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Grant Agreement no: 304842

The NanoBio4Trans project

A new nanotechnology-based paradigm for engineering vascularised liver tissue for transplantation

FP7-HEALTH-2012-Innovation-2

Medical technology for transplantation and bioartificial organs

Jenny Emnéus (Coordinator)

DTU

cellartis BY TAKARA BIO

BIOMODICS

LUXCEL BIOSCIENCES

university of groningen

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Partners

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BY TAKARA BIO

Partner 3: Biomodics,
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BIOMODICS

Partner 4: Luxcel Biosciences
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Partner 5: University of Groningen
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Elisabeth Verpoorte, Geny Grothuis

university of groningen

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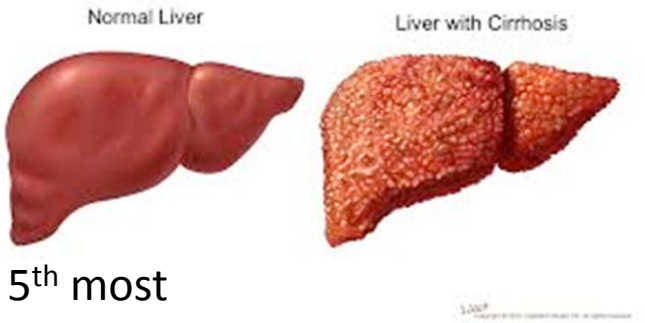
Facts about liver Failure & disease



~ 6 % of the EU population suffer from liver failure /diseases

70.000 Europeans are dying from chronic liver failure - the 5th most common cause of death in the EU.

Non-alcoholic fatty liver is predicted to increase and become the most common cause of advanced liver disease and liver failure in the 21st century due to increasing obesity and the increasingly aging population

The World Health Organization estimates that 10% of the world's population has chronic liver disease and that liver cirrhosis (scarred liver) will be the 9th most common cause of death in the western world by 2015.

➔ **Human suffering with substantial economic consequences**

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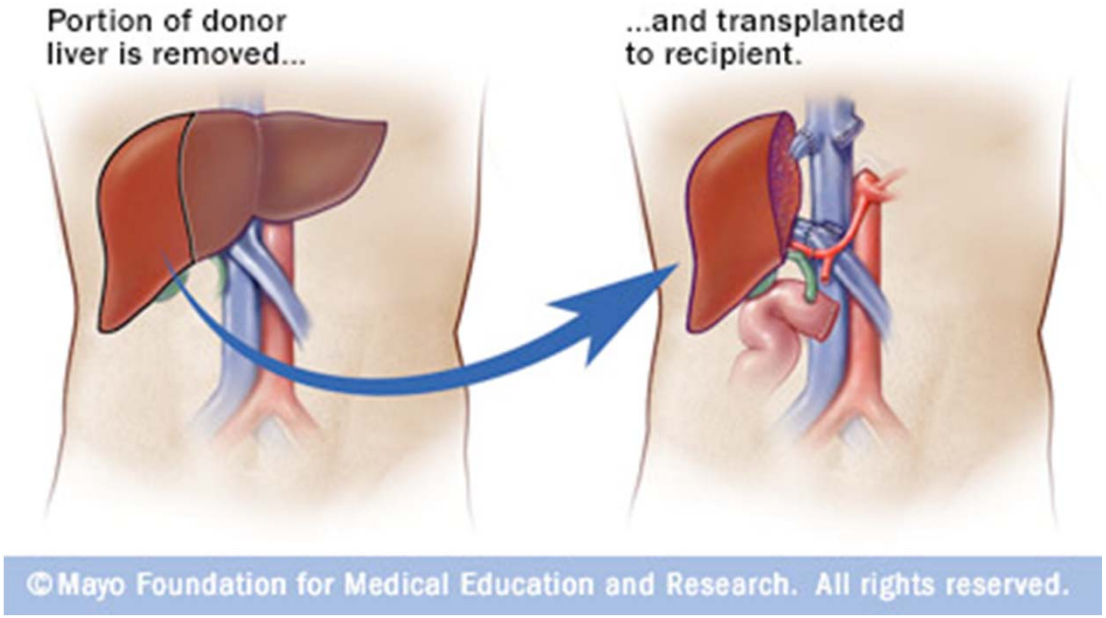
Actions

1. Liver transplantations

Conducted on patients with acute liver failure and chronic end-stage liver disease.

~6500 liver transplantations per year in EU
~230k€/transplant
~17k€/year follow up



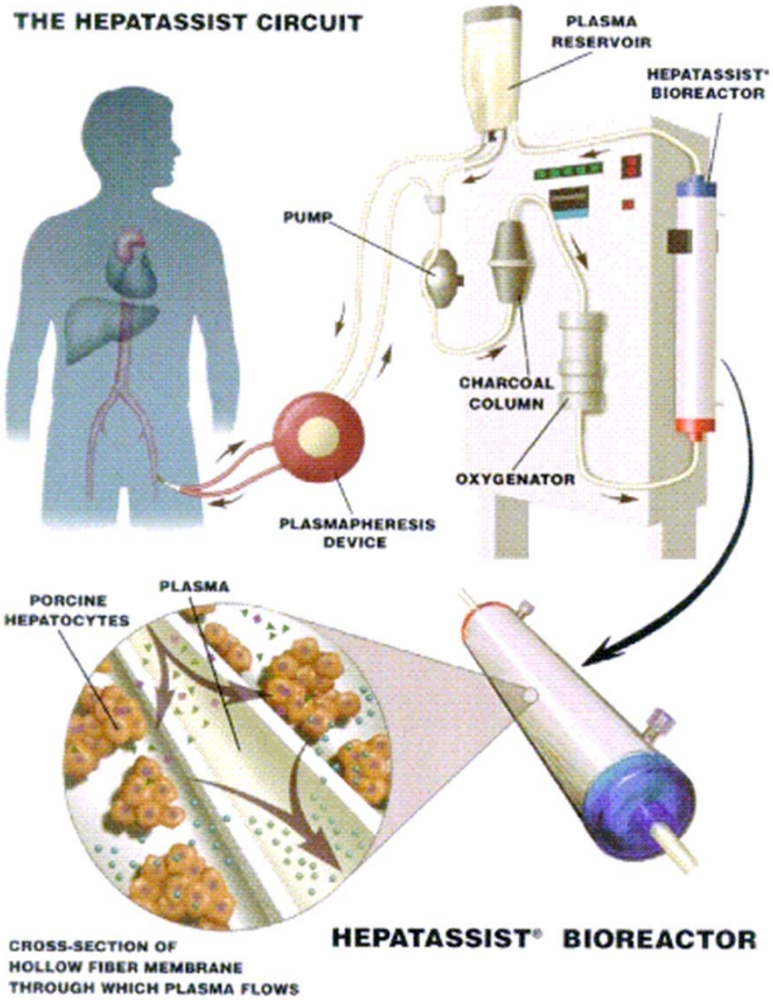
Actions

2. Liver Support Systems (LSS)

Extracorporeal Artificial Livers (EALs) and **Extracorporeal BioArtificial Livers (EBALs)** to bridge a patient to liver transplantation or to recover a patients liver from temporary failure.

Most common LLSs

Hollow fibre reactors with cultures of cryopreserved primary human or porcine hepatocytes, or hepatocyte cell lines



Motivation for NanoBio4Trans

B. Carpentier, A. Gautier, and C. Legallais, Artificial and bioartificial liver devices: present and future. Gut, 2009. 58(12): p. 1690-702.

“Special attention needs to be paid to identifying/isolating a readily available and functional source of cells and to improve hepatocyte entrapment.

“Bioreactor configurations that are not hollow-fibre based should be considered to improve both large scale cell culture prior to therapy and mass transfers during treatment. A better chance to develop should be given to these new and promising products”.

“This could be achieved by encouraging multi-disciplinary academic teams, as well as small and/or leading companies, to accompany such developments”.

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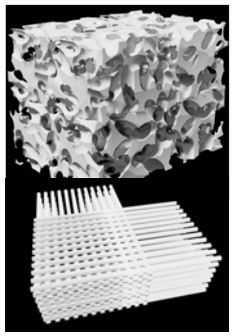
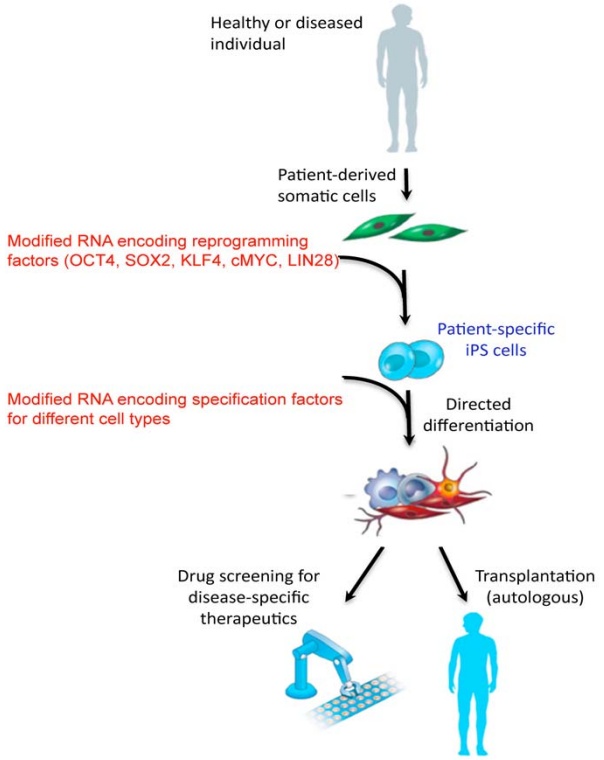
Three central SME technologies

➔ **Perfusable hybrid scaffold (PHS) for cell culture**

Responsibility: DTU, BioModics





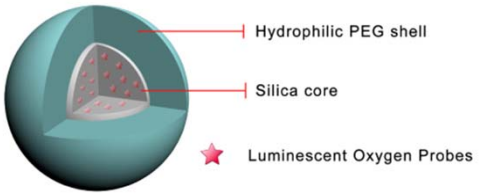
➔ **Human pluripotent stem cell (hPST) technology**

Responsibility: cellartis BY TAKARA BIO

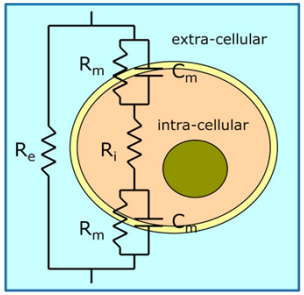
➔ **Sensor and bioassay technology**

Responsibility: LUXCEL BIOSCIENCES, DTU

Intra and extracellular O_2 sensing probes



Bioimpedance sensing of 3D cell growth



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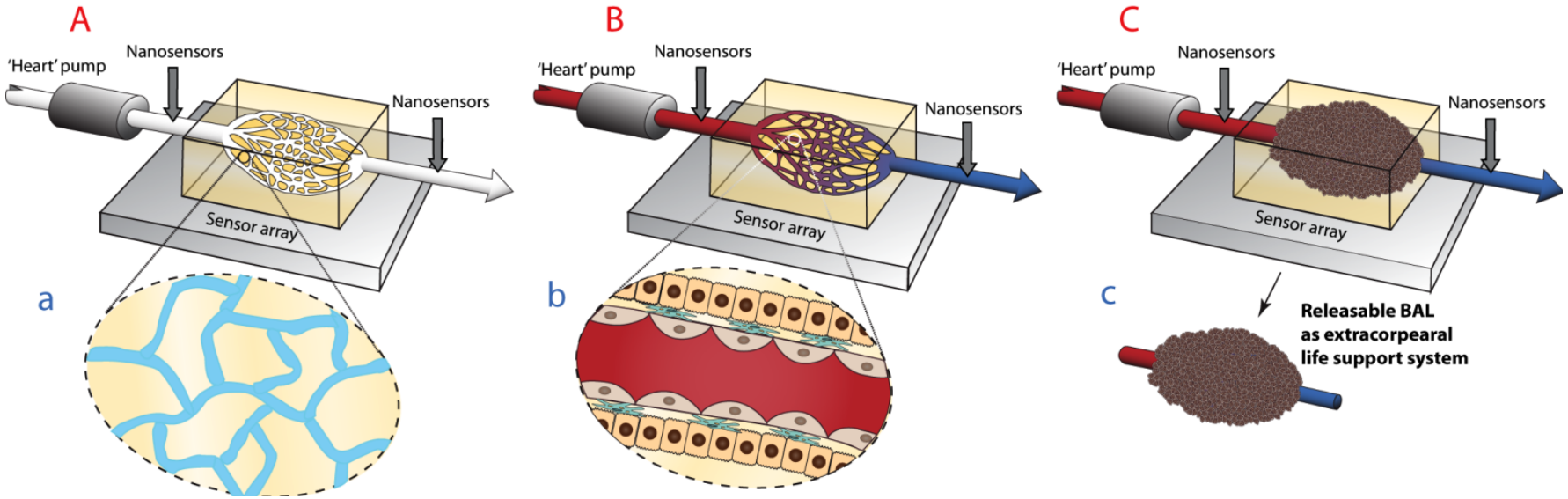
These three technologies should be merged into a bioartificial liver (BAL) support system

