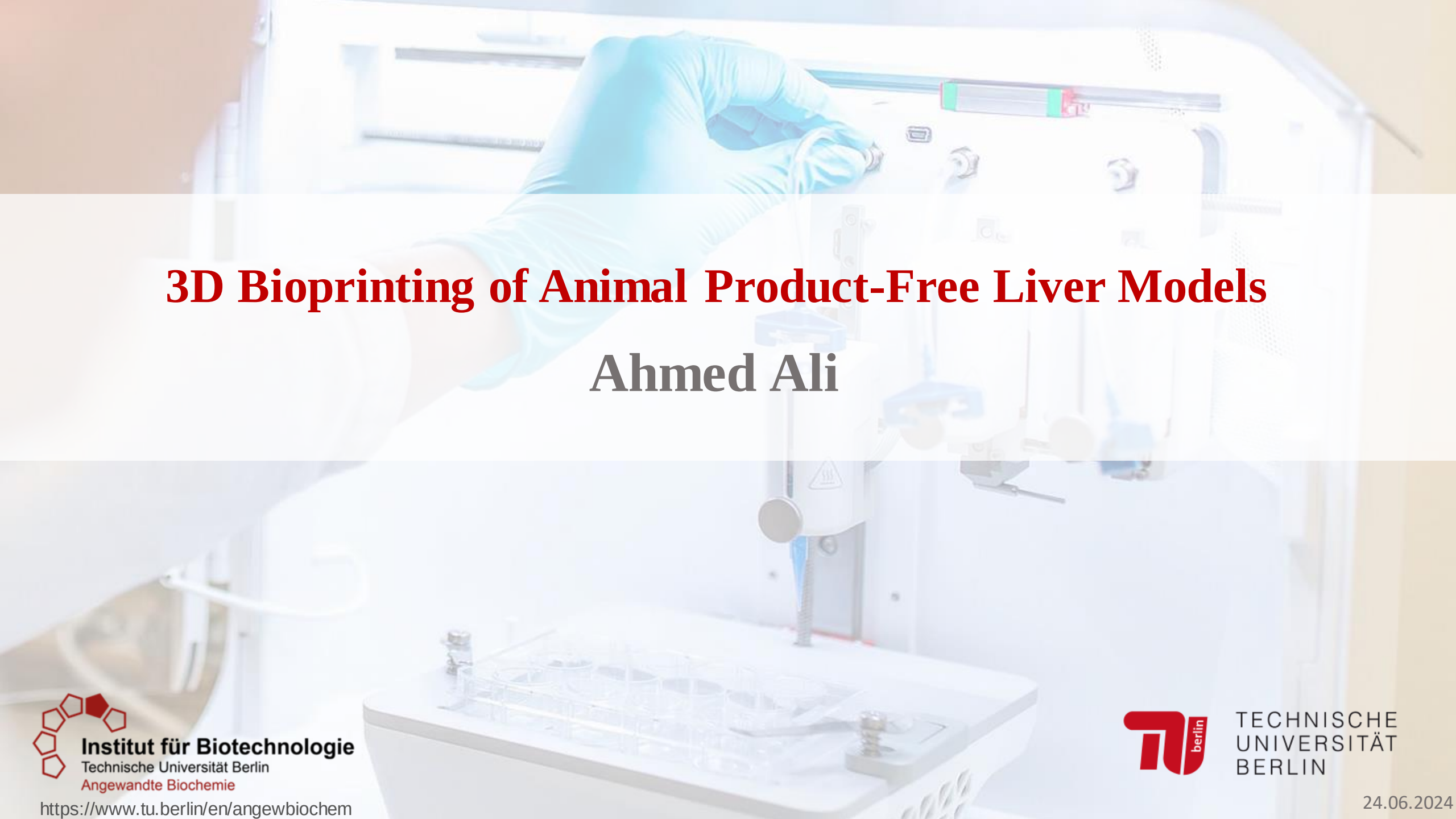

3D Bioprinting of Animal Product-Free Liver Models

Ahmed Ali

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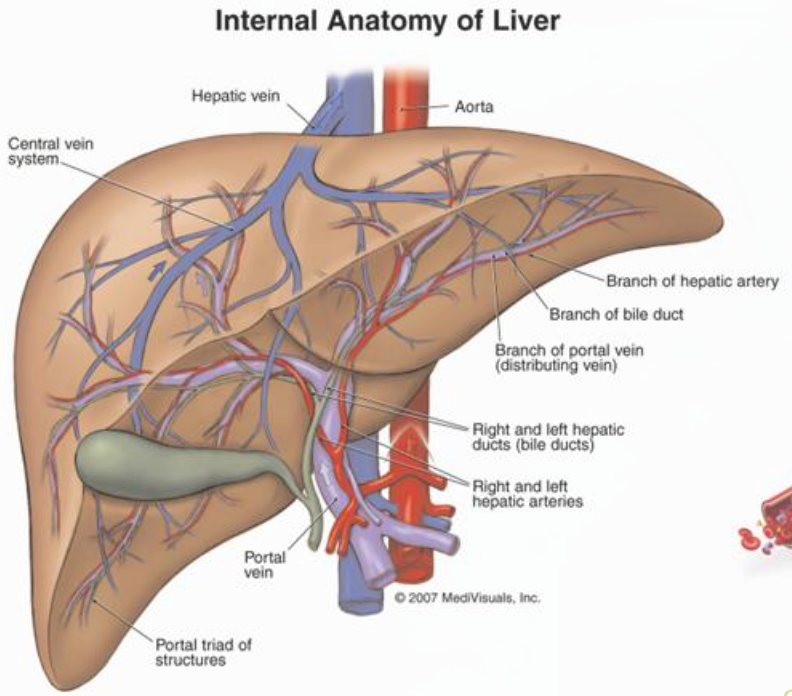
TECHNISCHE UNIVERSITÄT BERLIN

24.06.2024

Liver functions



Metabolism

- Carbohydrates
- Lipids
- Nitrogen Compounds

Production

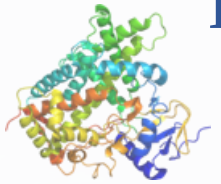
- Albumin
- Prothrombin
- Fibrinogen

Blood Homeostasis

- Homeostasis & Hemodynamics

Immunity

- Kupffer cells



Biotransformation

- Xenobiotics (CYP450)

- Fat absorption
- LDL metabolism

around 500 biochemical reactions [1]

(1) Ma, Liang, et al. Advanced Healthcare Materials 9.24 (2020): 2001517.

