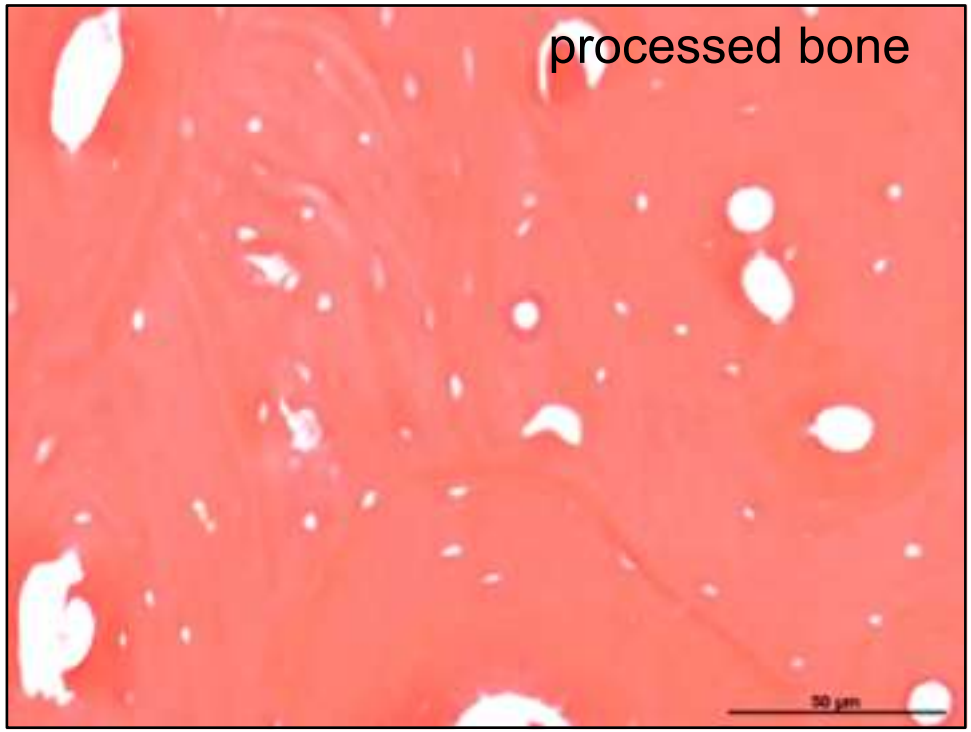
DAPI



Challenge: Bone needs to be de-calcified before cutting – takes a couple of weeks

H&E staining of porcine bone

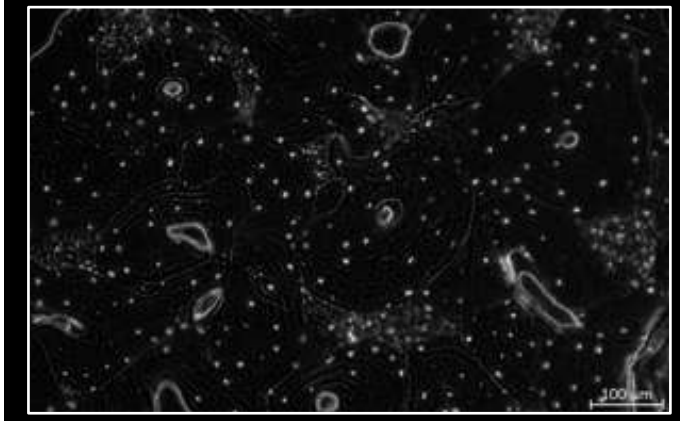


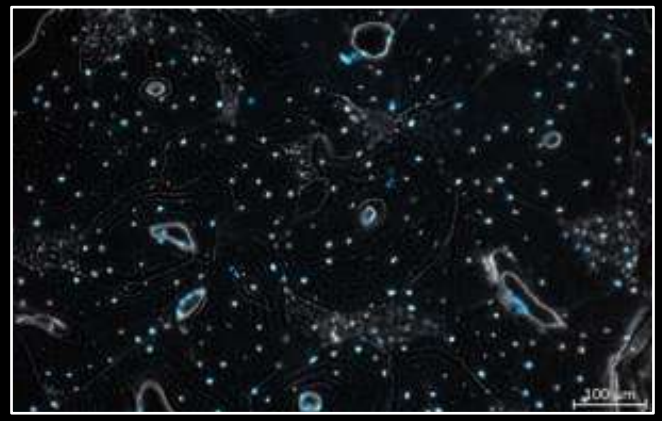


Lacunae with osteocytes

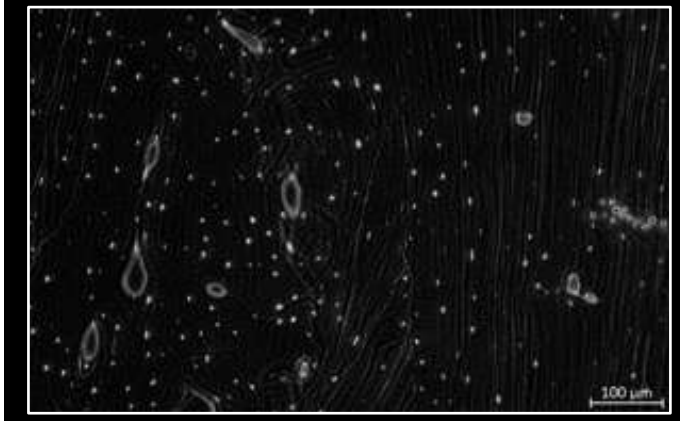
DAPI staining of porcine bone

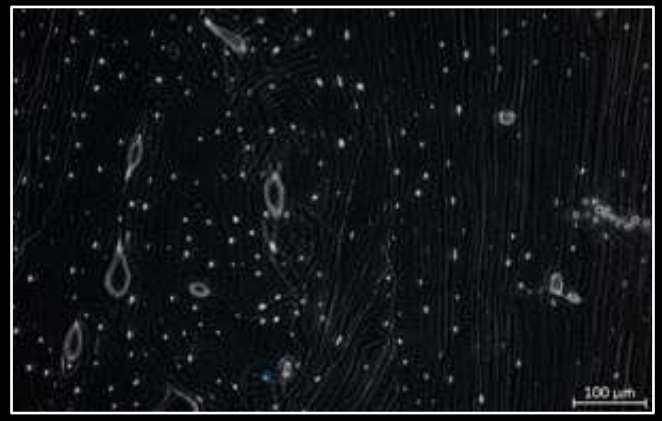
Non processed bone





processed bone





brightfield DAPI overlay

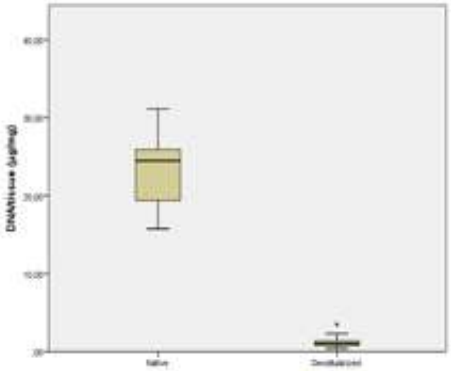
Nuclear material was successfully removed in bone and vessel. Matrix proteins and structure are preserved in both tissues.

Evaluation of decellularization

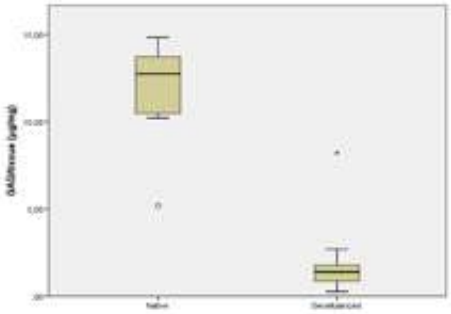
- Biochemical assays:
    - DNA content
    - GAG content
    - Hydroxyproline content (surrogate for collagen)

Biochemical assays on decellularized blood vessel

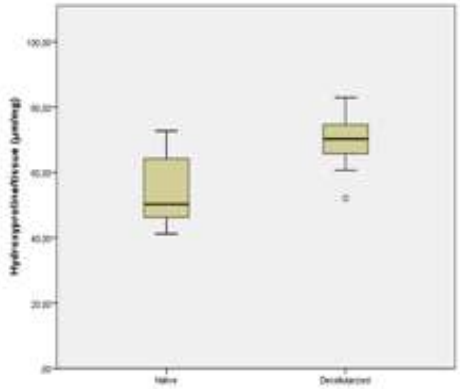
- DNA quantification:
  - DNA content decreased by 95% compared to native samples.



- GAGs quantification
  - GAG content decreased by 90% compared to native samples.