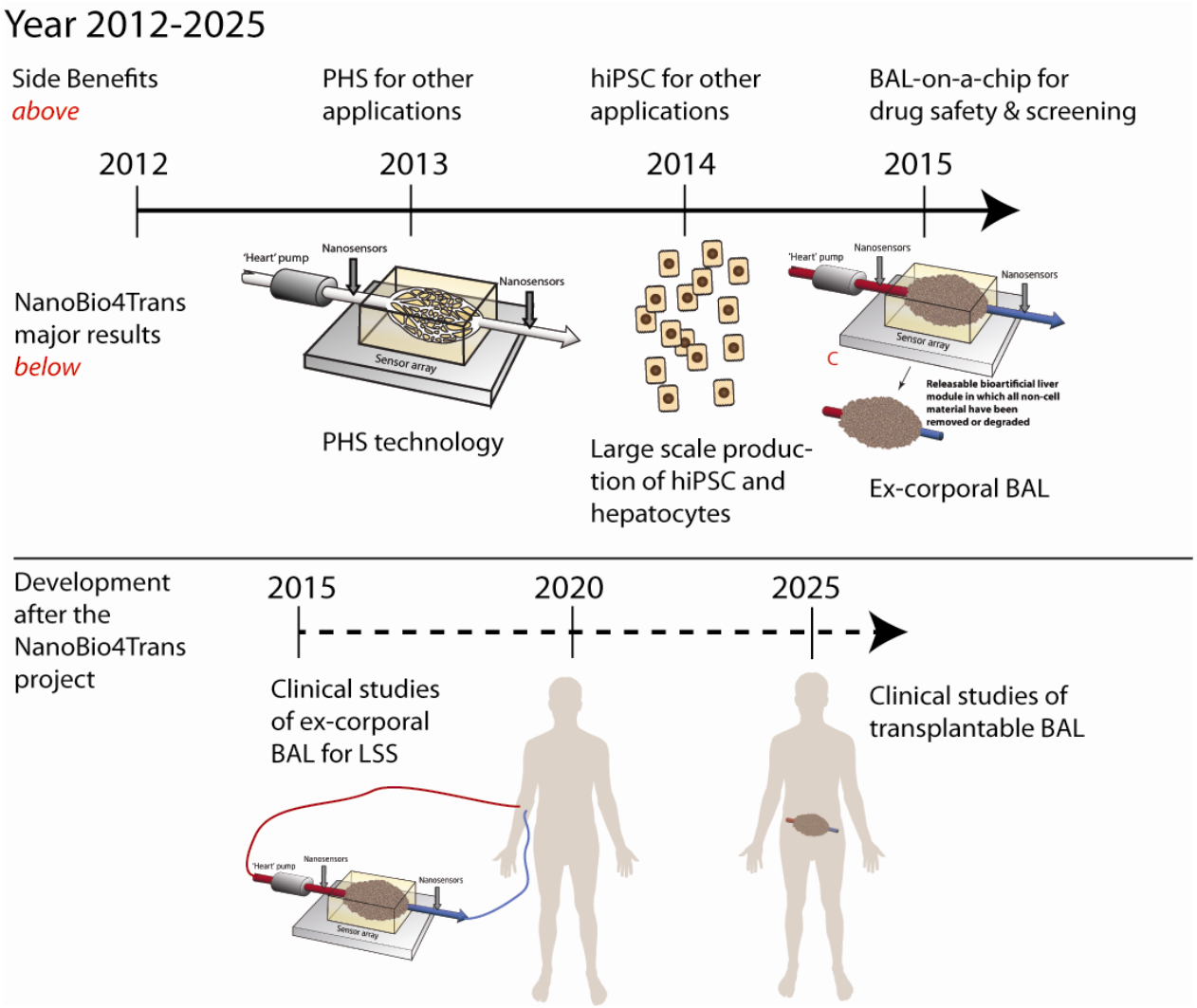
➔ **BAL development and Technology integration** – Responsibility: DTU



….that should be validated against real liver tissue

➔ **Assessment of BAL function** – Responsibility: university of groningen

Timeline of NanoBio4Trans



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Technological and Scientific Objectives

1. Optimization of hPSC production, characterization, up-scaling and industrial banking, hPSC library

2. Establishing and optimizing directed differentiation into hepatocytes

3. Developing and optimizing perfusion based 3D human liver tissue culture systems

4. Developing scalable vascularized perfusable hybrid scaffold (PHS) structures with primary highly branched unidirectional channel networks and secondary arbitrary porous networks enclosing hydrogel micro deposits (HMD) using various fabrication processes.

5. Developing and applying intra and extra cellular optical and bioimpedance sensing strategies, multi-parametric imaging and bioanalysis of cells, tissues and organs (integrity of vasculature, viability, O_2, pH, liver function, differentiation markers, etc).

6. Integrating PHSs and sensing systems into perfusion based BAL support systems

7. Adaption and upscaling of optimised 3D growth and differentiation protocols to the BAL support system for growth of BALs with dimensions in the order of cm^3 to dm^3.

8. Developing reference Liver-on-a-chip system to be used as the gold standard for validation of BAL support system.

9. To perform comparative studies of developed BAL and BAL-on-a chip systems with the Liver-on-a-Chip reference system, investigating potential differences in properties and functionality using the developed and integrated bioimaging, bioassay and HPLC protocols.

10. To perfuse the BAL with human blood plasma and test its ability for clearance of ammonia and bilirubin and production of certain key proteins.

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Overview of work

- **Develop Perfusable Hybrid Scaffolds (PHS)**
  - requirements for mimicking the in vivo environment
  - how we make 3D scaffolds

- **Develop Human induced Pluripotent Stem Cell (hiPSCs)**
  - upscaling of hPSCs
  - differentiation media
  - frozen media and cultures

- **Technology Integration & BAL development**
  - bioreactors and fluidics
  - pumping system
  - O_2 sensing intra and extracellular probes
  - Bioimpedance

- **Assessment of BAL function**
  - comparison to real liver tissue

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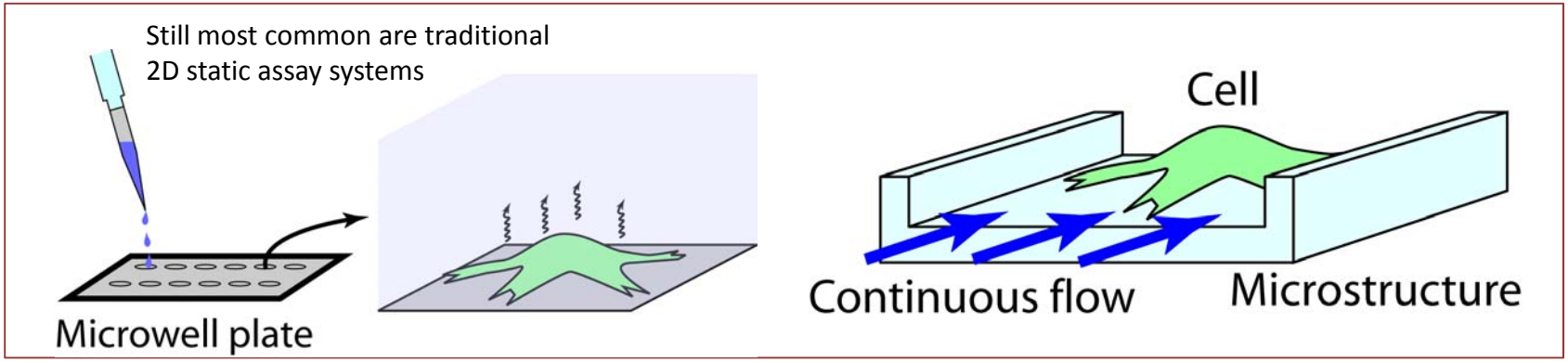
Perfusable Hybrid Scaffolds - PHS

Requirement for mimicking *in vivo* environment

Trend to develop systems that better mimic the *in vivo* environments of cells

1. Perfusion/Microfluidic *versus* Batch culture systems



Plus

- ❑ Continuous supply of fresh cell medium and removal of waste products
- ❑ Precise control of the chemical environment and supply of reagents

→ Minimized stress due to a more constant environment than in batch

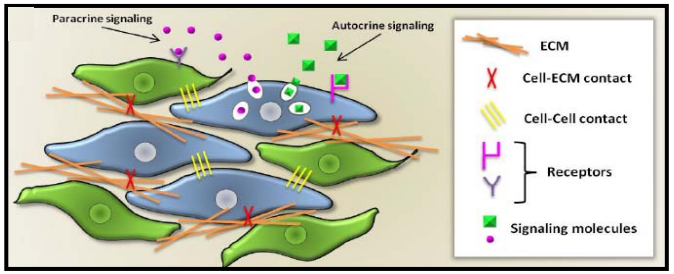
Minus

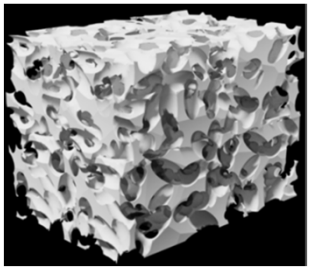
- ❑ Non-established in the scientific community with few or no commercial products available.
- ❑ Not well characterised how fluidics affect the cellular response

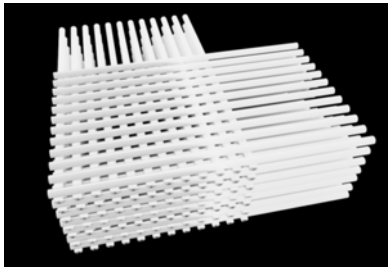
Perfusable Hybrid Scaffolds - PHS

Requirement for mimicking *in vivo* environment

2. Moving from 2D to 3D culture systems







Plus

- ❑ Better mimic of the *in-vivo* cell microenvironment
- ❑ Recapitulates the tissue–tissue interfaces, spatiotemporal chemical gradients, and mechanical microenvironments
- ❑ Essential for applications in bioartificial organ development and reliable drug screening

Minus

- ❑ 3D cell/tissue samples are several mm thick and highly scattering
  → Challenge to image for conventional microscopy
- ❑ Most cells reside within 200 µm from nearest blood vessel
  → Challenge to upscale and create vascular like flow channel networks in 3D