Hepatotoxicity

- The liver is a primary target for systemic toxicity caused by chemicals, drugs and natural toxins.
- Drug-related liver toxicity accounts for more than 50% of all clinical cases of acute liver failure [1].
- Responsible for 6% of all liver-related deaths and for 7% of all liver transplantations [2].
- Drug-induced liver injury is a major reason of drug failure during pre-marketing and post-marketing phases (29% of all drug withdrawals) [3].

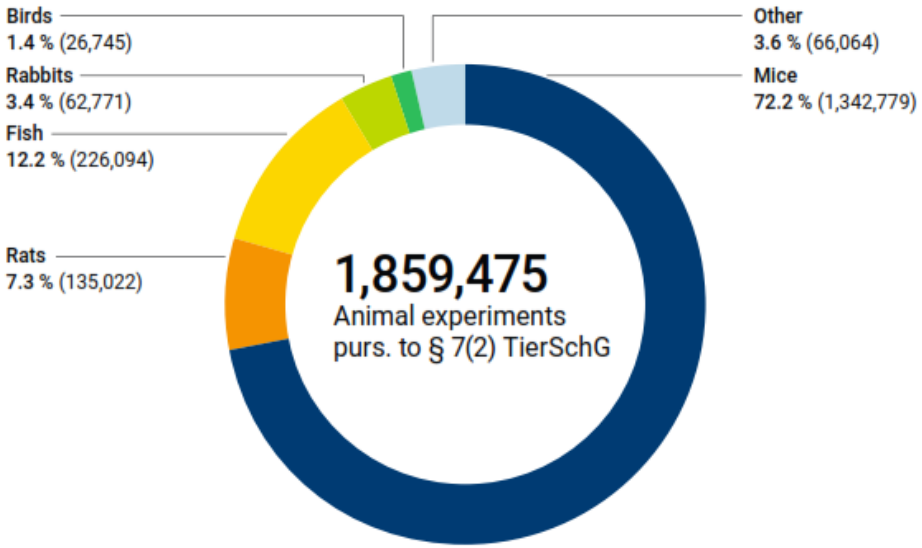
(1) Goldberg D.S., et al. Gastroenterology. 2015;148:1353–1361.e3.
(2) Germani G., et al. J. Hepatol. 2012;57:288–296.
(3) Lee W.M. Clin. Liver Dis. 2013;17:575–586.