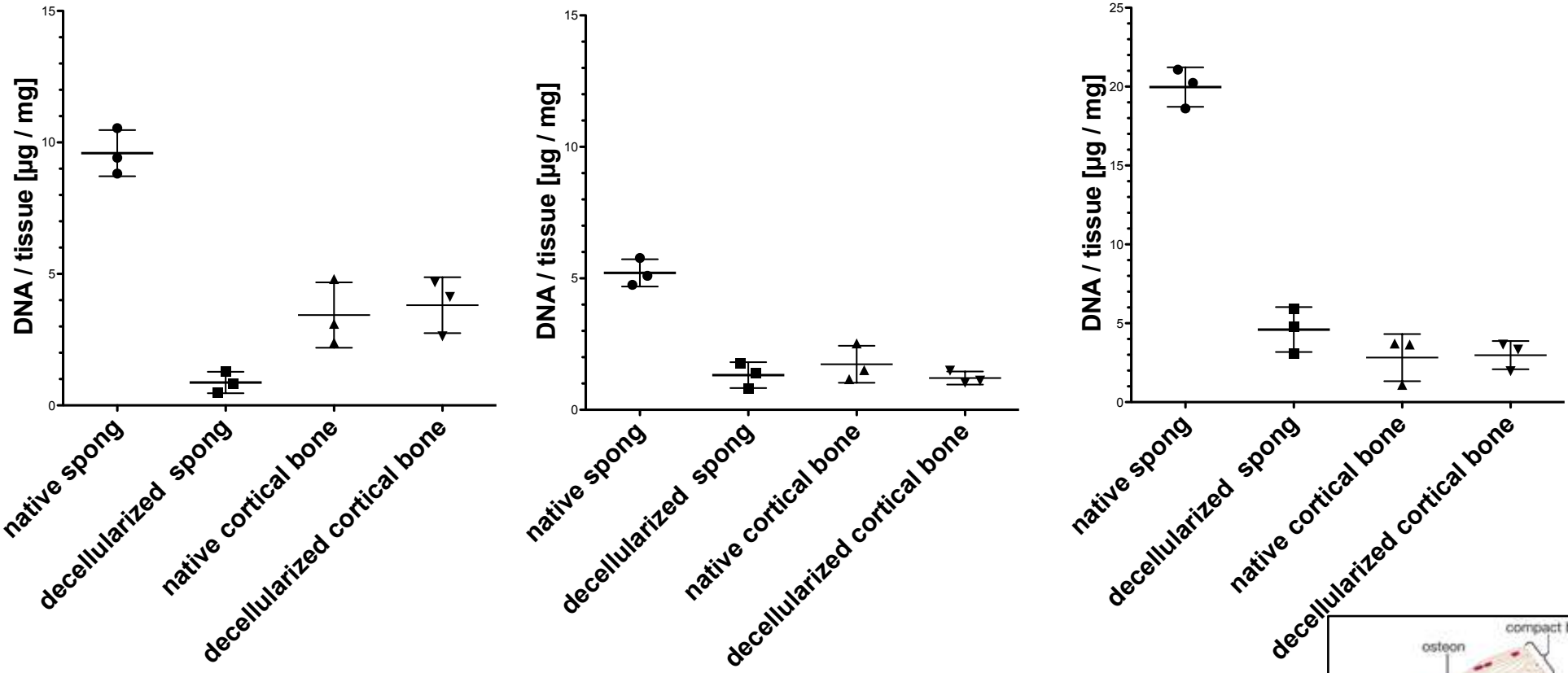


- Hydroxyproline quantification
  - Hydroxyproline content increased by 28% compared to native samples

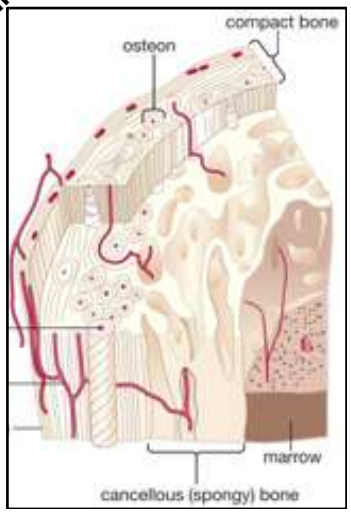


DNA quantification in bone tissue

3 independent experiments

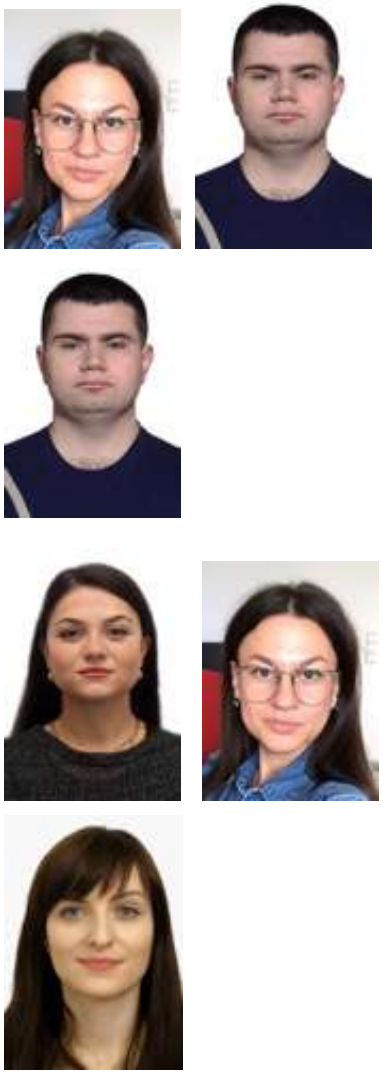


Reduction of DNA content is dependent on the type of bone. In compact bone no DNA reduction was observed compared to native bone. In spongy bone DNA reduction was detected.



Cell culture

- General training for the students with no experience in cell culture
- Isolation of primary cells:
    - Mesenchymal stroma cells from porcine bone marrow
    - Chrondrocytes
- Cultivation of endothelial cells
- Cultivation of fibroblasts



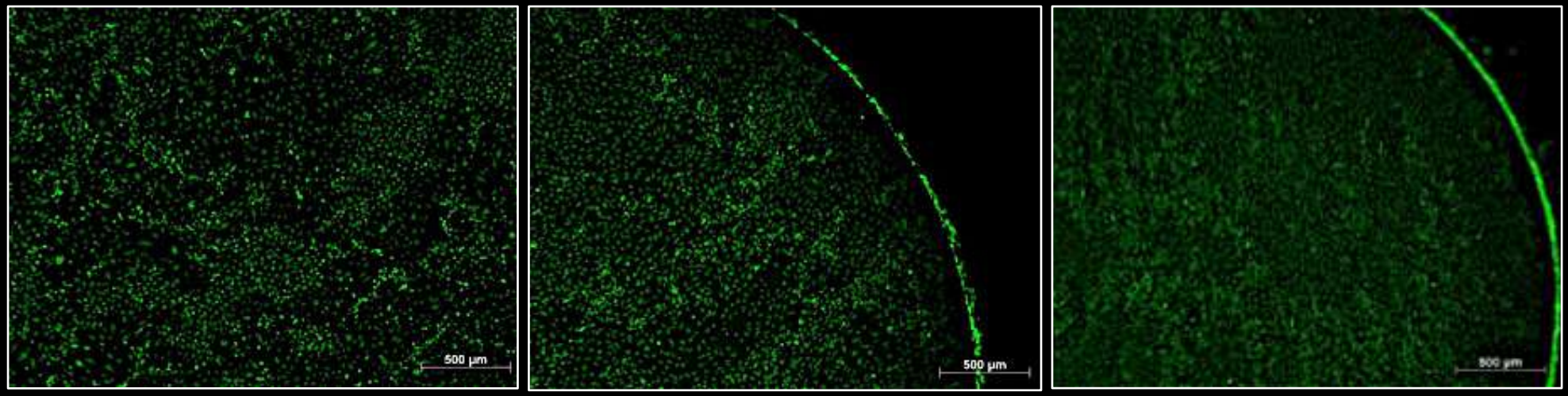
Cytotoxicity and Biocompatibility assays

- Evaluating the matrix after decellularization

- Cytotoxic effects of nanoparticles on filaments from amniotic membrane

Seeding of human endothelial vein endothelial cells on decellularized blood vessels

Calcein stain (labeling living cells), day 6



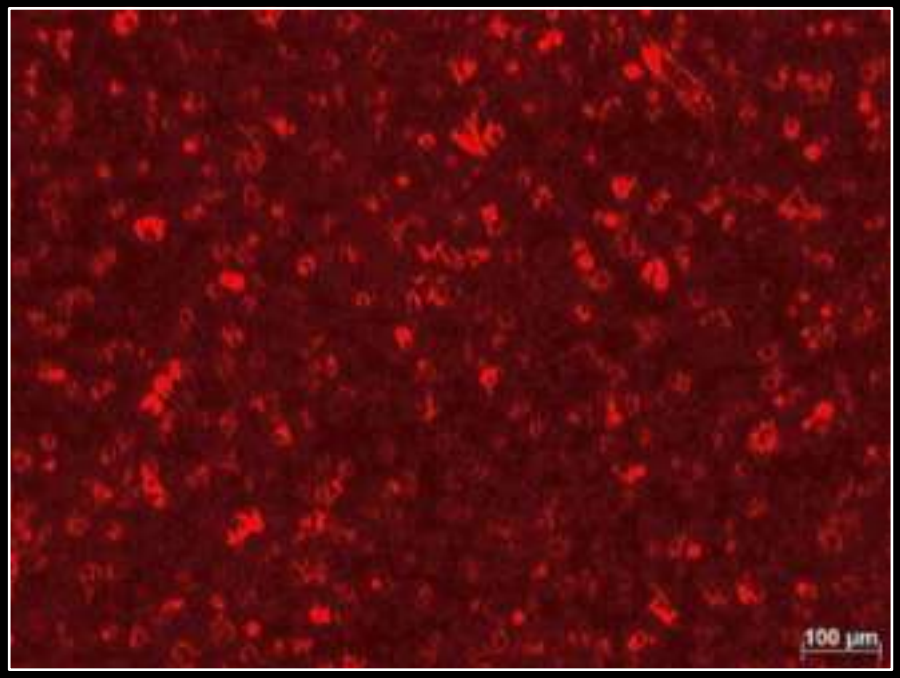
Control
(cells on tissue culture plastic)

Cells on decellularized blood vessel matrix

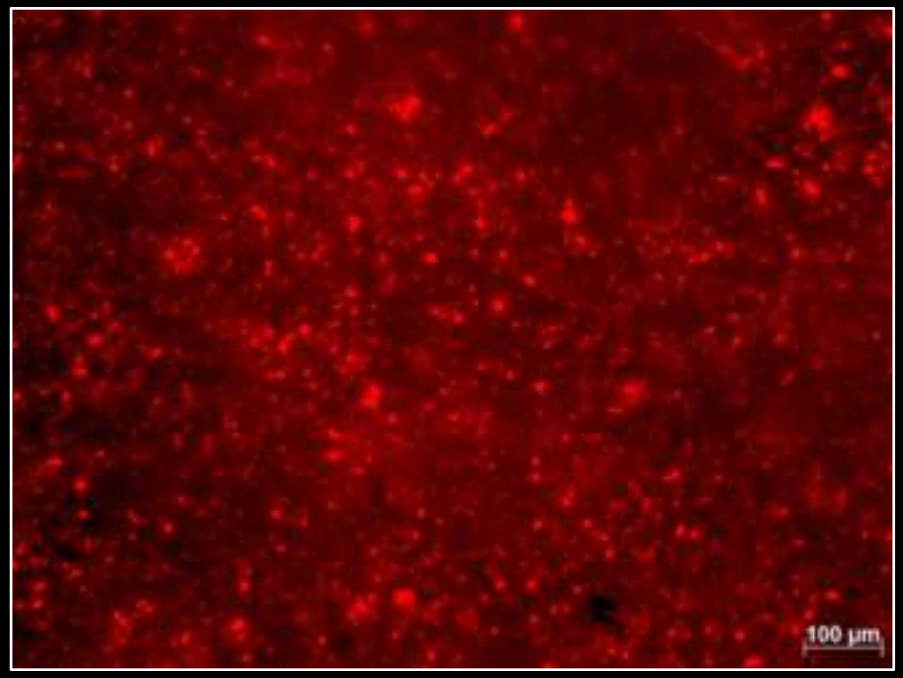
➡ Endothelial cells adhere to the luminal surface and proliferate.