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Perfusable Hybrid Scaffolds - PHS

Scaffold requirements

- Structural and mechanical support for cell growth
- Mechanical strength especially for scaling up
- Perfusable with extensive channel networks that allows growth of blood vessel and supply of nutrients and oxygen to cells in a 3D environment
- Interconnected pores between channels
- Biocompatible and potentially biodegradable

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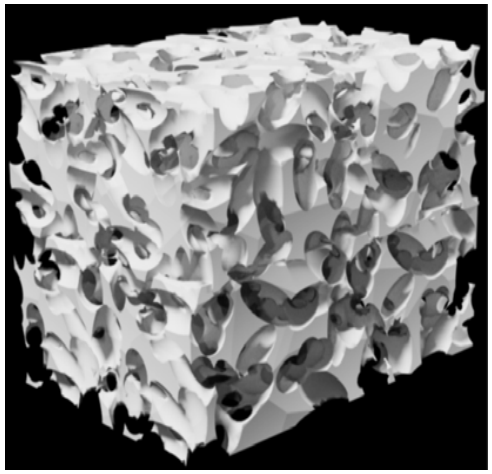
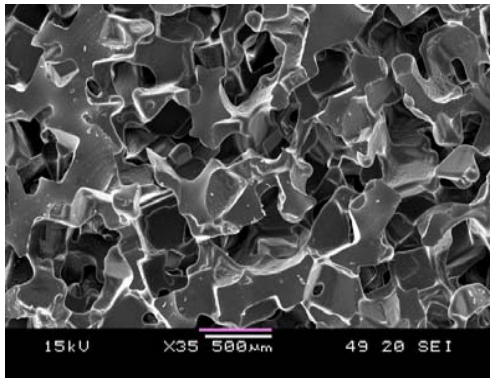
Fabrication of PHS

DTU

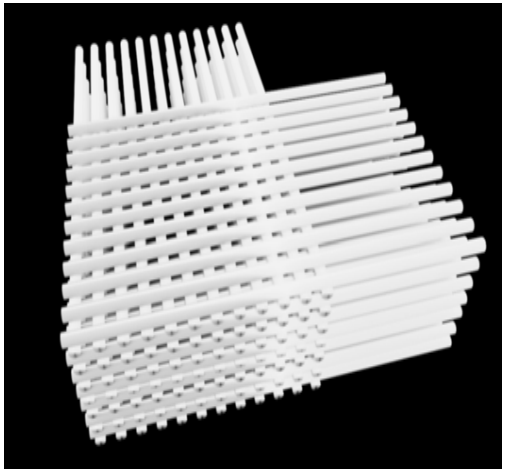
Combining 3D printing, elastomer casting and sugar/salt leaching procedures

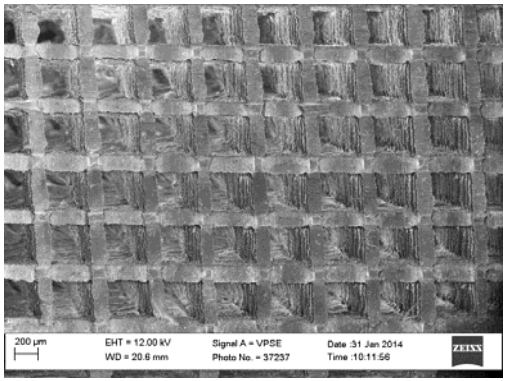
Random porous channels



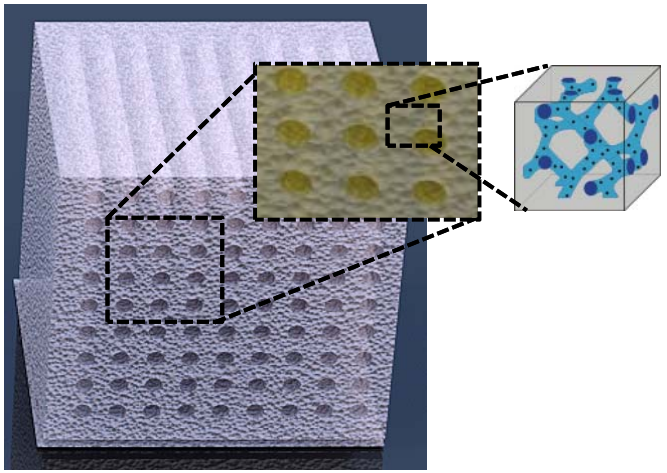


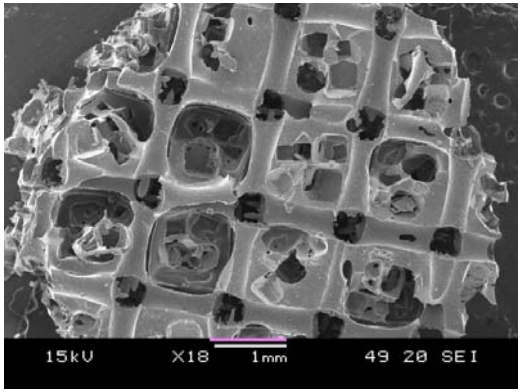
Structured porous channels





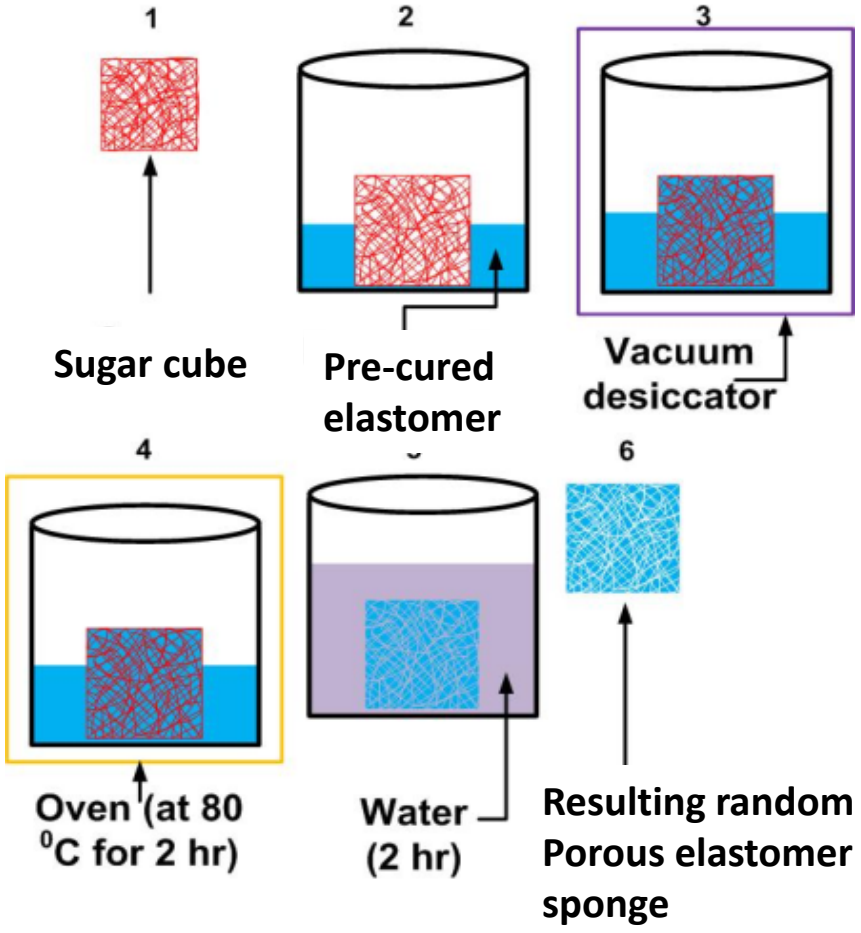
Combined random & structured porous channels
+ IPN of hydrogel micro deposits



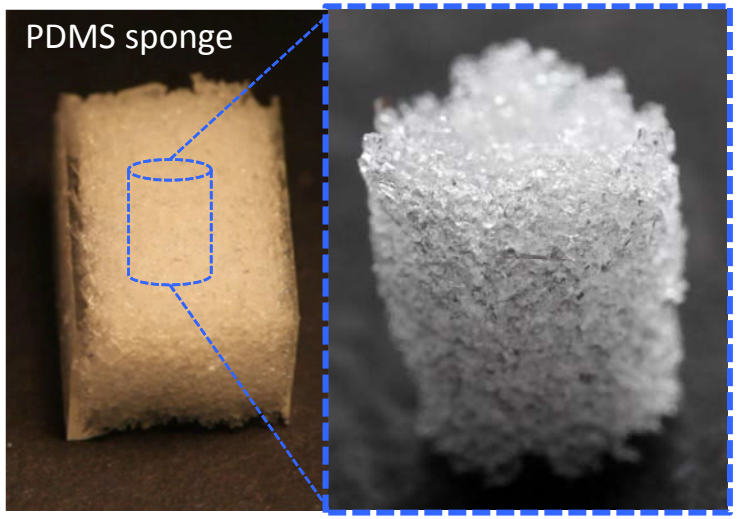


Fabrication of PHS
Random porous scaffolds

Fabrication method using sugar/salt leaching process



Cha & Kim. Biomed. Microdevices 2011, 13:877–883



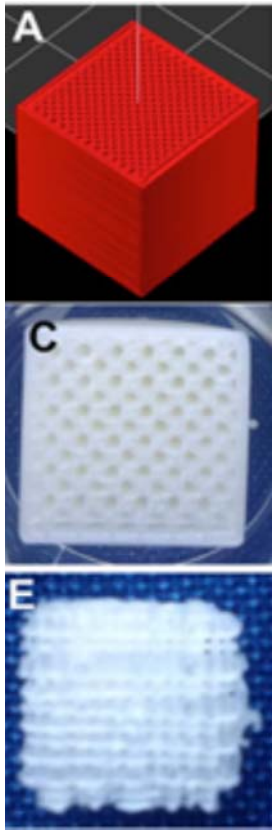
Fabrication of PHS

Structured porous scaffolds

3D printing of sacrificial mould

Polylactic acid (PLA)

Polyvinyl alcohol (PVA)