Testing models

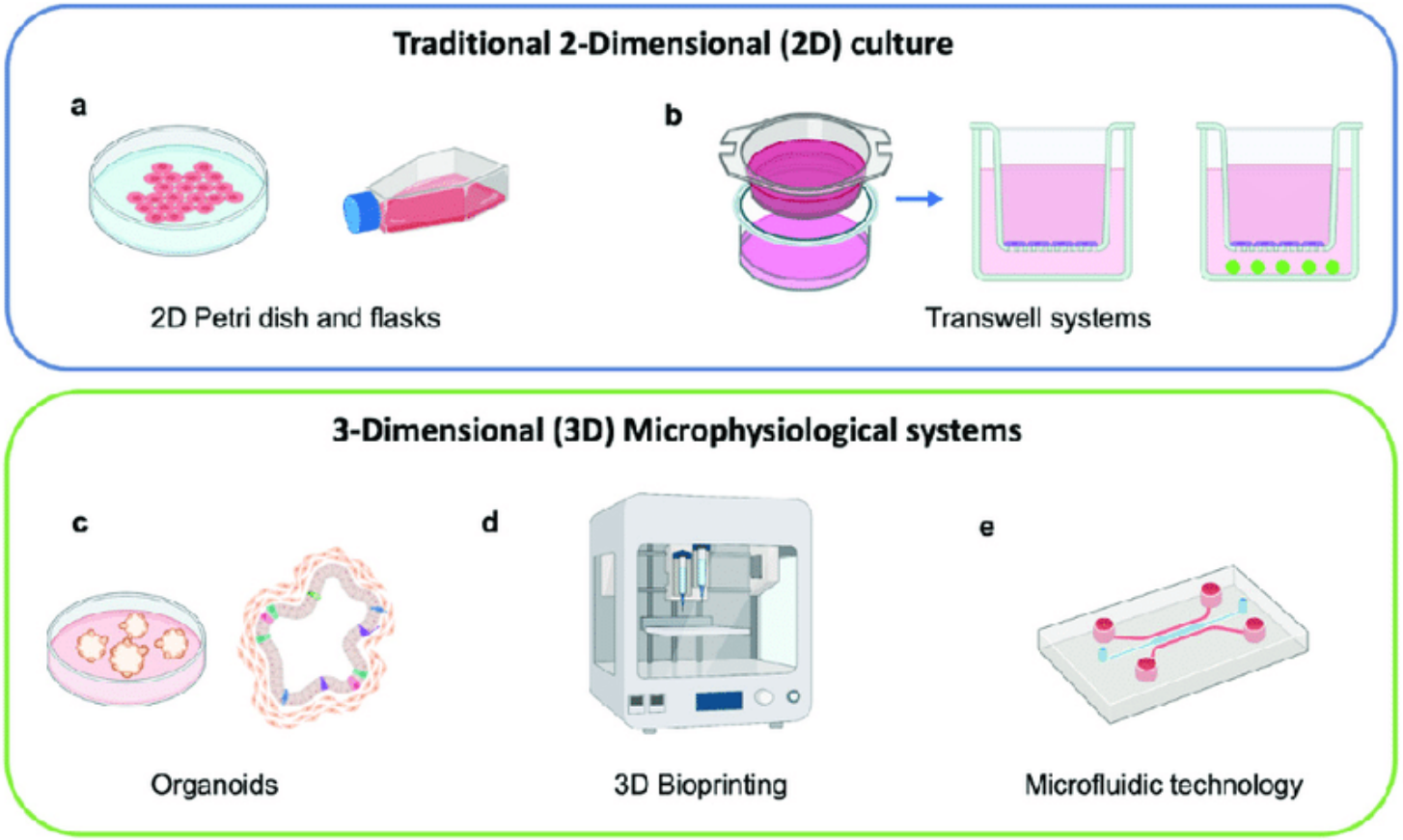
Animal testing

Animal species used in 2021

for animal experiments pursuant to § 7(2) TierSchG



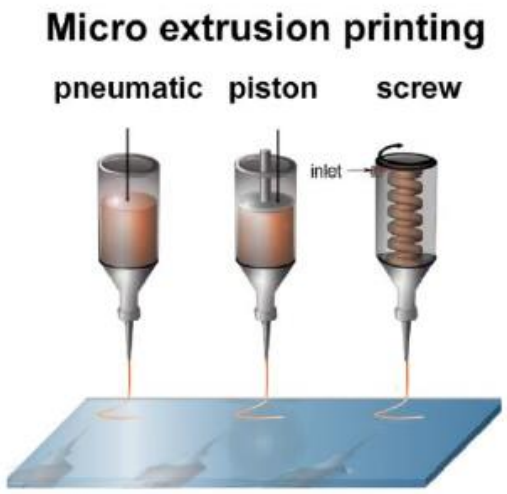
In vitro models

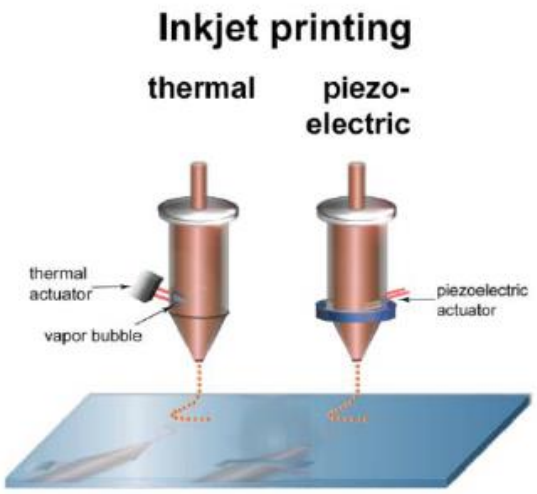


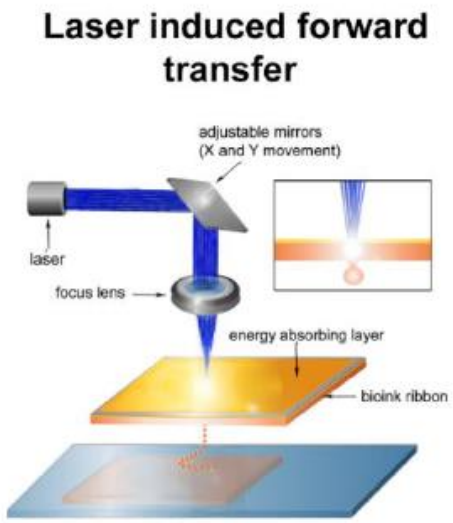
New approach methodologies (NAMs)

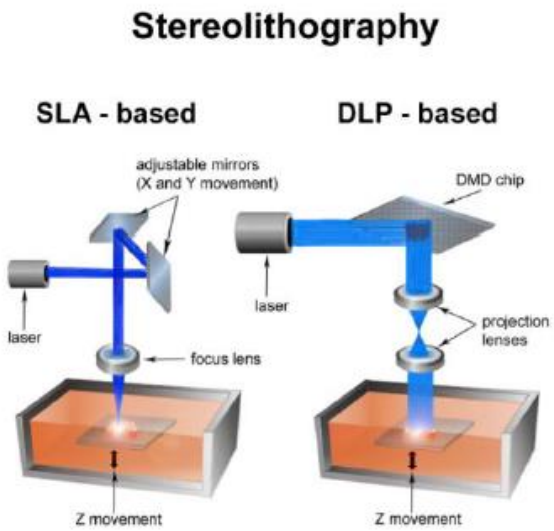
3D bioprinting

3D bioprinting is an additive manufacturing technique, which involves the addition or deposition of a bioink in a layer-by-layer fashion to create 3D structures like tissues and organs.









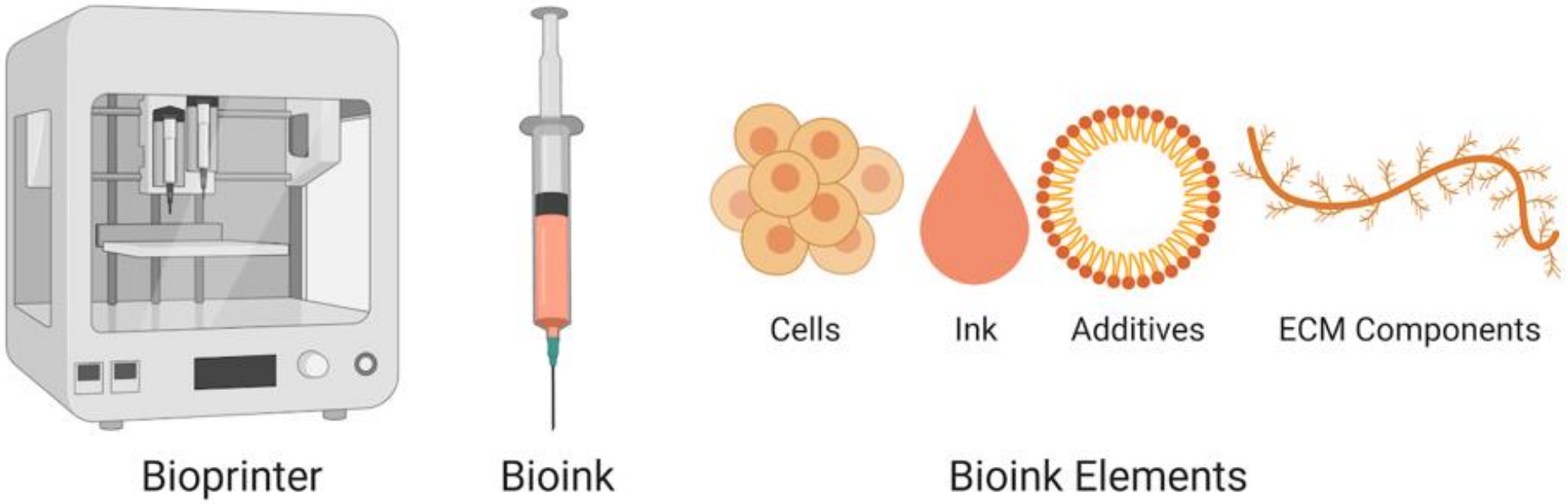
Berg, J. and Kurreck, J. (2021), *ALTEX*, 38(2), pp. 269–288.

3D bioprinting

Bioinks are soft biomaterials loaded with living cells.

Bioinks must combine two properties:

- They must have low initial viscosity to be printable.
- Following the printing process they must immediately become stiff to maintain the structure.