Seeding of labeled mesenchymal stroma cells from bone marrow (MSC) on decellularized bone

Decellularized bone piece in culture medium



Control (cells on tissue culture plastic)

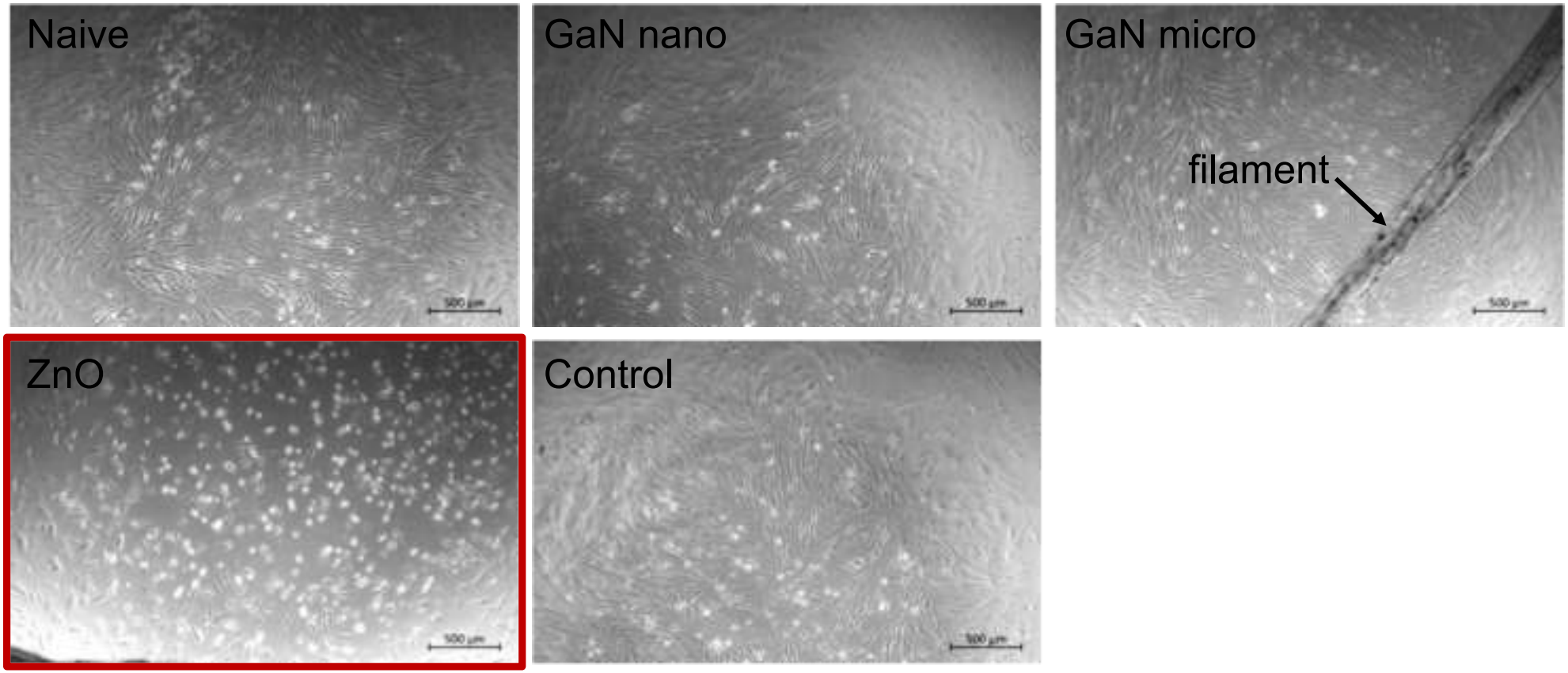


Cells on bone matrix

MSCs adhere to the bone matrix.

Biocompatibility of filaments from amniotic membrane coated with nanoparticles

- human foreskin fibroblasts (hFF) were cultivated with a defined amount of filaments



Filaments do not have a negative effect on hFF. With ZnO coated filaments a reduction in cell number compared to controls is observed (as expected).

Alternative approach:

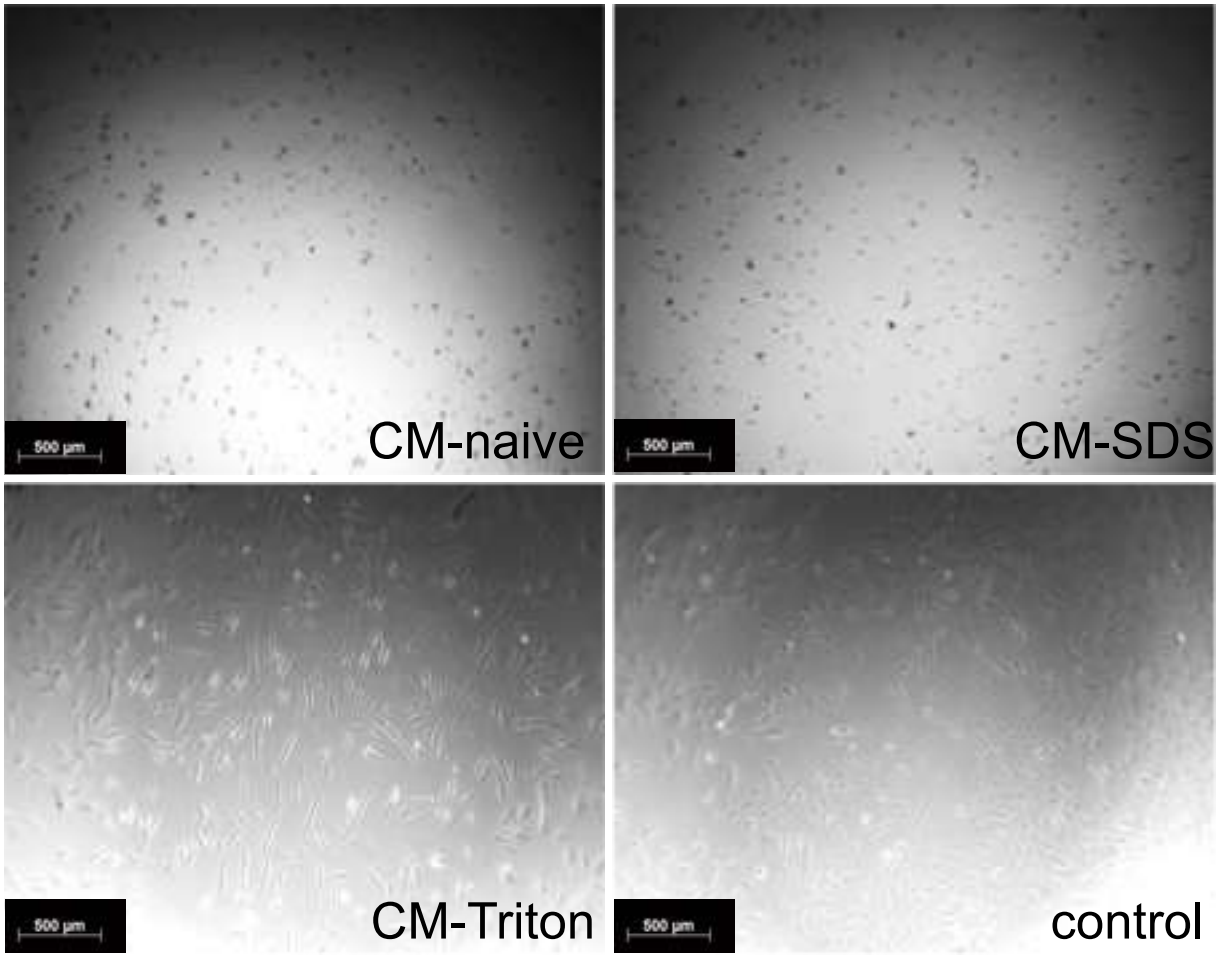
Decellularization of AM

- Concern: immunogenic potential of used human AM, which still contains cells

- Decellularization: Triton
  SDS
  (performed in Moldowa)