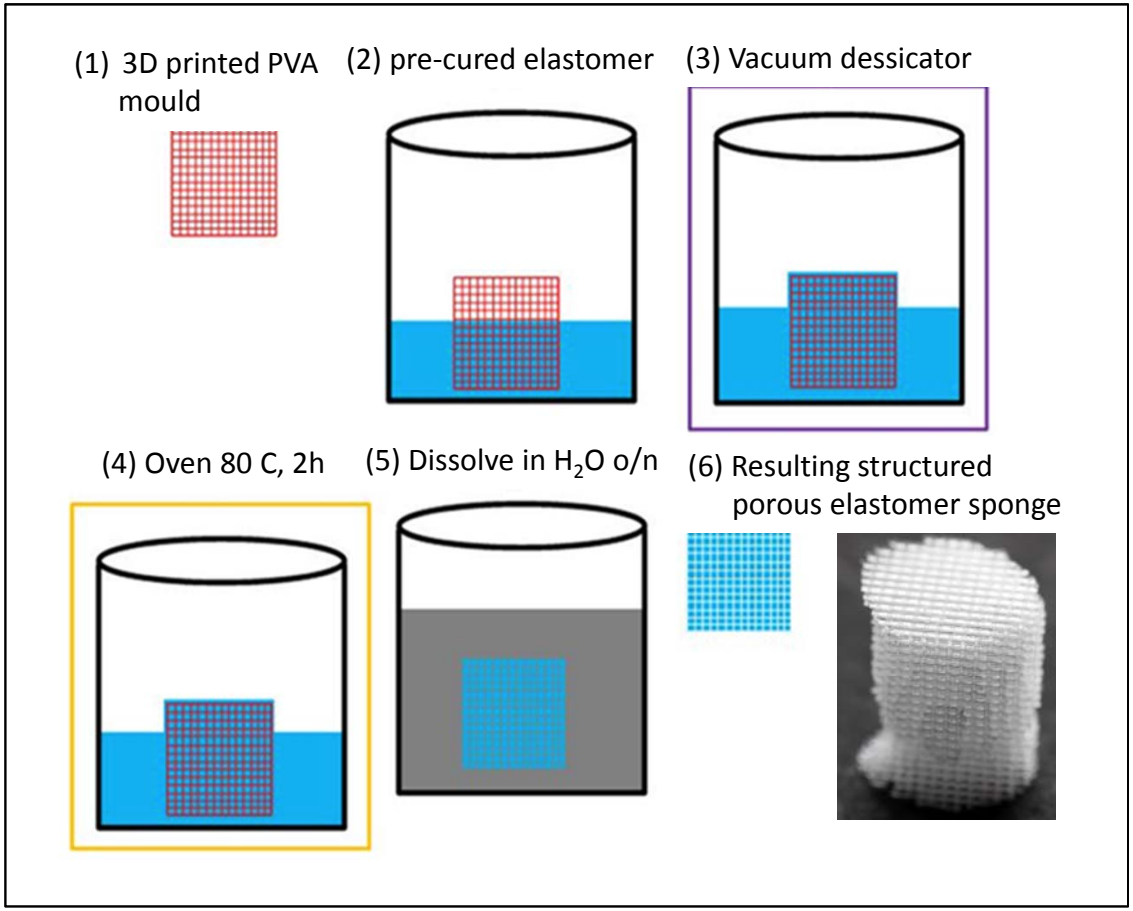


Computer design

3D printed sacrificial mould

Casted elastomer scaffold

Fabrication process using leaching of sacrificial mould



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Fabrication of PHS

Combined random & structured porous scaffolds

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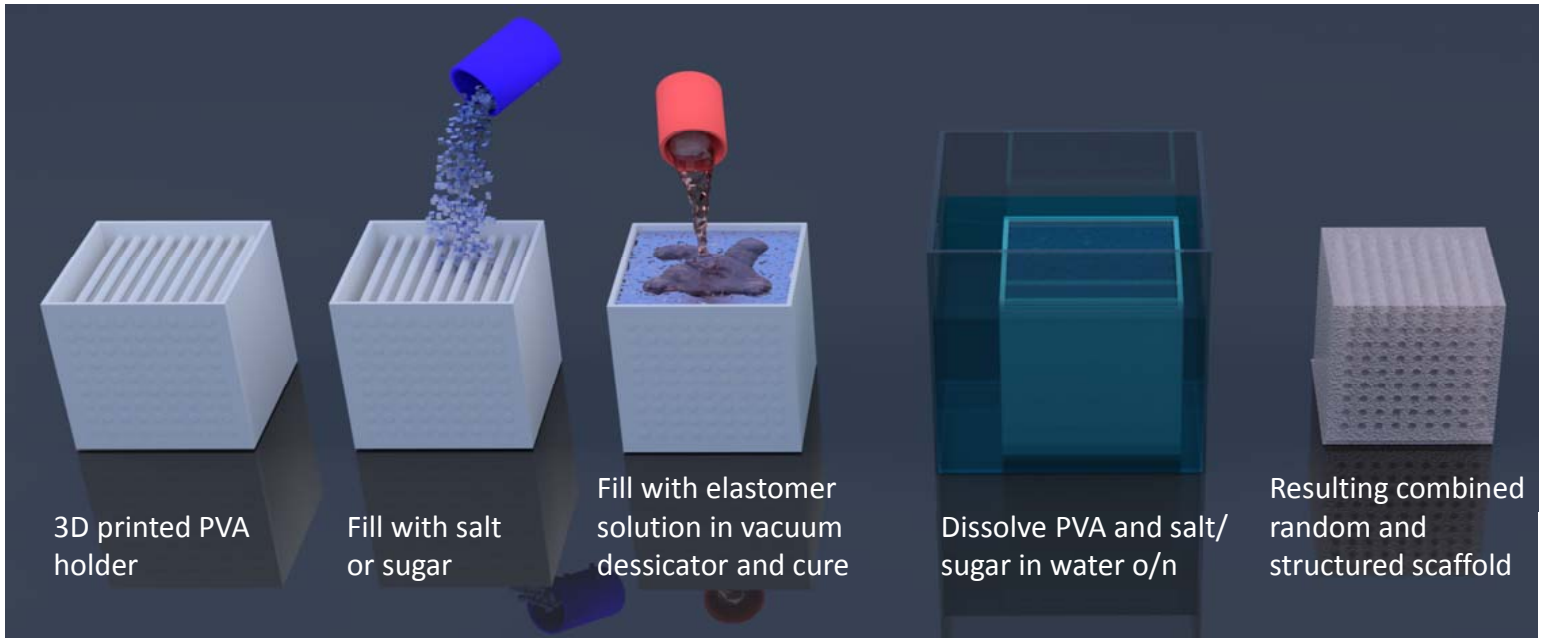
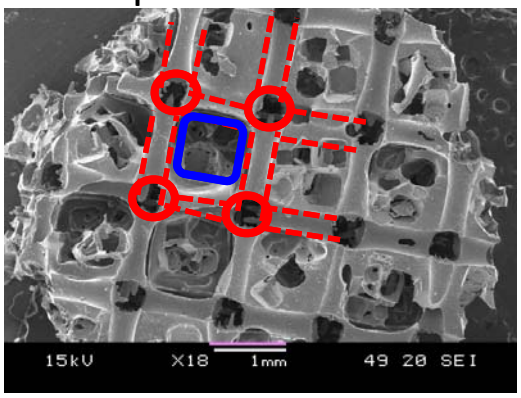
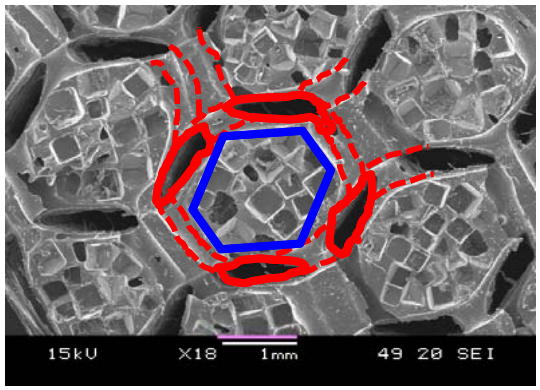
Fabricated through the combination of **3D printing of sacrificial PVA mould and salt/sugar leaching process**



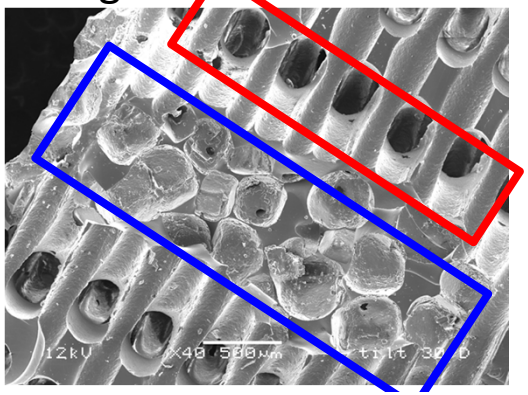
Wood pile structure



Hexagonal structure



Hexagonal sideview



Structured channels

Random pores

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Fabrication of PHS

Different materials

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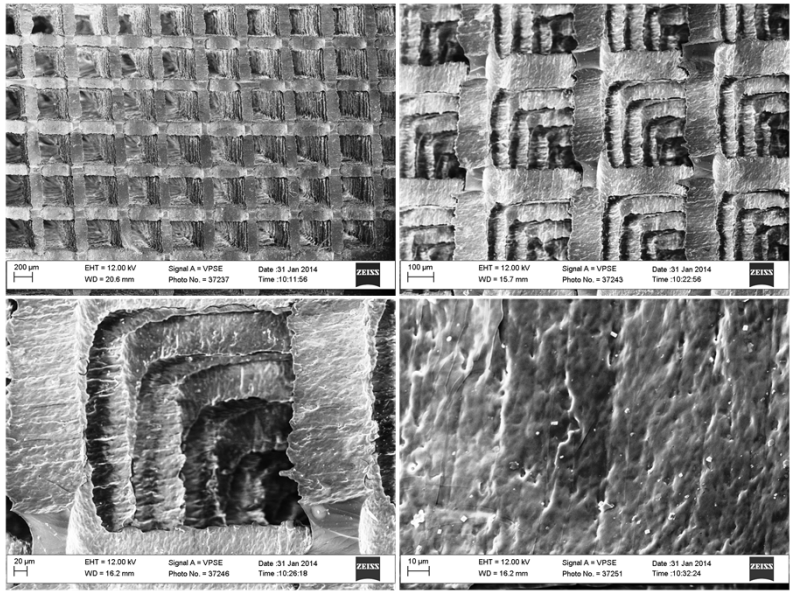
Elastomer: Silicone, Polydimethylsiloxane - PDMS

Silk: Combined silk and collagen scaffolds (D. Kaplan (T**u**ft Univ., USA)

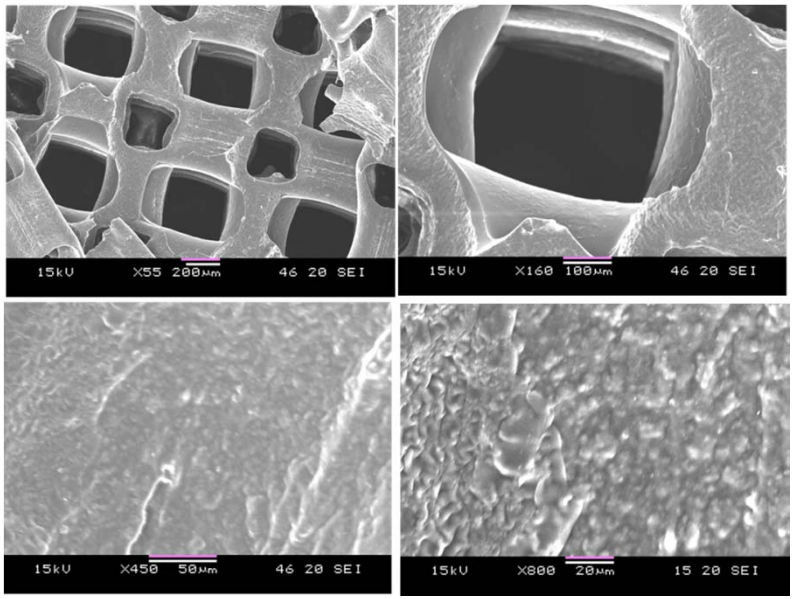
Hydrogels: Gelatin, Gelatin methacrylate hydrogel (GelMa)

Polyhydroxyethylmethacrylate (PHEMA), PEG-PHEMA etc.