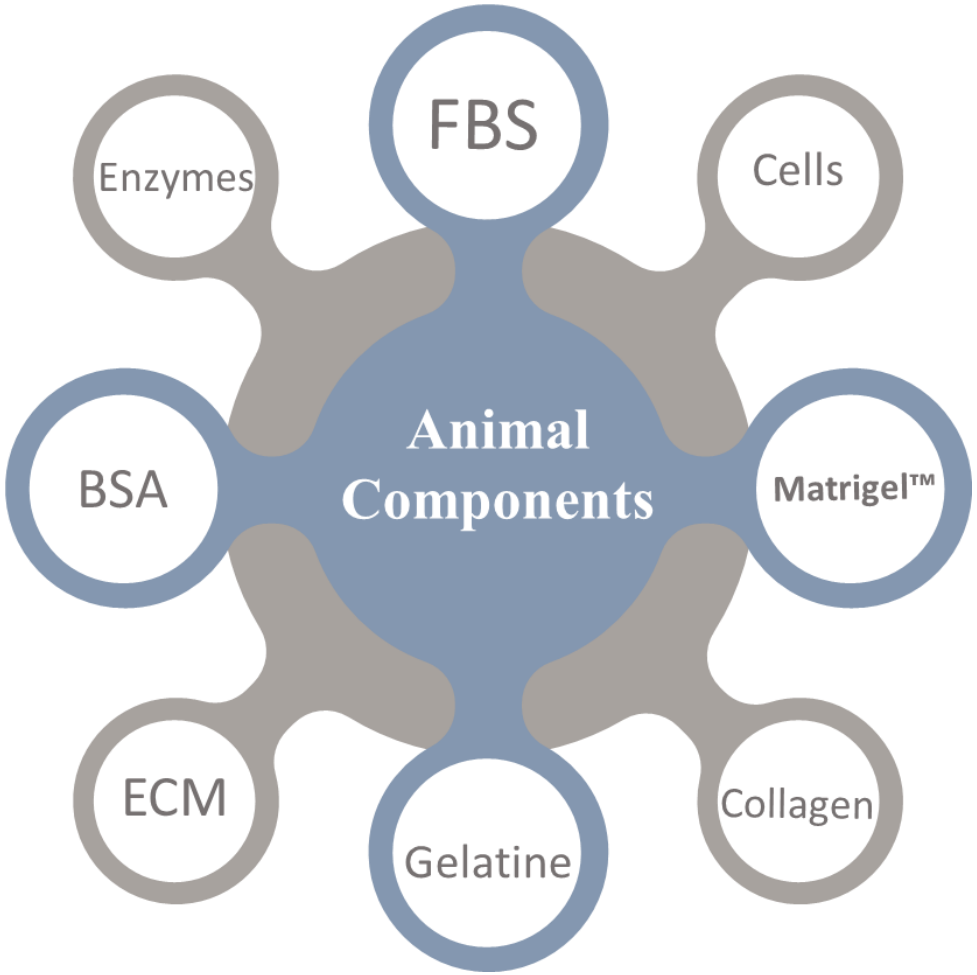
Animal-derived materials

Antibodies

Proteins

Basement membrane Extract

Fetal bovine serum



Do we really need a chimeric model? Why should we avoid animal components?

Why should we avoid animal components?

Animal Welfare

A 3-month-old ≈150 mL FBS

A 6-month-old ≈ 350 mL FBS

A 9-month old ≈ 550 mL FBS

The total volume of FBS used worldwide can be estimated to be around 500.000 L per year, which requires puncturing of 1 million fetuses. [1]

Scientific Problems

Unknown composition

Batch to batch variation[2]

PROFILE OF FETAL BOVINE SERA

	Average	Range	Number of Lot Samples
Endotoxin	0.356 ng/ml	0.008–10.0	39
pH	7.40	7.20–7.60	40
Hemoglobin	11.3 mg/dl	2.4–18.1	17
Glucose	125 mg/100 ml	85–247	43
Sodium (Na^+)	137 meq/l	125–143	43
Potassium (K^+)	11.2 meq/l	10.0–14.0	43
Chloride (Cl^-)	103 meq/l	98–108	43
Blood urea nitrogen	16 mg/100 ml	14–20	43
Total protein	3.8 g/100 ml	3.2–7.0	43
Albumin	2.3 g/100 ml	2.0–3.6	43
Calcium (Ca^{++})	13.6 mg/100 ml	12.6–14.3	43
Inorganic phosphorus	9.8 mg/100 ml	4.3–11.4	43
Cholesterol	31 mg/100 ml	12–63	43
Uric acid	2.9 mg/100 ml	1.3–4.1	43
Creatinine	3.1 mg/100 ml	1.6–4.3	43
Total bilirubin	0.4 mg/100 ml	0.3–1.1	43
Direct bilirubin	0.2 mg/100 ml	0.0–0.5	43
Alkaline phosphatase	255 mU/ml	111–352	43
Lactic dehydrogenase	864 mU/ml	260–1215	43
Serum glutamate oxalacetate transaminase 340	130 mU/ml	20–201	43
Selenium	0.026 μg/ml	0.014–0.038	25
Cortisol	0.5 μg/dl	<0.1–2.3	43
Insulin	10 μU/ml	6–14	40
Parathyroid hormone	1718 pg/ml	85–6180	41
Progesterone	8 ng/dl	<0.3–36	42
T3	119 ng/dl	56–223	41
T4	12.1 ng/dl	7.8–15.6	42
Testosterone	40 ng/dl	21–99	42
Prostaglandin E	5.91 ng/ml	0.5–30.48	37
Prostaglandin F	12.33 ng/ml	3.77–42.00	38
Thyroid stimulating hormone	1.22 ng/ml	<0.2–4.5	40
Follicle stimulating hormone	9.5 ng/ml	<2–33.8	34
Growth hormone	39.0 ng/ml	18.7–51.6	40
Prolactin	17.6 ng/ml	2.00–49.55	40
Leutinizing hormone	0.79 ng/ml	0.12–1.8	38
Vitamin A	9 μg/dl	<1–35	16
Vitamin E	0.11 mg/dl	<0.1–0.42	16

1) Gstraunthaler et al. Cytotechnology, 2013, 65, 791).

2) Price, Paul J., and Elizabeth A. Gregory. In vitro (1982): 576-584.

Why should we avoid animal components?