Biocompatibility of decellularized amniotic membrane

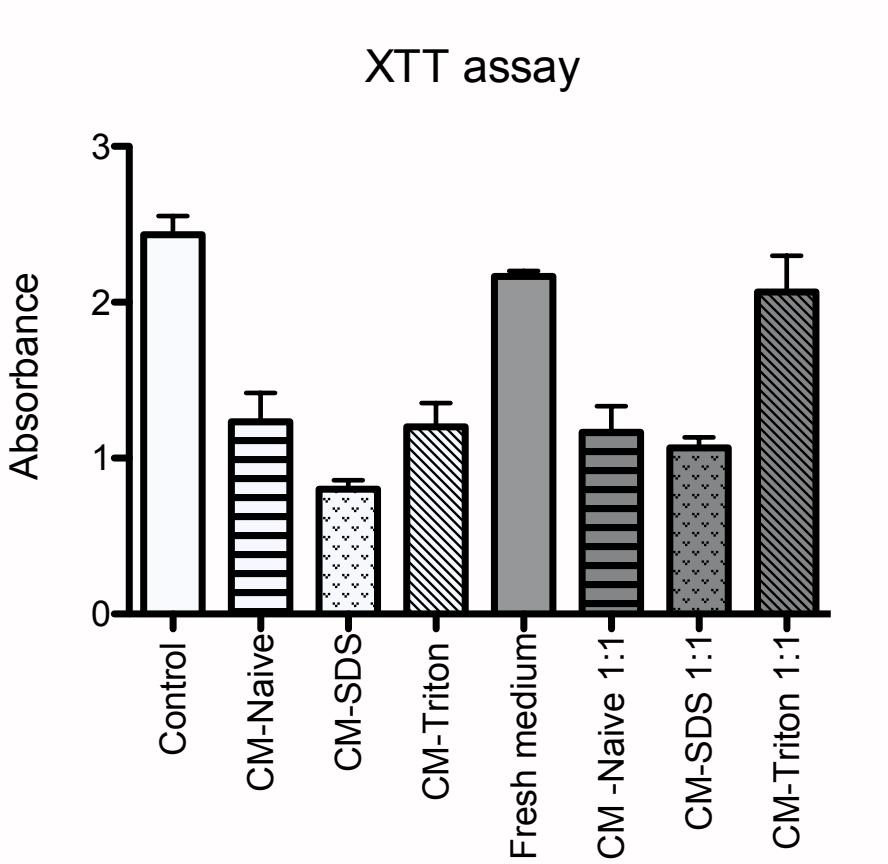
- Amniotic membrane was decellularized with SDS or Triton and dried
- Biopsy punches were made (same circular area)
- Pieces were incubated in medium (same volume) to retrieve conditioned medium (CM)
- hFF were seeded and cultivated in CM
- Pictures were taken after 24h



Cytotoxic effects are observed with CM from naïve AM and SDS decellularized AM.

Biocompatibility of decellularized amniotic membrane

- XTT assay:
  - hFF were seeded into 96 well plate and cultivated
  - Medium was replaced by CM or a mixture of CM/fresh medium (1:1)
  - XTT assay was performed



Cytotoxic effects were observed with CM of naïve, SDS and Triton decellularized AM. CM from Triton decellularized AM mixed with fresh medium reached control levels.

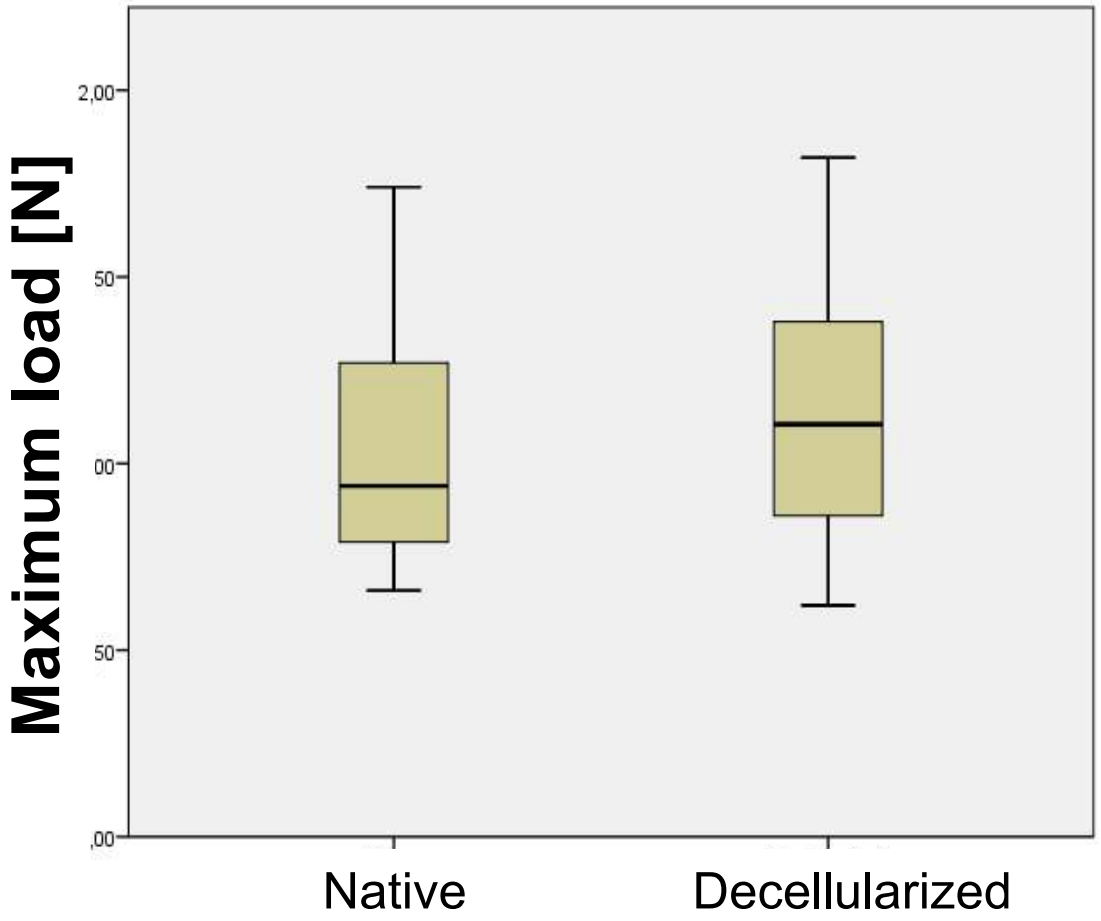
Mechanical testing

- Evaluating the mechanical properties after decellularization
  e.g. suture retention

- Evaluating the mechanical properties after coating of filaments with nanoparticles

Testing device from Instron
(located at NIFE)

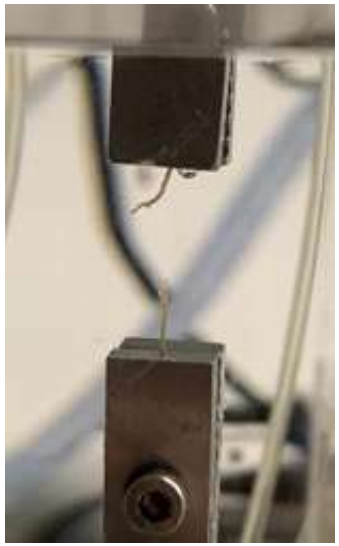
Suture retention strength of decellularized carotid artery



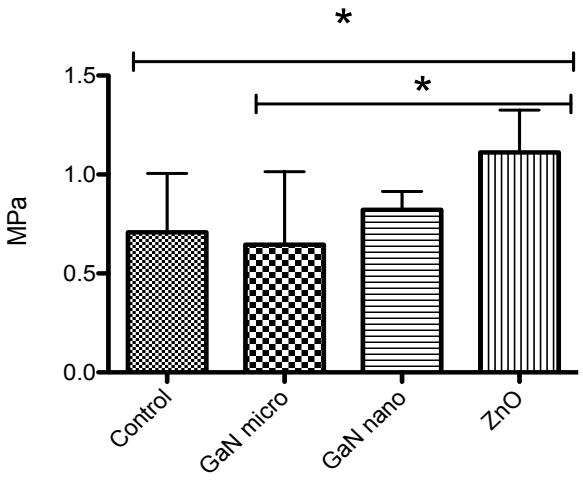
n=9 for native group and n=8 for decellularized group.

➡ No statistically significant difference between untreated and decellularized samples.

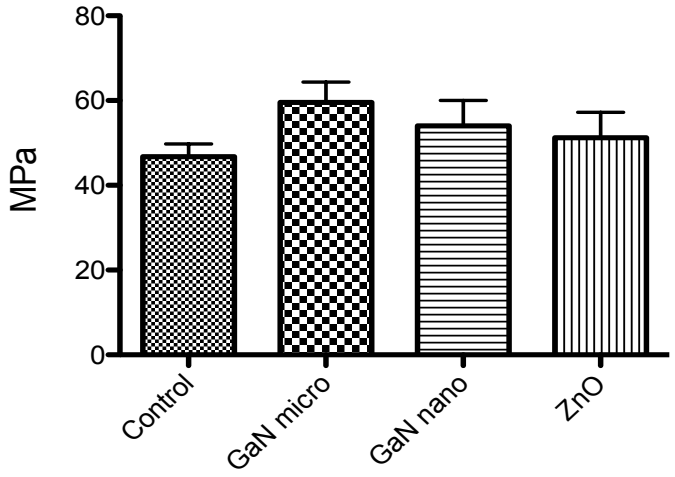
Mechanical testing of filaments



Tensile Stress at Break (MPa)



Tensile Stress at Maximum Load (MPa)



➡ No tremendous differences observed between filaments with different coating.

N=6, * = p-value < 0.05

Mechanical testing cartilage tissue

- compression with a force of 100N on 8 mm in diameter tissue disk, with a speed – 0.1-0.5 mm/min.