PDMS casted on 80% infill printed PVA



PHEMA casted on 50% infill printed PVA



Fabrication of PHS with Interpenetrating Polymer Networks (IPN) Impregnation with doxocycline

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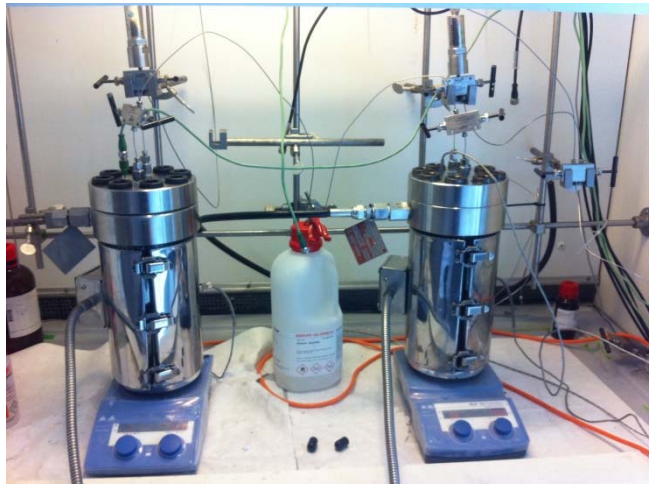
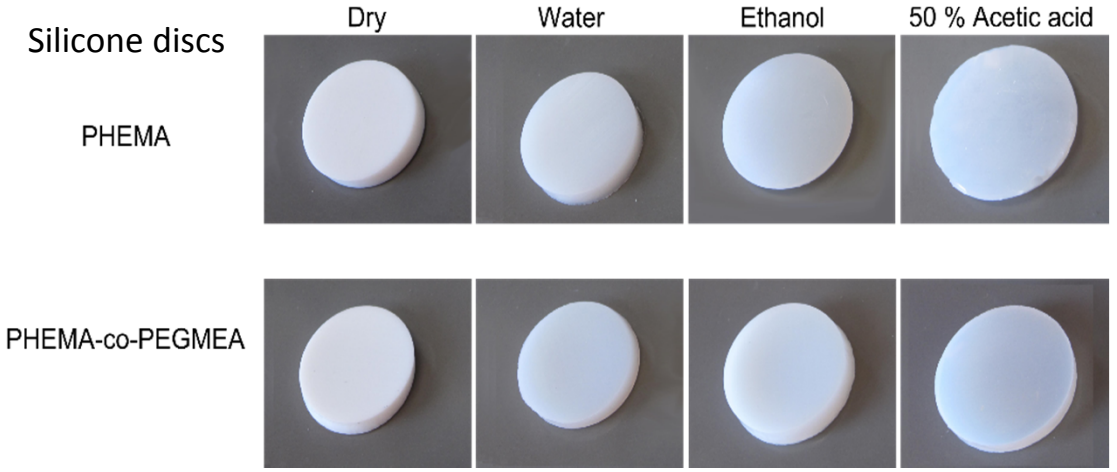
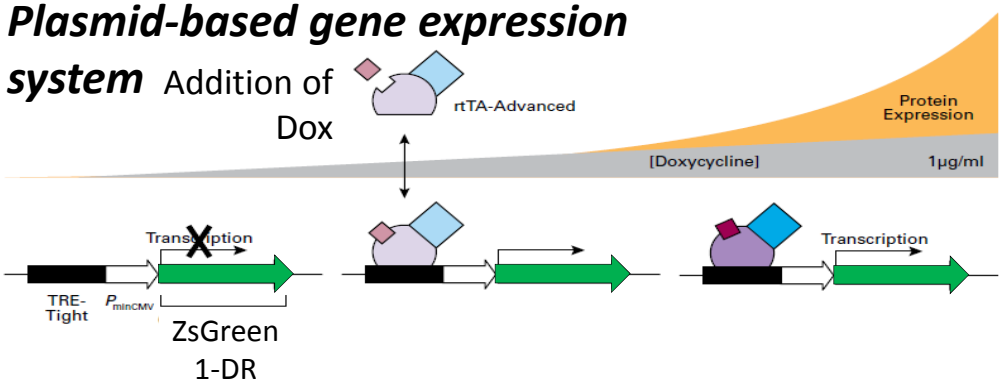
BioMODICS DTU





Plasmid-based gene expression system Addition of Dox



By impregnation of silicon scaffolds with doxycycline a green fluorescent protein will be expressed

Doxycycline release from IPN with HEMA-co-PEGMEA

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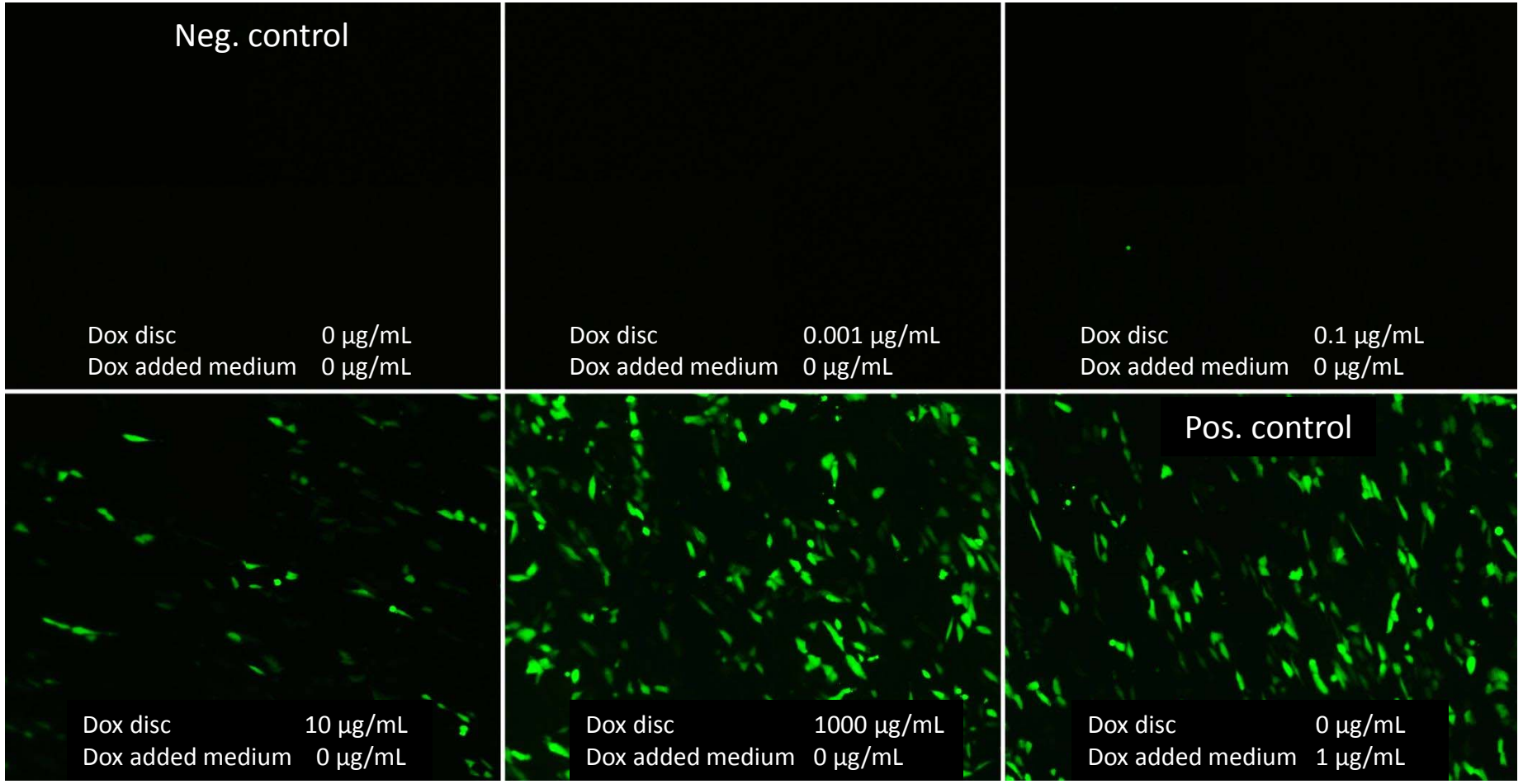
BioModics DTU

Scaffolds: PE4062|Poly[HEMA-co-PEGMEA480] **Cells:** HeLa-Tet On cells from Clontech

Gene expression: 24 hours



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hPSCs technology

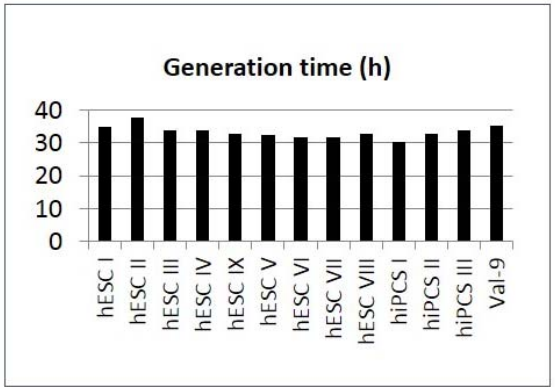
cellartis BY TAKARA BIO

- Optimising culture system for mass production of human pluripotent stem cells (hPSCs), upscaling and banking
- Systematically screening and optimizing culture conditions enabling differentiation and maturation of hPSC-derived hepatocytes
- Optimising the differentiation in 3D and flow

Stable culturing system: DEF-CS

The Culturing System: DEF-CS

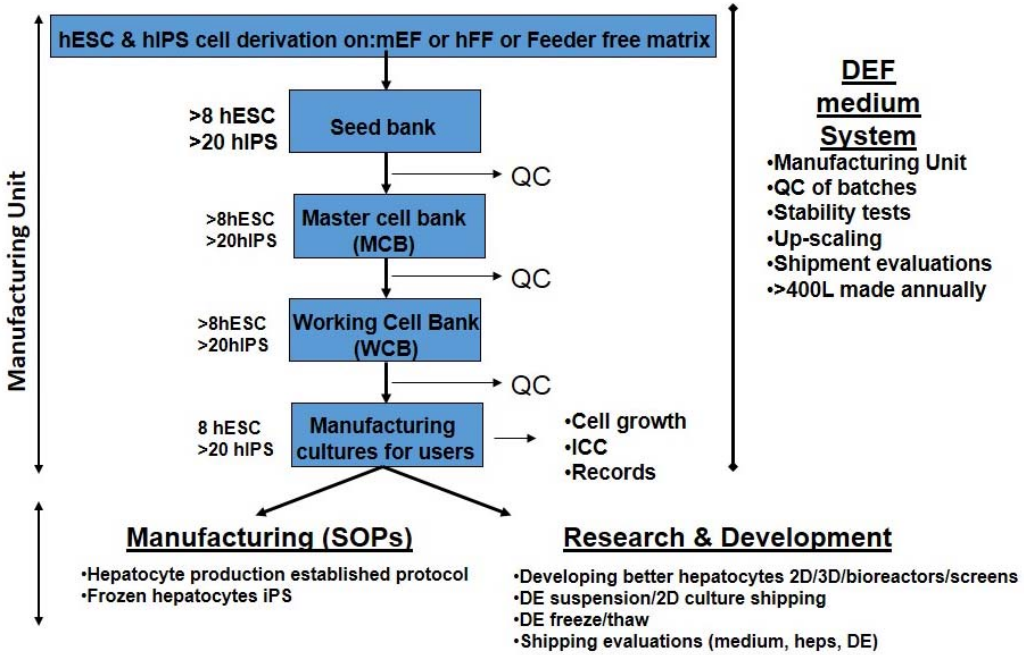
- "***De***fined, ***f***eeder-free ***c***ulturing ***s***ystem"
- Completely defined cell culture medium and coating
- Enzymatic single cell propagation
- Karyotypically normal (> p30)
- Retain the capacity to form all 3 germ layers (> p30)
- Continue to express pluripotent markers (> p30)



- Undifferentiated cells (hESC and hIPSC) can successfully be passaged and expanded in Cellartis DEF medium system.

- The doubling times of several different hESC and hiPSC lines have been tested and the variation between cell lines is very low.

Optimized robust feeder free stem cell culturing system



- hESC and hiPSC are transferred to the DEF system.
- Established cells are frozen in seed banks, followed by quality control of the seed bank.
- Master cell banks (MCBs) and working cell banks (WCBs) are established following the optimized passage procedures in Cellartis manufacturing unit.
- The manufacturing unit follows strict SOPs and supplies research groups and customers with undifferentiated cells for further research and development.