Animal W

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A 6-month-old ≈ 3

A 9-month old ≈ 5

The total volume of FI can be estimated to be per year, which requir million fet

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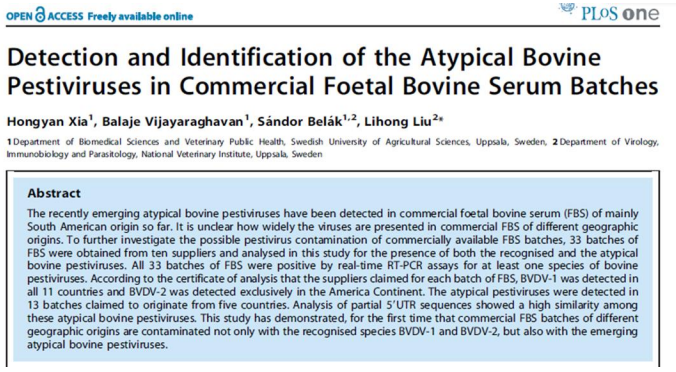
Safety

May be contaminated with viruses, mycoplasms, fungi, and prions



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Detection and Identification of the Atypical Bovine Pestiviruses in Commercial Foetal Bovine Serum Batches

Hongyan Xia[1], Balaje Vijayaraghavan[1], Sándor Belák[1,2], Lihong Liu[2]*

1 Department of Biomedical Sciences and Veterinary Public Health, Swedish University of Agricultural Sciences, Uppsala, Sweden, 2 Department of Virology, Immunobiology and Parasitology, National Veterinary Institute, Uppsala, Sweden

Abstract

The recently emerging atypical bovine pestiviruses have been detected in commercial foetal bovine serum (FBS) of mainly South American origin so far. It is unclear how widely the viruses are presented in commercial FBS of different geographic origins. To further investigate the possible pestivirus contamination of commercially available FBS batches, 33 batches of FBS were obtained from ten suppliers and analysed in this study for the presence of both the recognised and the atypical bovine pestiviruses. All 33 batches of FBS were positive by real-time RT-PCR assays for at least one species of bovine pestiviruses. According to the certificate of analysis that the suppliers claimed for each batch of FBS, BVDV-1 was detected in all 11 countries and BVDV-2 was detected exclusively in the America Continent. The atypical pestiviruses were detected in 13 batches claimed to originate from five countries. Analysis of partial 5'UTR sequences showed a high similarity among these atypical bovine pestiviruses. This study has demonstrated, for the first time that commercial FBS batches of different geographic origins are contaminated not only with the recognised species BVDV-1 and BVDV-2, but also with the emerging atypical bovine pestiviruses.

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2) Price, Paul J., and Elizabeth A. Gregory. In vitro (1982): 576-584.

Why should we avoid animal components?

Anim

A 3-month-

A 6-month-

A 9-month

The total volum
can be estimate
per year, which
mill

1) Gstraunthaler et al. Cyt

2) Price, Paul J., and Elizab

Supplier	Sample ID	Origin	BVDV-1	BVDV-2	BVDV-3
A	A1	Australia	+	−	−
	A2	Brazil	−	−	+
	A3	USA	+	+	−
	A4	USA	+	+	−
	A5	USA	+	+(ns)	−
B	B1	Australia	+	−	+
	B2	Australia	+	−	+
	B3	Australia	−	−	+
	B4	Canada	+	+(ns)	+
	B5	Mexico	+	+	+
	B6	USA	+(ns)[a]	−	+
C	C1	USA	+	−	−
	C2	USA	+	−	−
D	D1	USA	+	+	−
E	E1	Canada	+	+	−
	E2	EU	+	−	−
	E3	New Zealand	+	−	−
	E4	South American	+	−	+
	E5	USA	+	+	−
F	F1	Brazil	+	+	+
G	G1	Australia	−	−	+
	G2	Brazil	−	−	+
H	H1	Australia	+	−	+
	H2	Mexico	+	+(ns)	−
	H3	USA	+	−	+
J	J1	South Africa	+	−	−
K	K1	Canada	+	+	−
	K2	Colombia	+	−	−
	K3	Denmark	+	−	−
	K4	Dominican Republic	+	−	−
	K5	France	+	−	−
	K6	Mexico	+	−	−
	K7	Unidentified	+	−	−

[a]Sequence has not been determined.

doi:10.1371/journal.pone.0028553.t001

S

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B

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Insulin	10 μU/ml	6–14	40
Parathyroid hormone	1718 pg/ml	85–6180	41
Progesterone	8 ng/dl	<0.3–36	42
T3	119 ng/dl	56–223	41
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Testosterone	40 ng/dl	21–99	42
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Why should we avoid animal components?

Fraud

ATLA 42, 207–209, 2014

Comment

"These products may contain added adult bovine serum albumin (BSA) of United States origin, water, and/or cell growth promoting additives. For

In 2011, GE Healthcare (a unit of General Electric Co.) acquired PAA Laboratories, Linz, Austria. In April 2013, GE Healthcare published a product information to customers, stating that batches of fetal bovine serum (FBS) produced at PAA facilities from March 2008 to March 2013 are subject to label non-conformances, i.e. that:

From this, it can be concluded that the use of serum in cell culture may involve a number of disadvantages: a) serum in general is an ill-defined supplement in culture media, with high qualitative and quantitative, geographical and seasonal batch-to-batch variations; b) FBS may contain adverse factors, like endotoxins, mycoplasma, viral contaminants or prion proteins; c) there are animal

Gstraunthaler, et al. ‚Alternatives to laboratory animals 42.3 (2014): 207-209.