Young modulus (MPa)

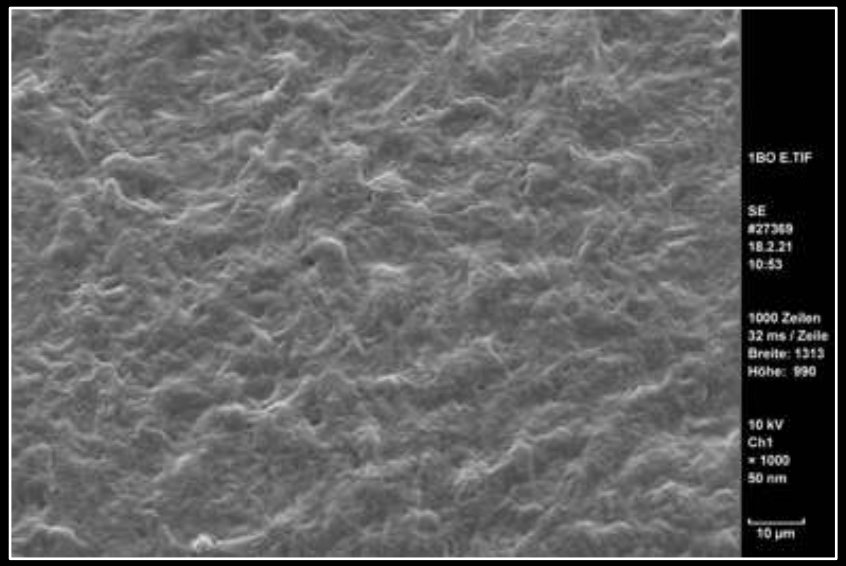
3 months old pig cartilage		6 months old pig cartilage	
S1	5.00	S1	4.59
S2	5.96	S3	1.67
S3	6.41	S4	1.77
S4	4.33	S5	4.51
S6	5.49	S6	4.76
S7	4.15	S7	5.18
S8	5.28	S8	4.86
S9	3.73	S9	5.18
S10	6.24		
Mean	**5.18**	**Mean**	**4.07**
STDV	**0.95**	**STDV**	**1.47**

Scanning electron microscopy (SEM)

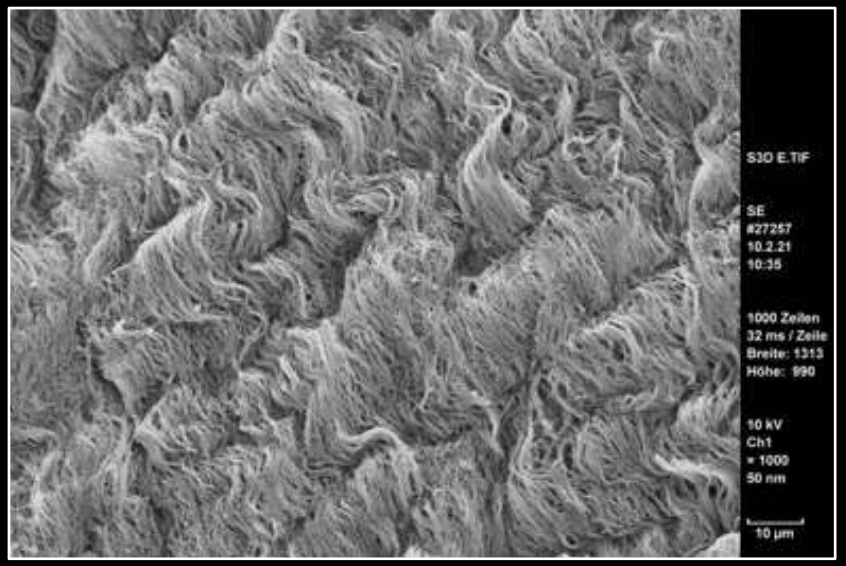
- Analysis of the structure of the surface of decellularized tissues
- Analysis of the surface of filaments

SEM of blood vessels

Luminal surface of native carotid artery



Luminal surface of decellularized artery

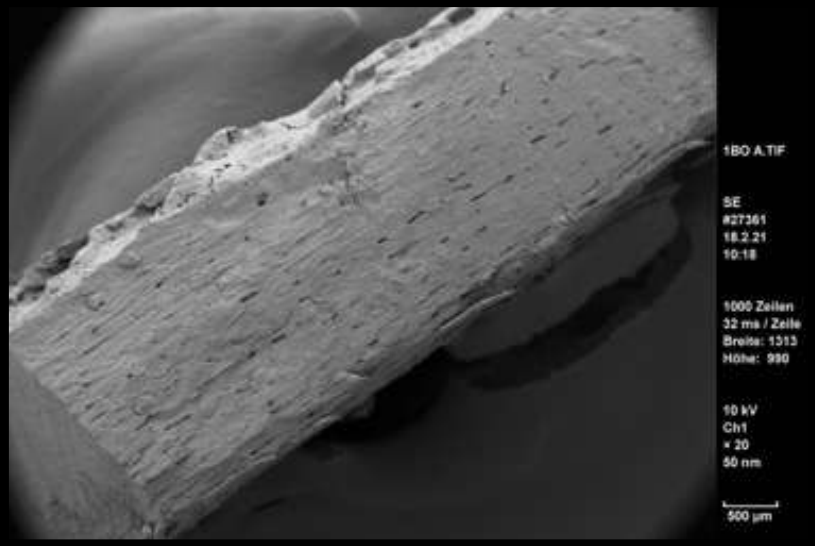


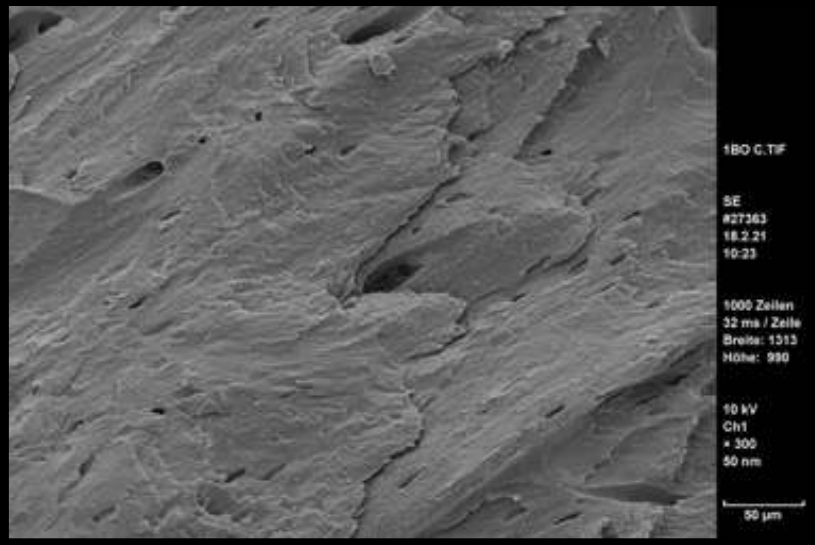
➡ Endothelial cell layer is removed. Fibrous material is exposed after decellularization.

SEM of bone

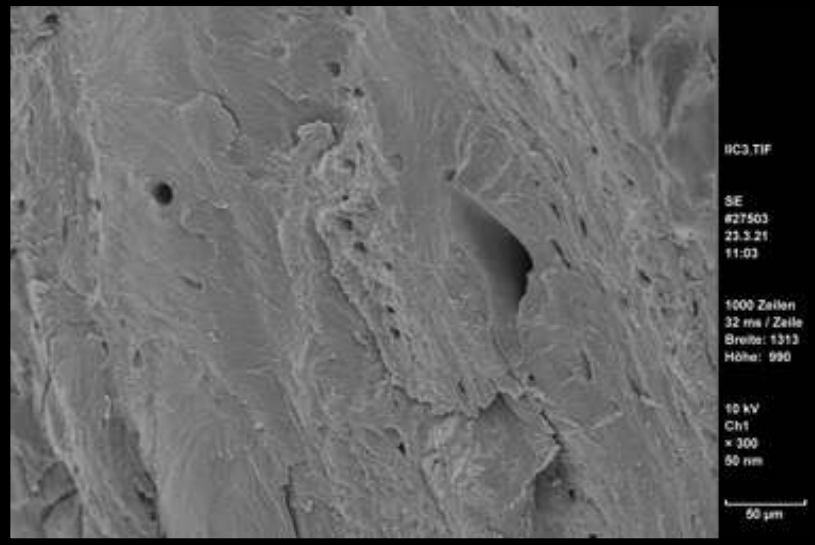
Challenge: Preparation of sample – freeze fracture

native cortical bone





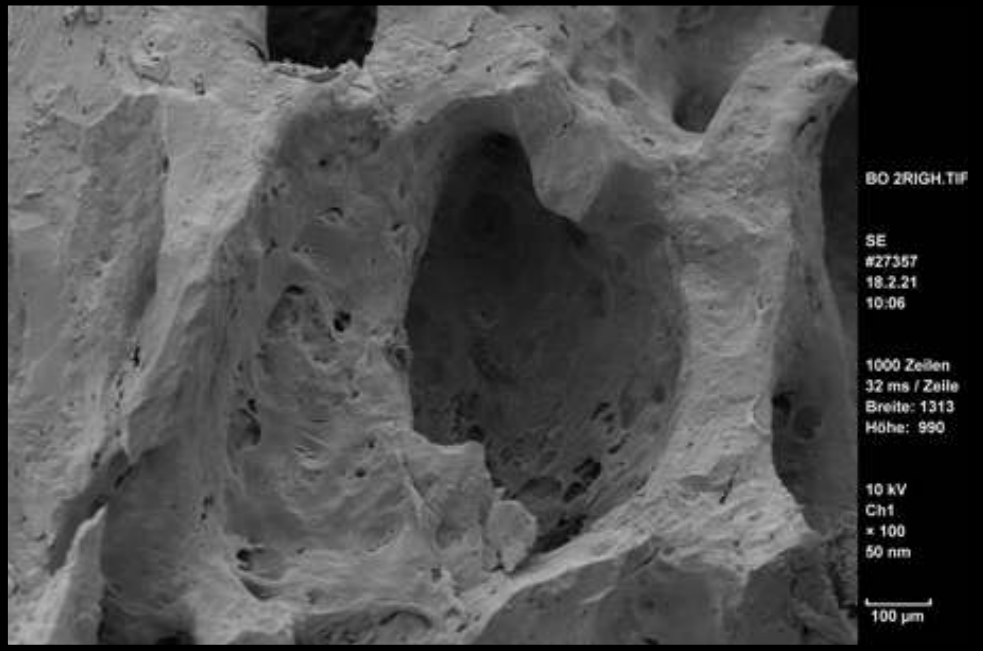
processed cortical bone



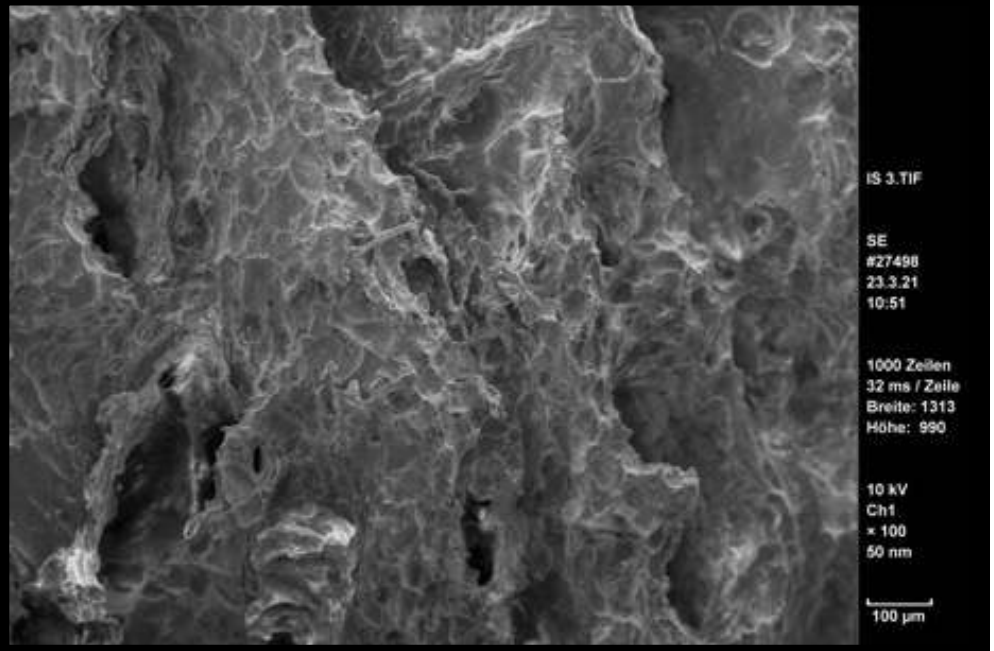
➡ Cortical bone seems to be not affected by the treatment.