The stability and robustness of the whole system has been verified by using >8 hESC lines and >20 hiPSC lines.

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Differentiation of cells to hepatocytes in 3D and under flow

cellartis BY TAKARA BIO

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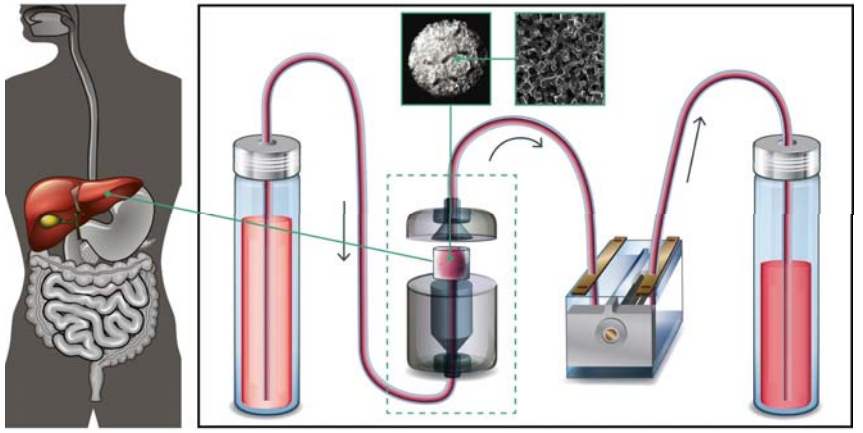
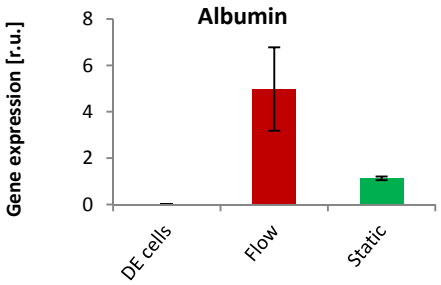
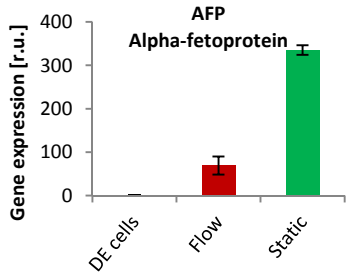
- Large scale 3D production of hPSC-derived hepatospheres has been established approaching 10^9 cells in 500 ml

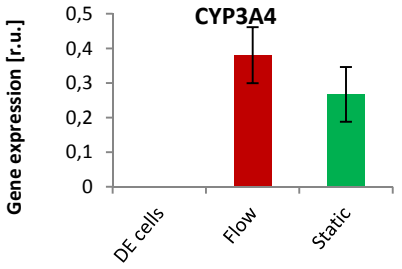
- Differentiation to hepatocytes under flow conditions. Cells are cultured in 3D PHS scaffolds perfused with medium for 25 days

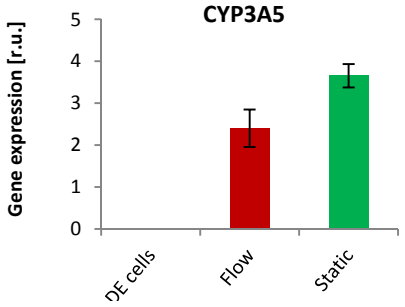


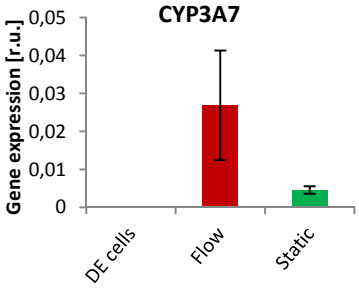
Cells differentiated under flow have the similar and sometimes better mRNA expression of hepatocyte markers compared to conventional 2D static cultured cells, ***details in poster***

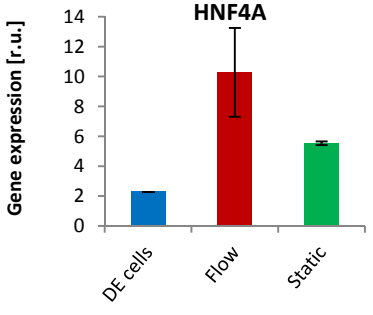












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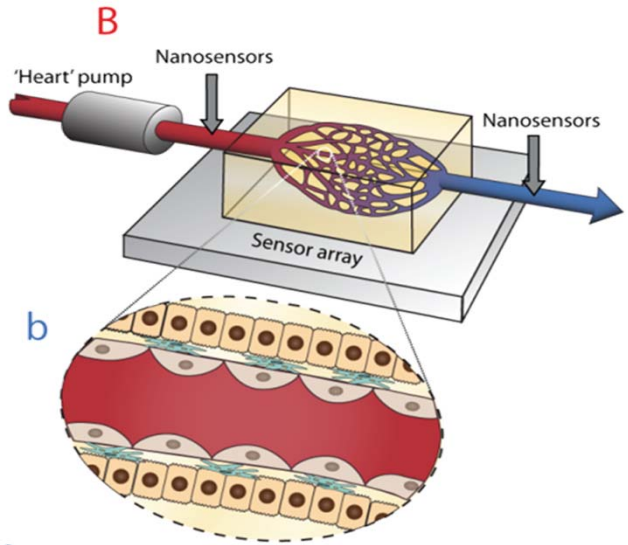
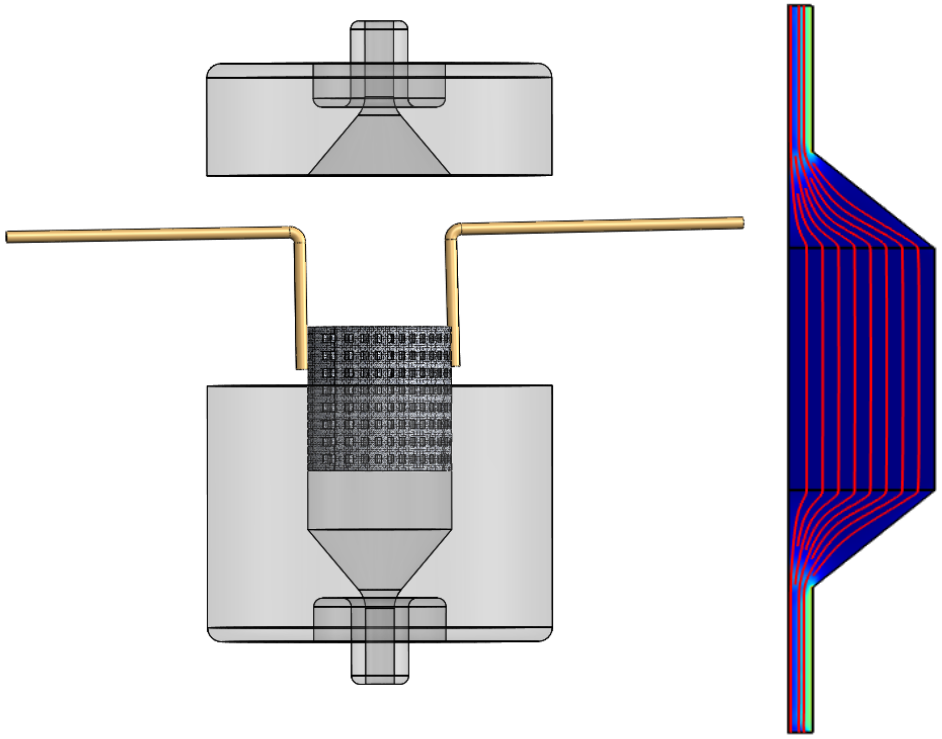
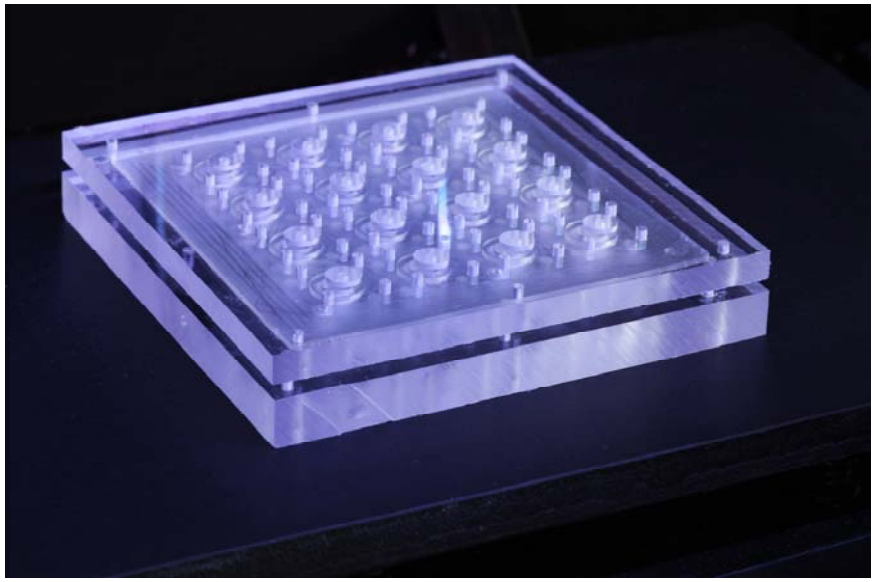
BAL support system

- Fabrication of system components for BAL support system
- Enable high through-put optimisation
- Integration of sensors
- Apply the system for optimisation of differentiation of hPSC
- Develop Liver-on-a-Chip systems for metabolic investigations



Perfusable 3D bioreactor design





- Perfusion bioreactor
- Polycarbonate
- Fabrication – Micromilling
- Pt –electrodes for bioimpedance

- Array of 16 perfusable bioreactors
- One lid for all bioreactors or individual lid for time point experiments