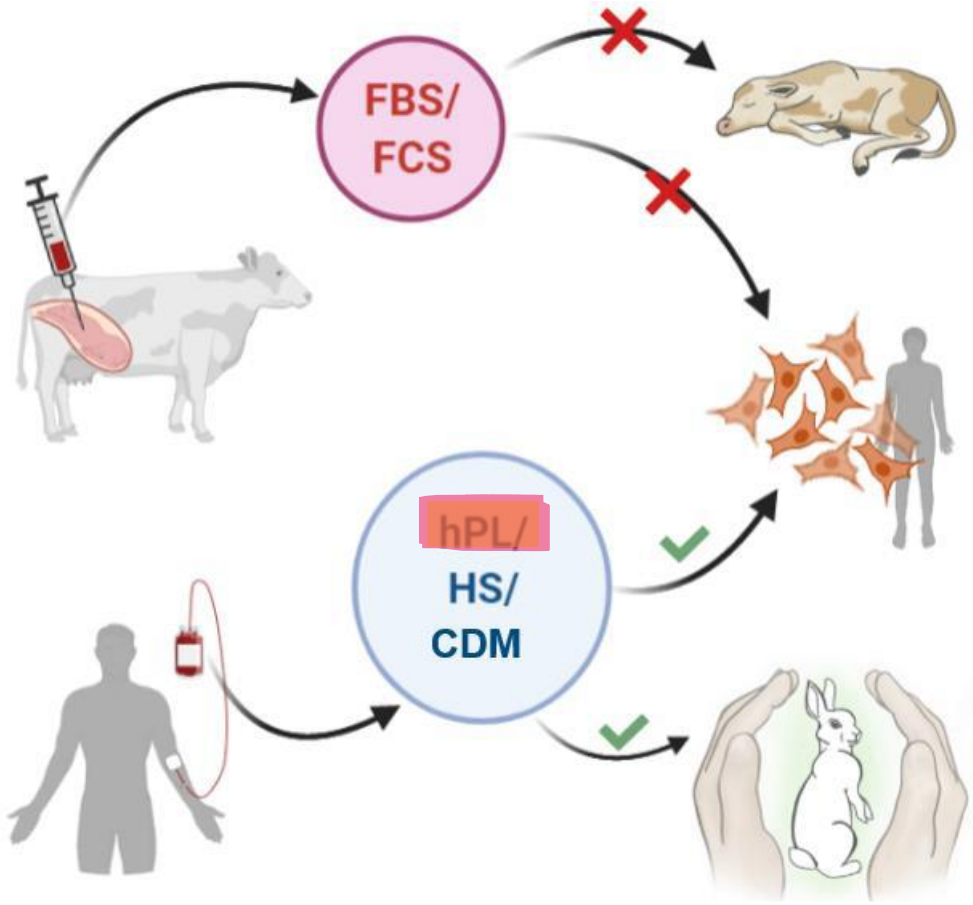
Alternatives to FBS

Human Platelet Lysate

- **hPL** is obtained from donated blood that can no longer be used for medical purposes.

Issues:

- Possible contaminations despite screening of donated blood samples.
- How can we cover higher demand?
- Ethical issues of blood donations.
- Batch-to-batch variations



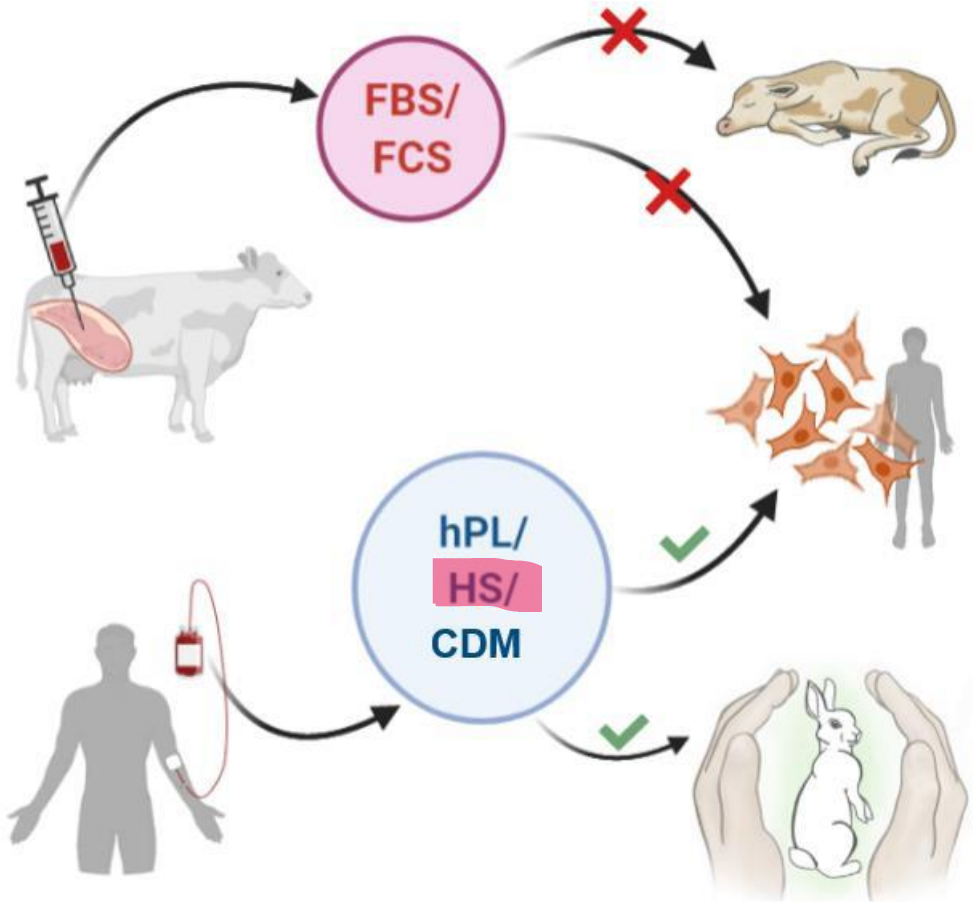
Alternatives to FBS

Human Serum

- **HS** serves as a more physiologically relevant supplement for culturing human cells compared to animal-derived sera such as FBS.

Issues:

- Possible contaminations despite screening of donated blood samples.
- More expensive due to limited supply.
- Ethical issues of blood donations.
- Batch-to-batch variations



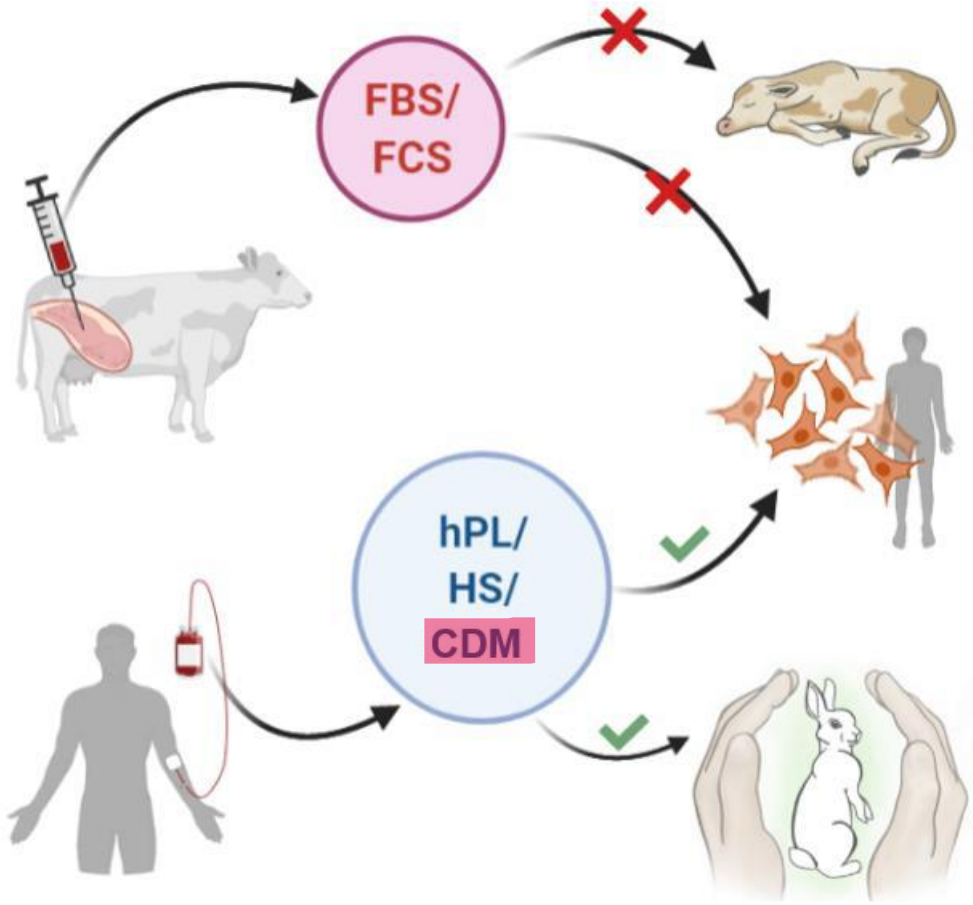
Alternatives to FBS

Chemically defined medium (CDM)

- **CDM** has completely defined composition, allowing for reproducibility and control over experimental conditions.