SEM of bone

native spongy bone

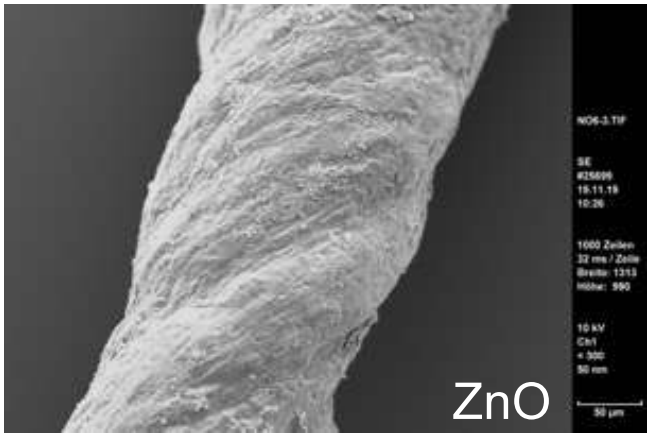


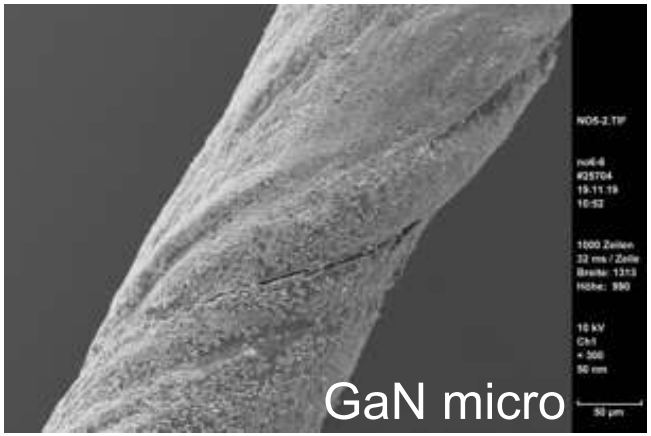
processed spongy bone

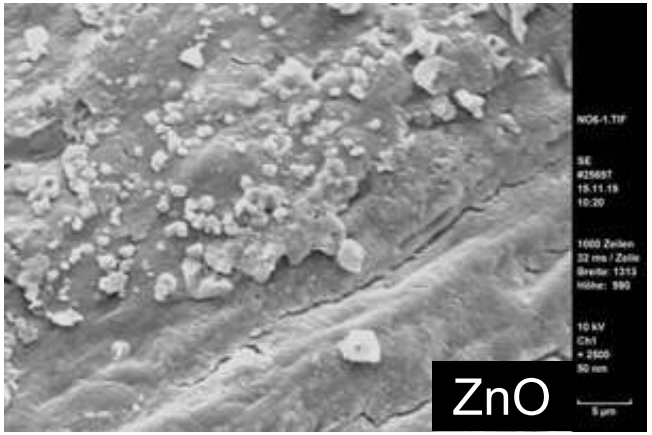


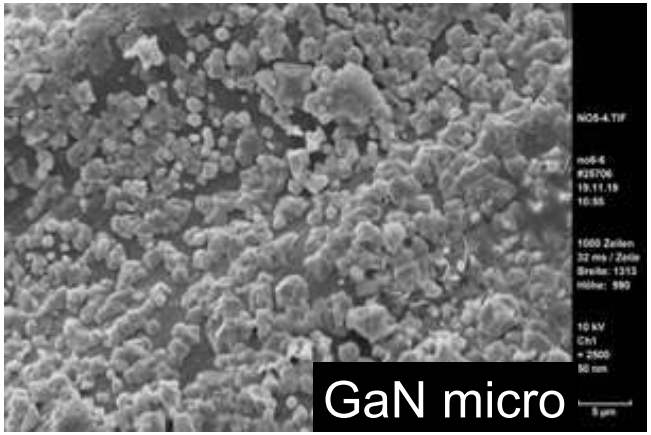
➡ Spongy bone is affected by the treatment.

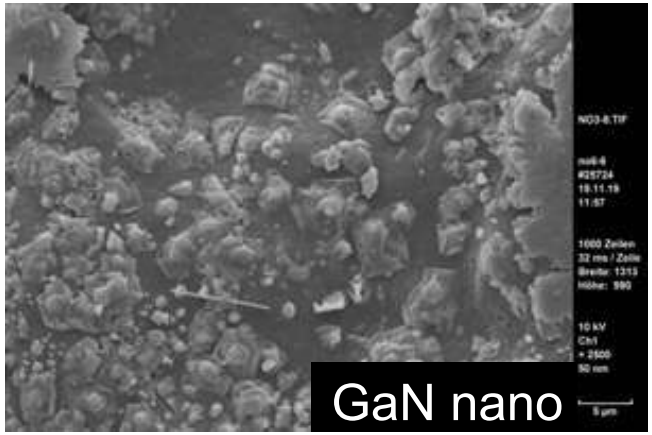
SEM of filaments dip-coated with different particles







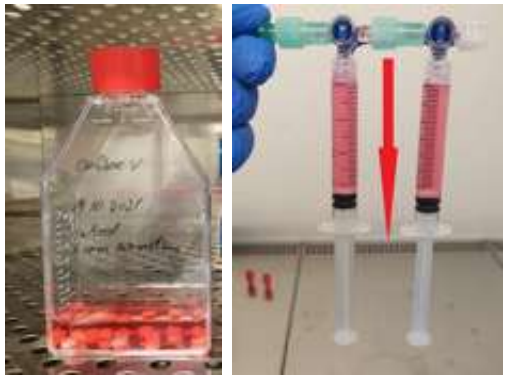




➡ Density of particles differs between different types of particles.
Need for optimization.

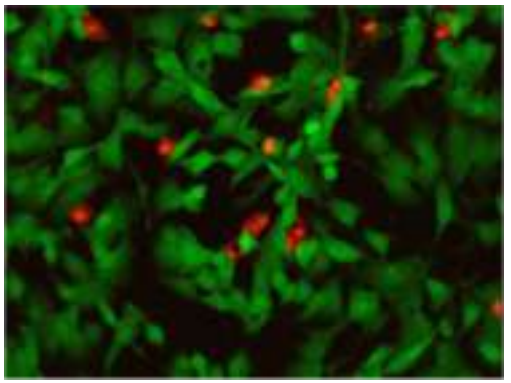
Challenge: Long term preservation of functional cartilage

- Prolonged cultivation in cell culture media



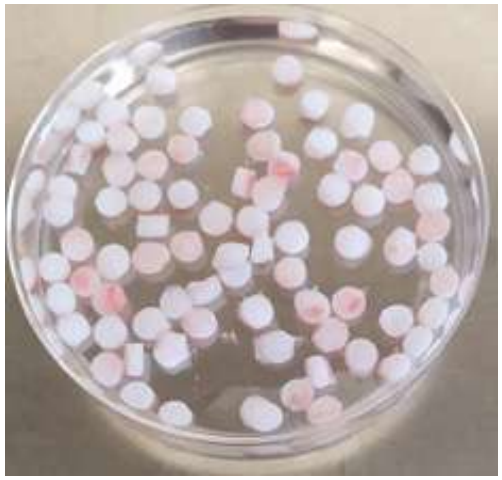
- Cryoconservation
  - Standard freezing protocol
  - Vitrification

- Read out: Live/dead assay (living cells appear green, dead cell appear red)

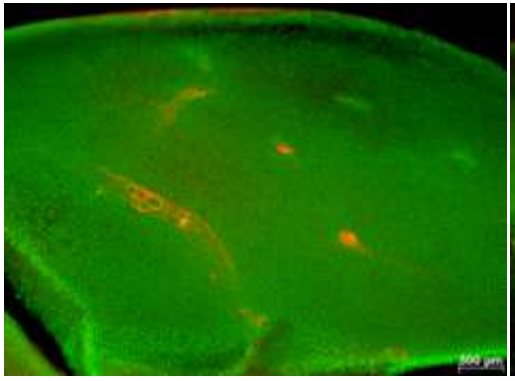


Long term preservation of functional cartilage

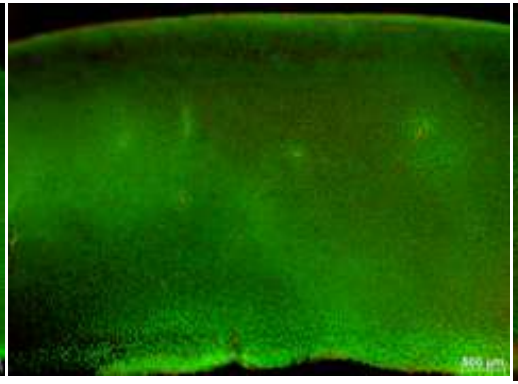
- Preparation of cartilage tissue from pig