BAL support system

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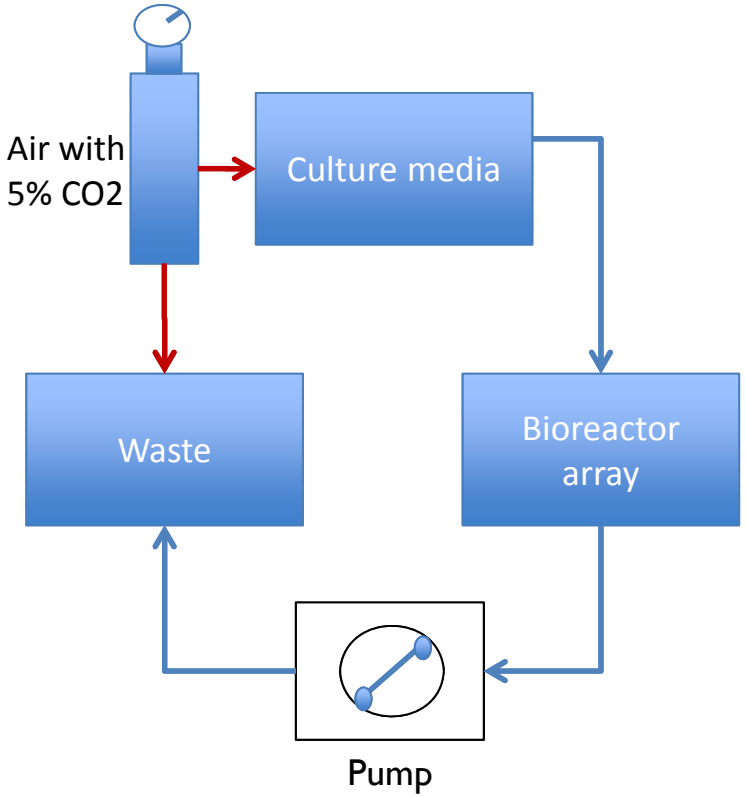
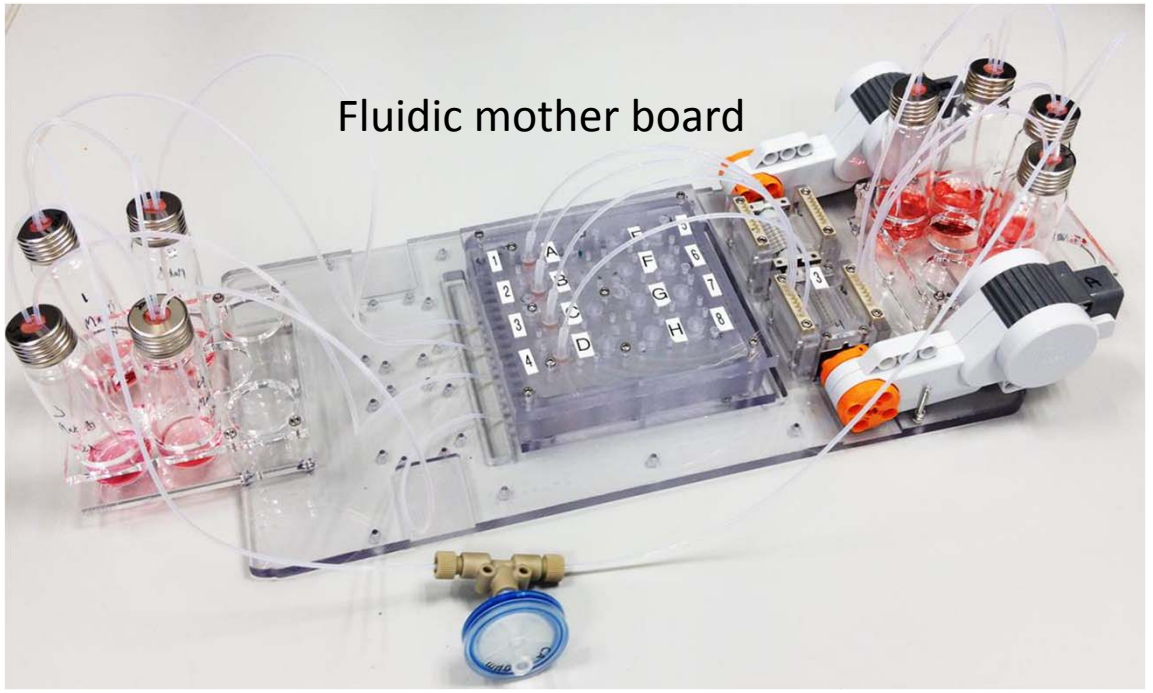
Experimental set up





- Perfusion from bottom–up in bioreactor
- 4 pumps allow 4 different flow rates to be tested
- Entire system placed in incubator

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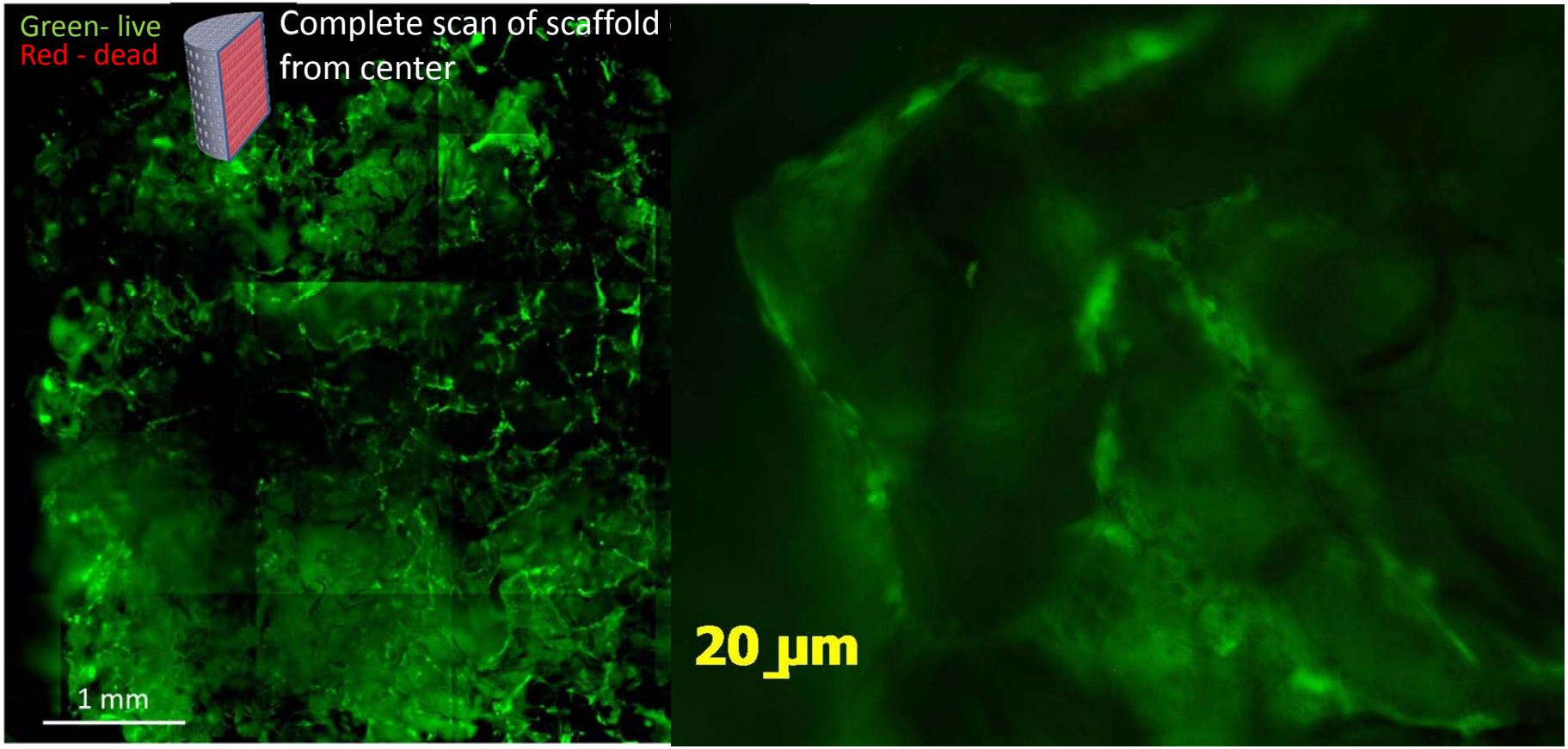
BAL support system

DE differentiation to hepatocytes

- 2.5 x 10^6 pre-differentiated hiPS (definitive endoderm-DE) per scaffold
- 22 days of differentiation from the DE stage
- Random porous scaffolds, 16 bioreactors, flow rate: 1 µl/min
- Evaluation of differentiation:
  Live/Dead staining & Gene expression of hepatocyte markers by RT-PCR



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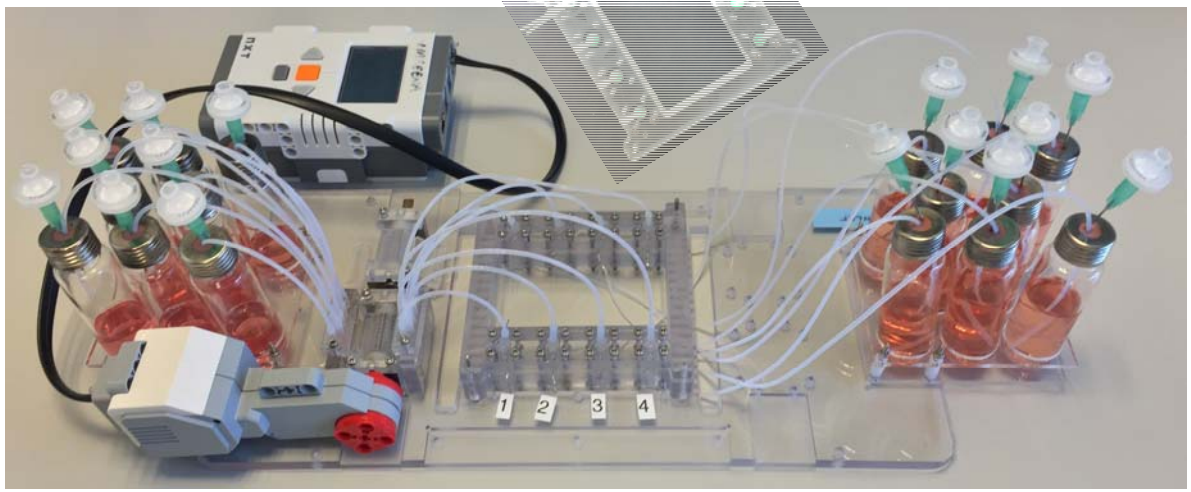
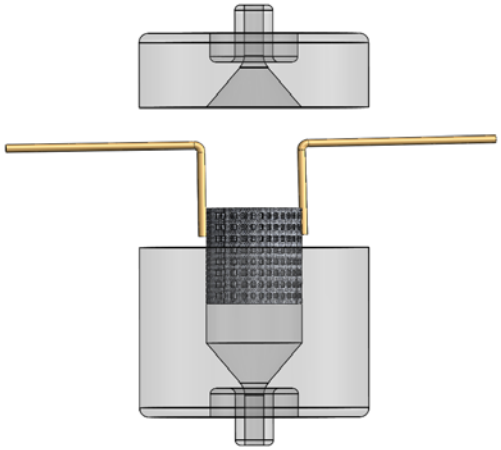
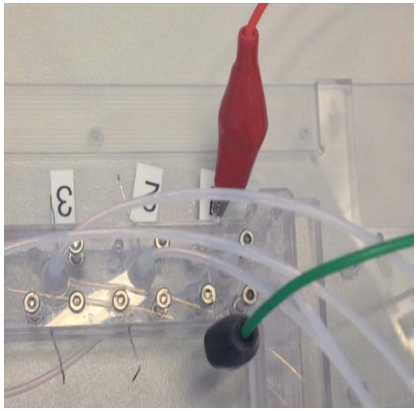
BAL support system
with integrated sensing

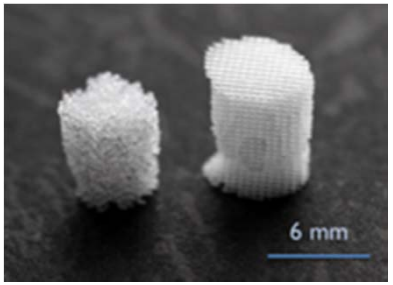
O_2 sensor polymer coating on inner surface of the bioreactor