Components of CDM:

- Amino Acids
- Vitamins
- Inorganic Salts
- Glucose or Alternative Energy Sources
- Lipids
- Trace Elements
- Growth Factors and Hormones



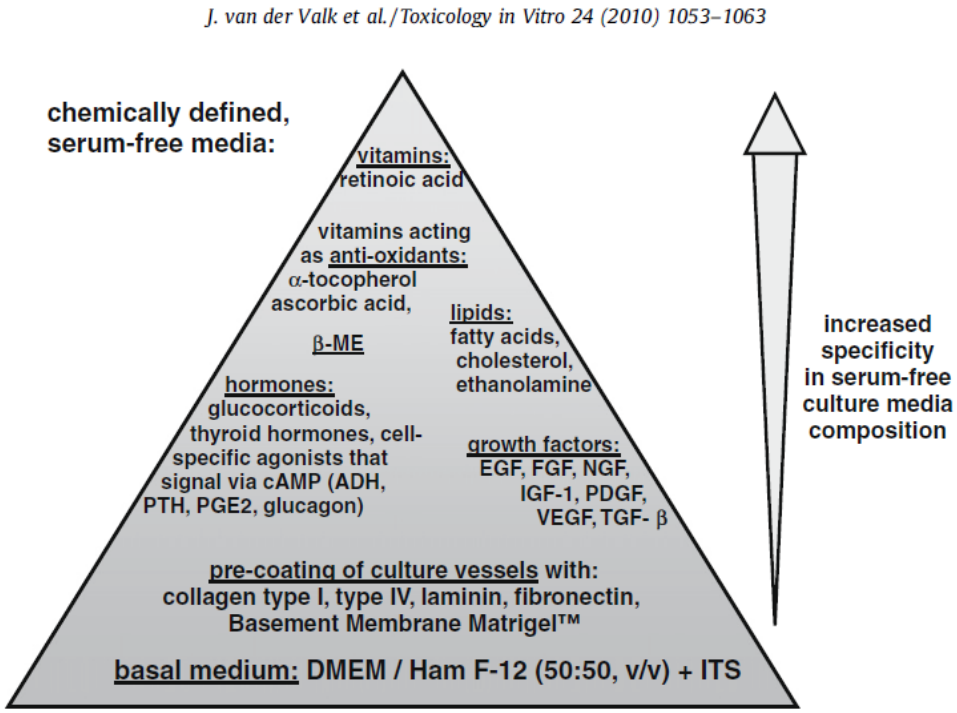
How to adapt cells?

Develop your own serum-free medium

1- Basal medium

- It is recommended to start a new formulation with a 50:50 (v/v) mixture of DMEM and Ham's nutrient mixture F-12

- the basal medium must contain an essential, so called, ITS supplement (insulin, transferrin and selenium).
  - **Insulin**
  - **Transferrin** is also an essential protein in culture medium where the main action is to transfer iron into the cells
  - **Selenium** is an essential trace element and acts in particular in selenoproteins which protect cells against oxidative stress



How to adapt cells?

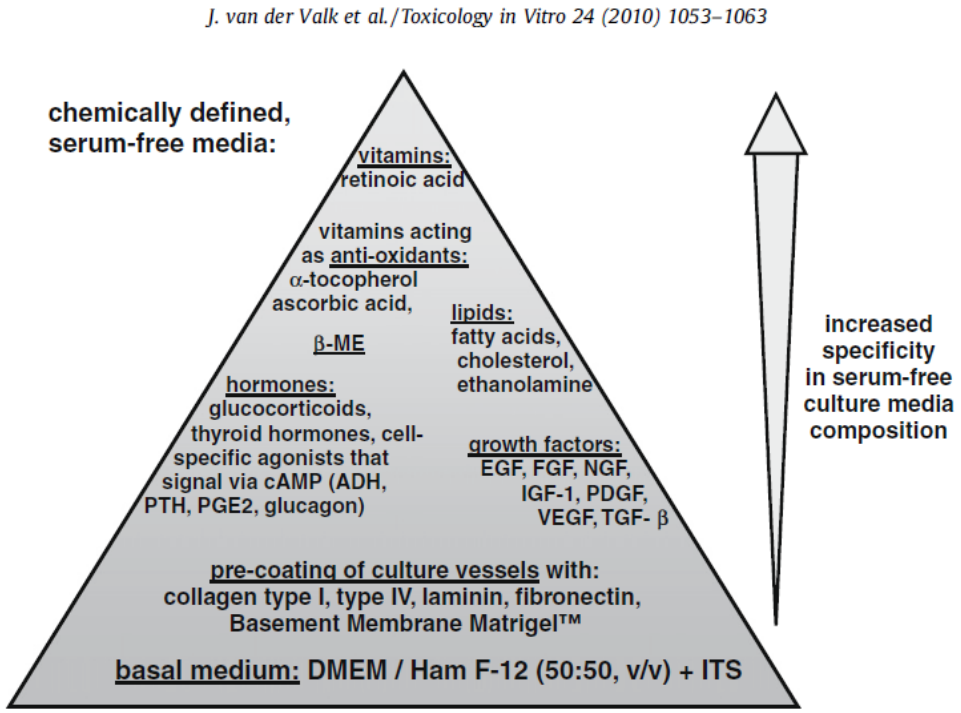
Develop your own serum-free medium

2- Supplements

- **Hormones**
  - Glucocorticoids (dexamethasone and hydrocortisone), triiodothyronine (T3)

- **Growth factors**
  - EGF, HGF, FGF-2,NGF,…etc

- **Protease inhibitors**
  - Protease inhibitors thus have a protective effect on cells, but are not essential. When no protease inhibitors are supplied, one should carefully assess the trypsin concentration.