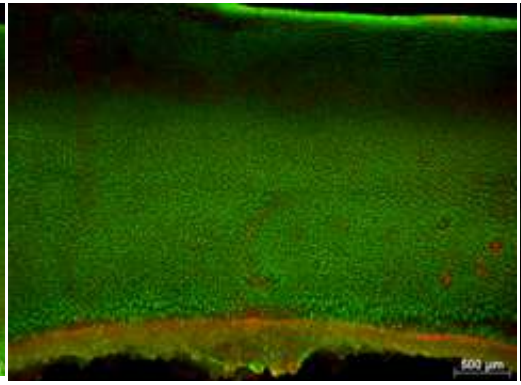
- Cultivation for 4 weeks in medium at 37°C



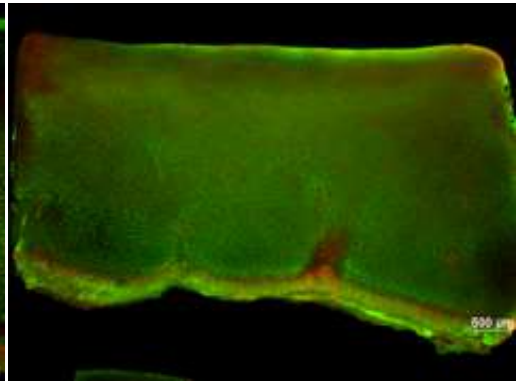
1 week



2 weeks

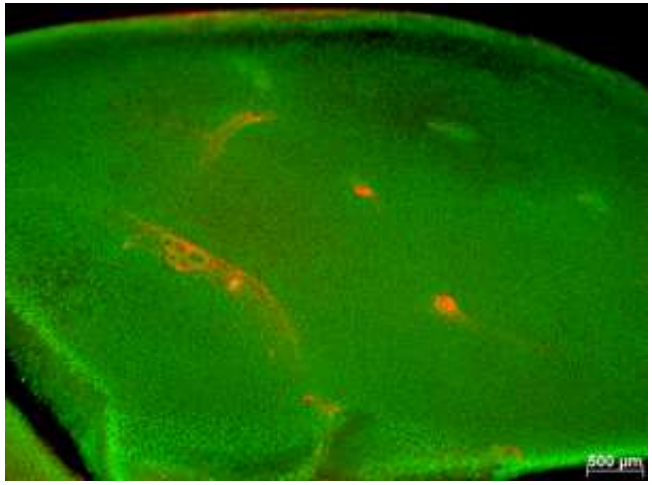


3 weeks

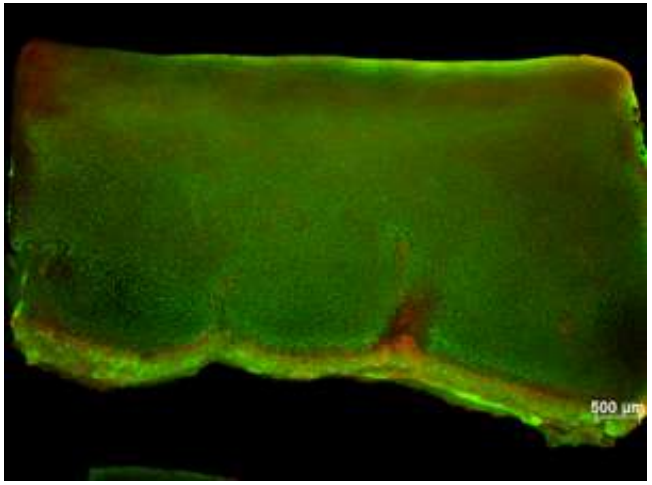


4 weeks

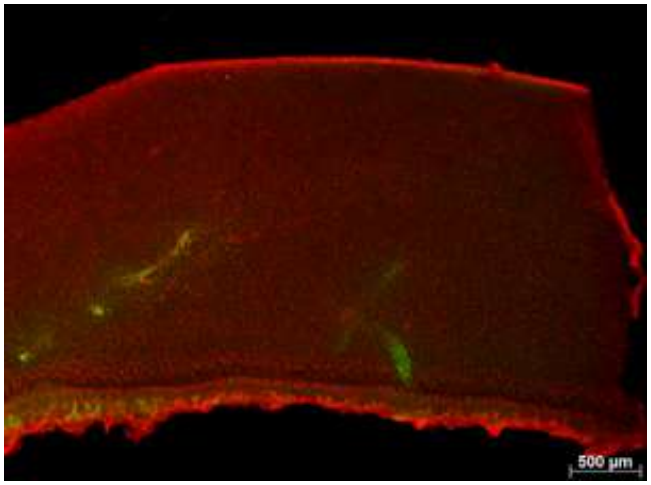
Long term preservation of functional cartilage



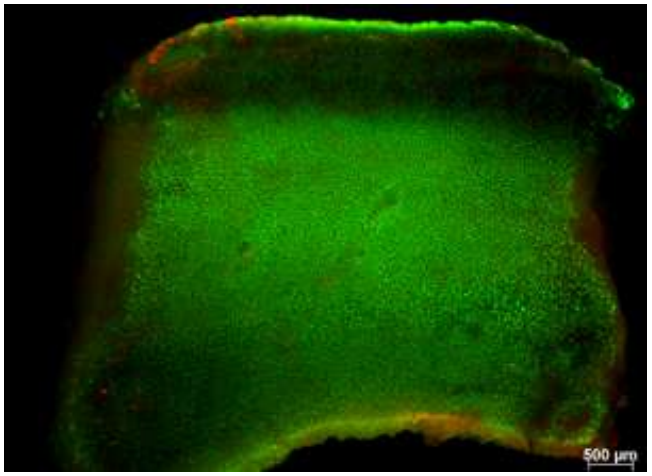
Control (directly after harvest)