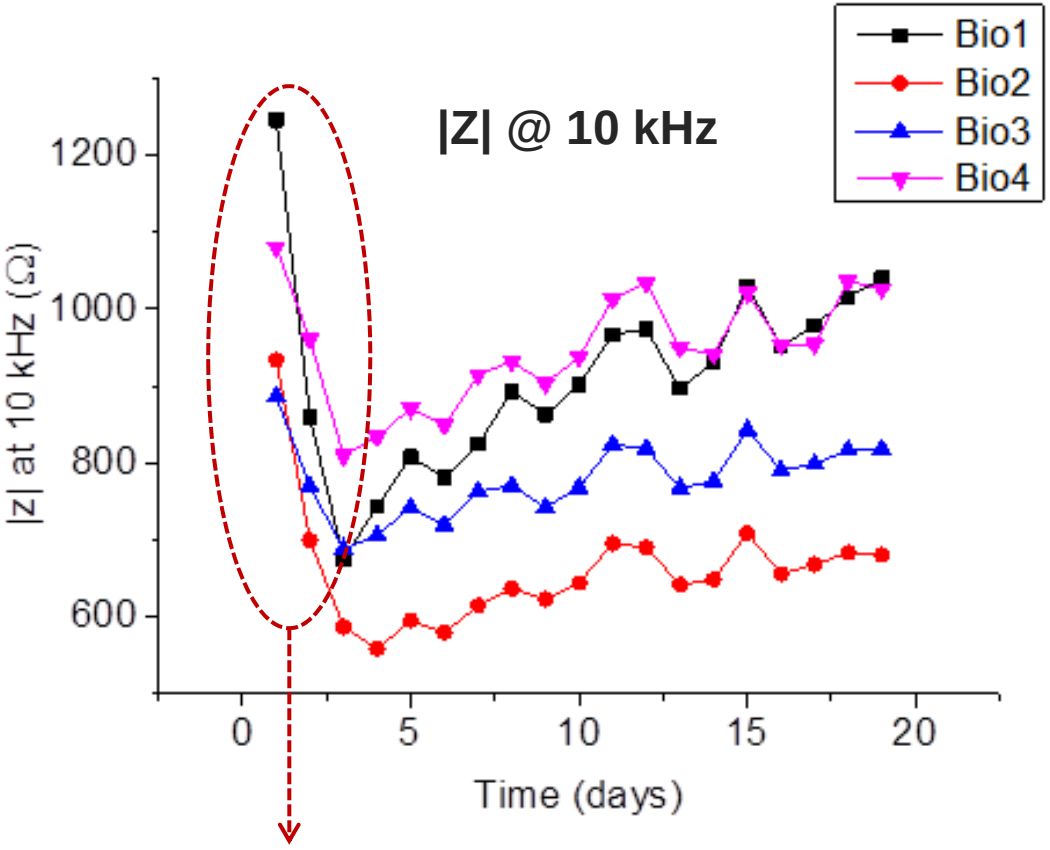
- Array of 8 bioreactors, allocating cylindrical random porous PDMS scaffolds (Ø=6 mm; H=5 mm)
- 2 Pt electrodes (Ø=0.4 mm; H=10 mm, full shaft conductive) on the scaffold sides
- 10 mV injected voltage
- 10 Hz < f < 1 MHz





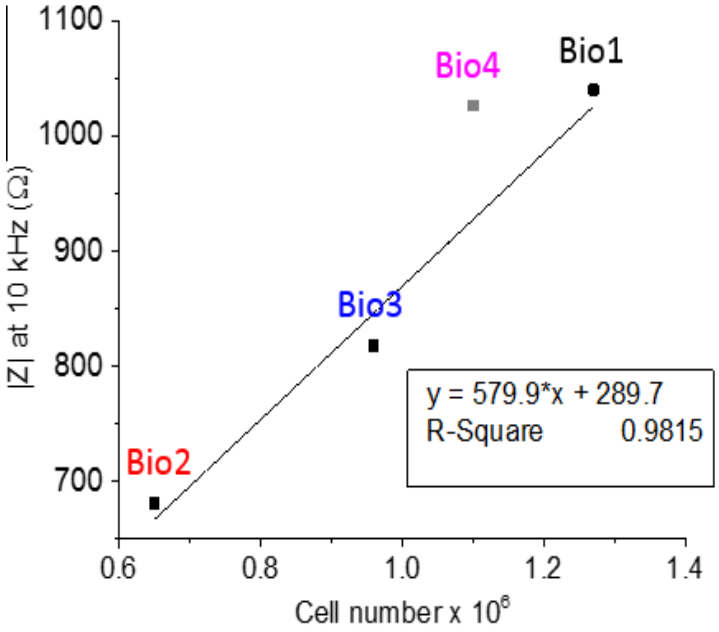
Bioimpedance from perfused culture for 19 days

- ❑ $2x10^6$ HepG2 cells/scaffold
- ❑ perfusion rate = 5 µL/min



Cell detachement after the 1st day in culture (just ~ 8.03 x 10^5 ± 2.43 x10^5 cells still attached)

Cell quantification with Picogreen assay



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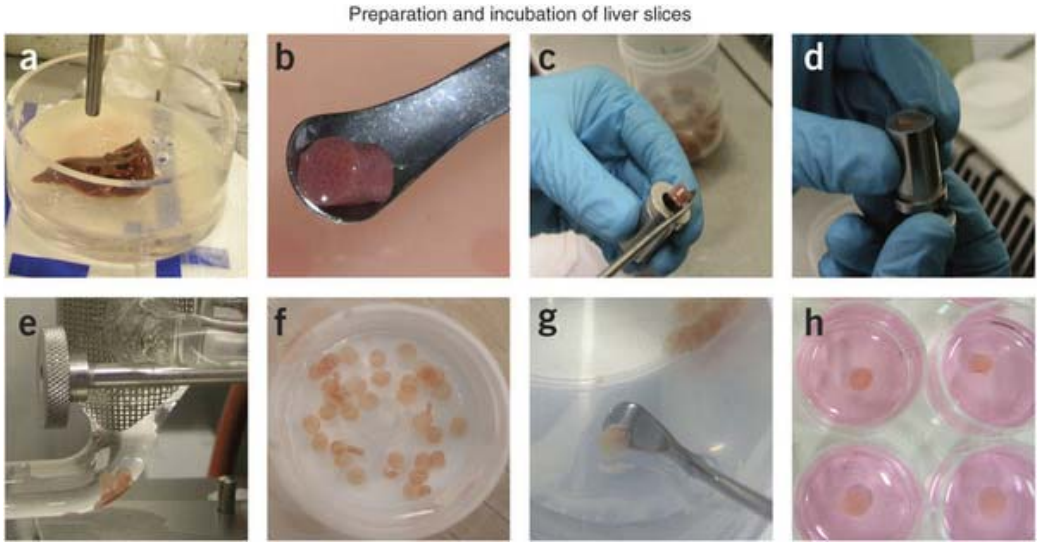
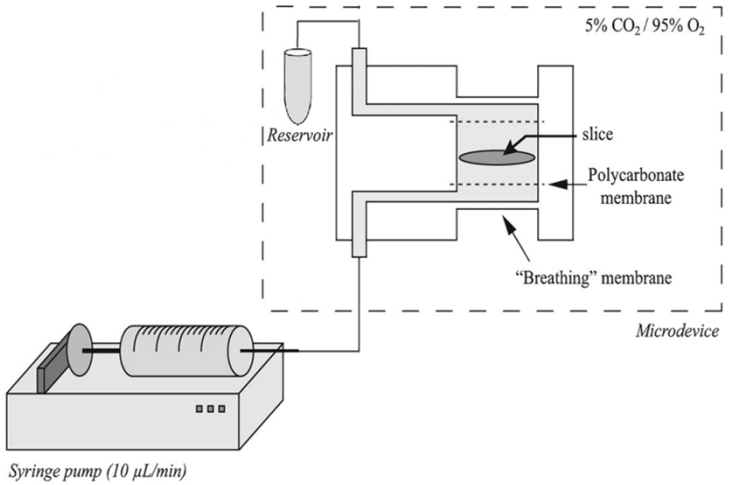
BAL assessment

using precision cut liver slices (PCLS)





Two incubation systems:

-static well plate

-perfused chip model

Human liver slices:

-all liver cell types are present

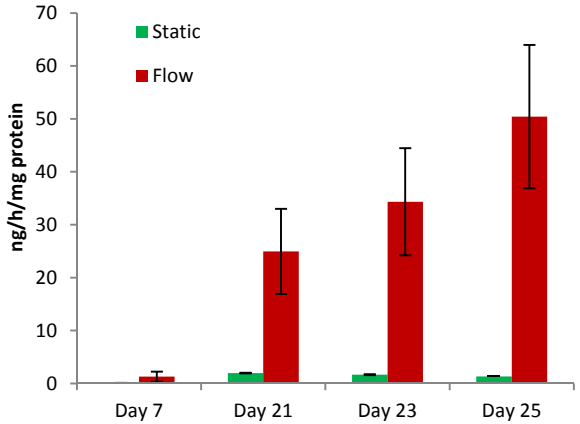
-hepatic microarchitecture is preserved

-drug metabolism function is well preserved

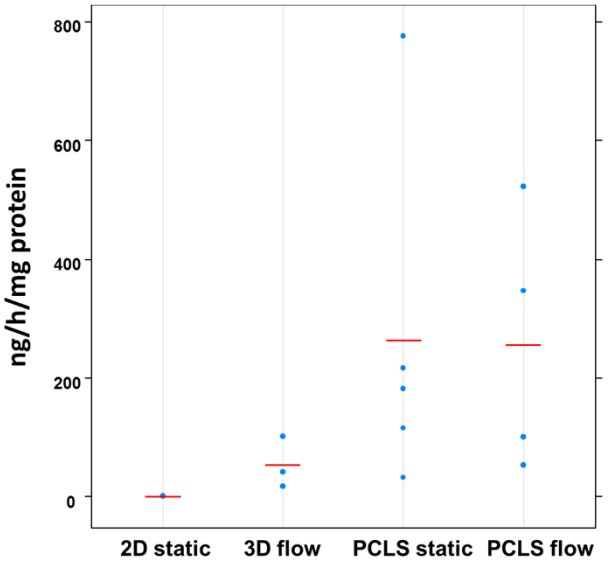
BAL assessment with PCLS - static and flow

Albumin Synthesis

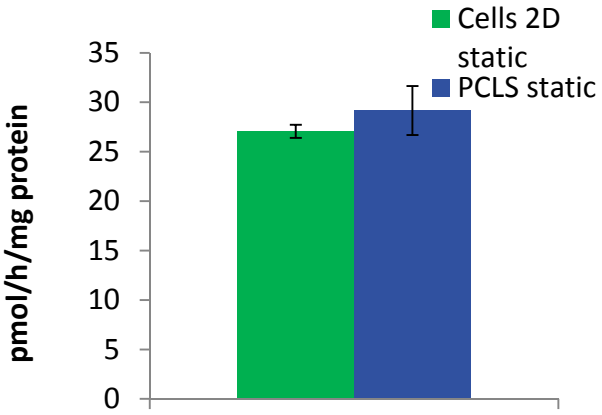
Differentiation of DE cells



Comparison of BAL with PCLS



Bile acid synthesis



Conclusions:

- **Albumin synthesis**: We see increasing albumin secretion during differentiation and maturation of DE to hepatocytes with higher secretion in flow than at static condition.
  Albumin synthesis: Secretion from the differentiated cells in flow is comparable to the liver slices, although some slice donors have very high secretion rate.
- **Bile acid synthesis.** No data for the flow samples, as they become too diluted, however, Bile acid synthesis of the differentiated cells is similar to the PCLS cultured at static conditions.

Phase I and Phase II metabolism – details can be found on poster

Phase I metabolism of cells in 3D PDMS scaffold is in most cases lower than the PCLS at flow conditions

Phase II metabolism was comparable (glucuronidation) to or higher (sulfation) than in PCLS.

Drug cocktail

CYP	Substrate	Metabolite
1A	Phenacetin	Paracetamol
2B6	Bupropion	OH-bupropion
2C19	Mephenytoin	4-OH-mephenytoin
2C9	Diclofenac	4-OH-diclofenac
2D6	Bufuralol	OH-bufuralol
3A	Midazolam	1-OH-midazolam

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Conclusion

- **Stem-cell derived hepatocytes are highly differentiated and most of their functions is comparable to the PCLS, which are considered as a good benchmark for the liver in vivo.**

- **Stem-cell derived hepatocytes cultured on 3D scaffold are promising for future development of BAL and BAL support system and**

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Acknowledgements

SEVENTH FRAMEWORK PROGRAMME

DTU

cellartis BY TAKARA BIO

BIOMODICS

LUXCEL BIOSCIENCES

university of groningen