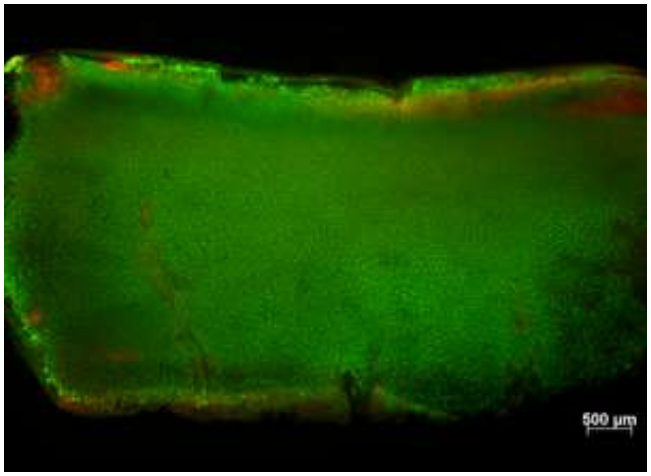
4 weeks, 37°C



4 weeks, 4°C



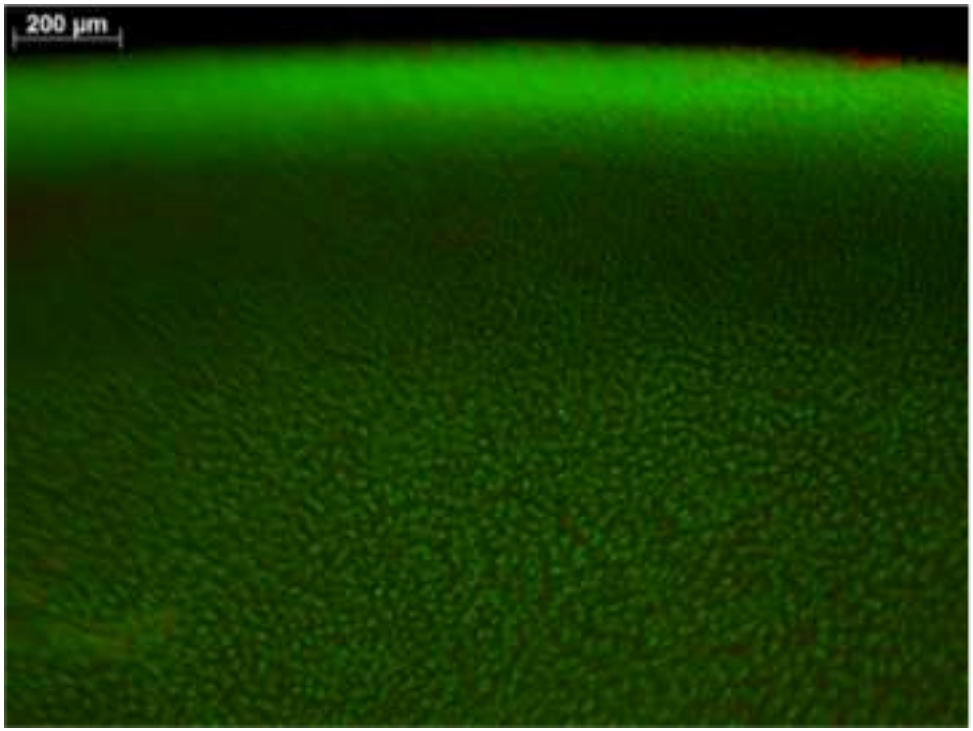
37°C, ultrasound stimulated



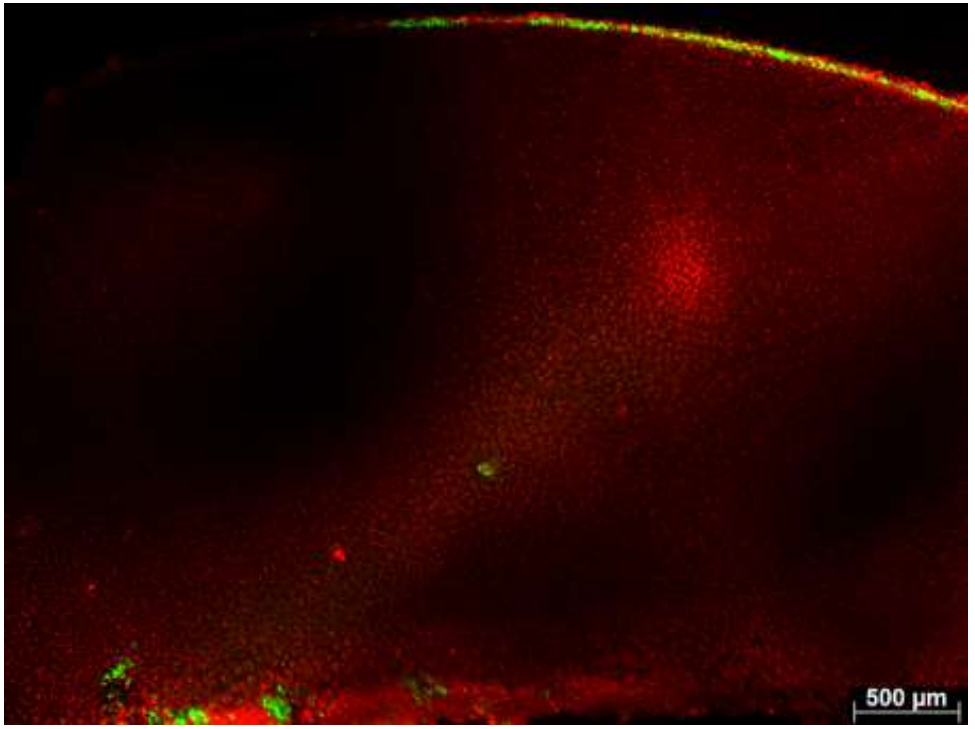
walking imitation

Long term preservation of functional cartilage

- Cryoconservation
    - Standard freezing protocol



Fresh harvested cartilage

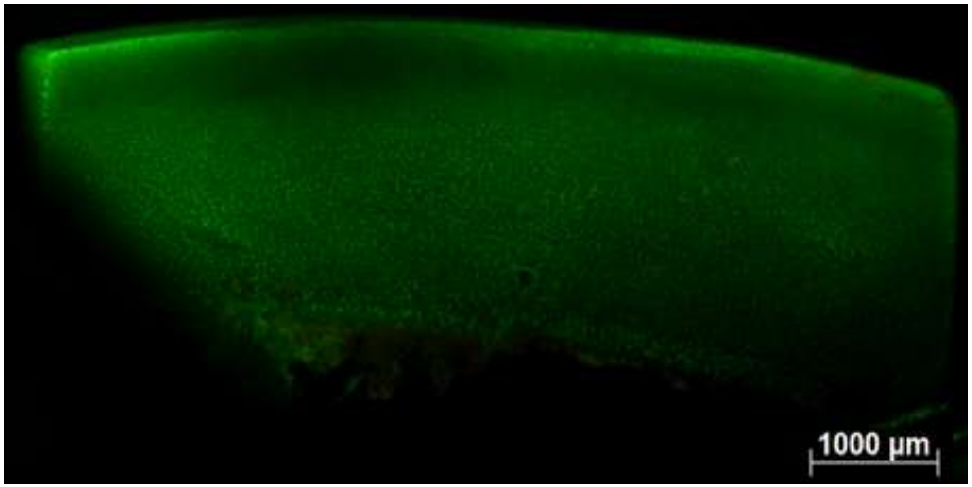


- 80°C, 1 week in standard freezing medium (10%DMSO)

Long term preservation of functional cartilage

- Vitrification (in cooperation with Prof. Wolkers, NIFE):
    - Vitrification is an alternative to cryopreservation by freezing that enables hydrated living cells to be cooled to cryogenic temperatures in the absence of ice formation.
    - Loading the cells or tissues with vitrification solutions (VS) is the most important step in vitrification protocol. Process of cells loading consist in replacing of intracellular/intracellular water with VS.
    - Because of their tolerance limits, cells cannot be directly exposed to a full-strength vitrification solution and therefore need to be exposed to serial dilutions of the vitrification solution at low temperatures.

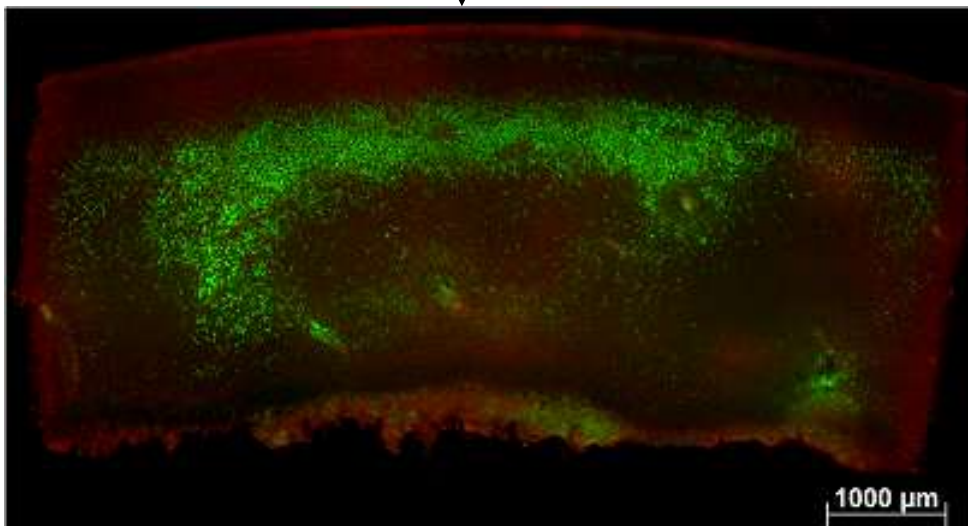
Vitrification of cartilage



Control (stored in cell culture medium)

First try of vitrification

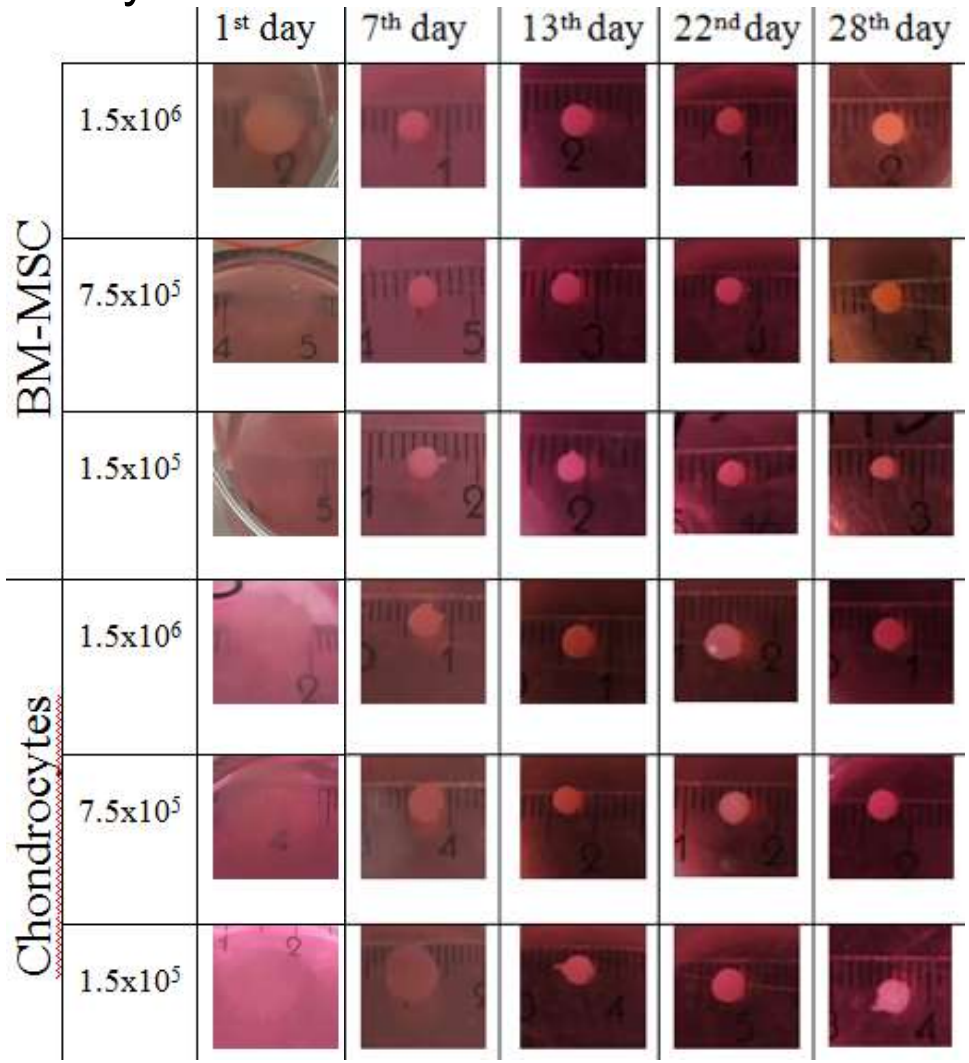
Several modifications

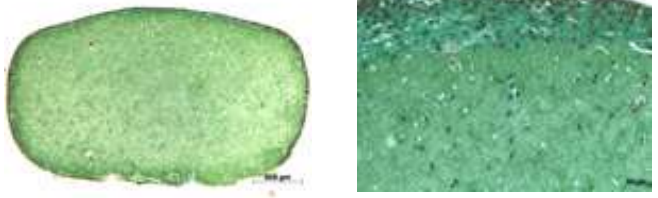


Finding of an appropriate modality of articular cartilage storage and maintain its functionality is a difficult task requiring resources and time.

In vitro cartilage generation

- Collagen was extracted from porcine Achilles tendon
- Generation of 2 mg/mL collagen hydrogels with MSC or chondrocytes





Summary

- Utilization of all techniques and machines available and necessary for the individual projects was made possible, also through cooperation with other institutes.

- Insights into the pitfalls of decellularization and complexity for developing protocols with appropriate controls were appreciated.

- Parts of the conducted research led to conference contributions, even recognized and awarded by the organizers of MedEspera-2022.

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Mikhail Magdai
Sanja Sladic
Daria Wehlage

NIFE
Prof. Wim Wolkers
Dr. Sugat Tuladhar

Thank you for your attention!

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