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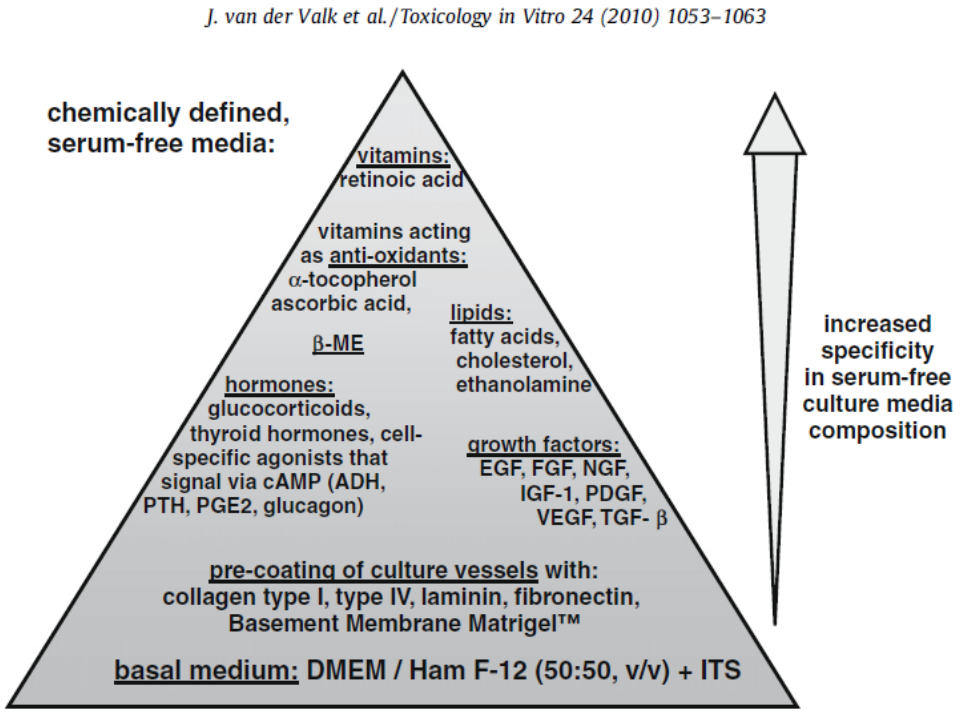
Develop your own serum-free medium

2- Supplements

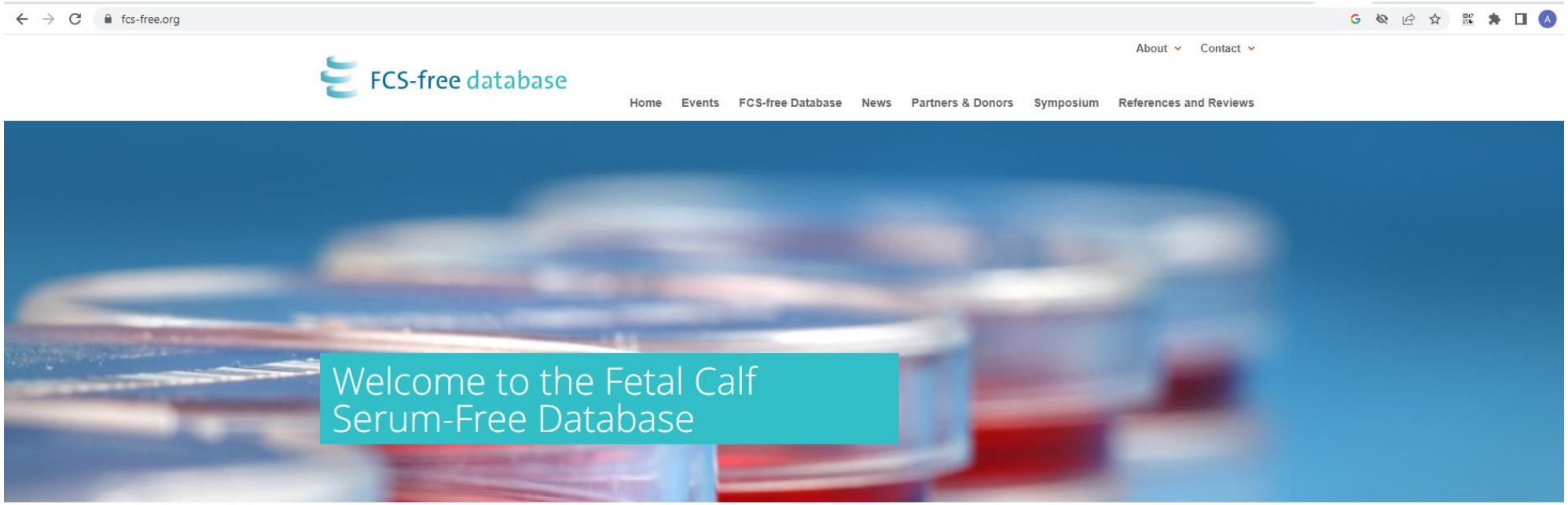
- **Shear force protectors**
  - Turbulence in bioreactors and perfusion cultures cause shear stress in cells
  - **Pluronic F68**

- **Proteins**
  - Proteins are carriers for different low molecular weight components and may facilitate cell adhesion
  - rHSA
- **Vitamins**
- **Amino Acids**
- **Glutamine**
- **Trace elements**
- **Attachment factors**



FCS-free database

FCS-free database https://fcs-free.org/



Universal medium

A new animal product free defined medium for 2D and 3D culturing of normal and cancer cells to study cell proliferation and migration as well as dose response to chemical treatment

Ólöf Birna Rafnsdóttir [a,b], Anna Kiuru [a,c], Mattis Tebäck [a], Nathalie Friberg [a], Philippa Revstedt [a], Johan Zhu [a,d], Sofia Thomasson [a,e], Agnieszka Czopek [a], Atena Malakpour-Permlid [a,f], Tilo Weber [g], Stina Oredsson [a,*]

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[c] Occupational and Environmental Dermatology, Skåne University Hospital, 214 28 Malmö, Sweden
[d] Clinical Microbiology and Infection Prevention and Control, Region Skåne, 221 85 Lund, Sweden
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Preparation of a universally usable, animal product free, defined medium for 2D and 3D culturing of normal and cancer cells☆

Tilo Weber [a], Jeffrey Bajramovic [b], Stina Oredsson [c,*]

[a] Animal Welfare Academy of the German Animal Welfare Federation, Neubiberg 85579, Federal Republic of Germany
[b] 3Rs Centre Utrecht, Utrecht University, Utrecht 3584 CJ, The Netherlands
[c] Department of Biology, Lund University, Lund 22362, Sweden

Table 1
Composition of the defined medium.

Medium component[a]	Concentration in medium
Basal medium: DMEM / Ham's F12	-
Optional extra buffer: HEPES	10 mM
Non-proteins	
All-trans retinoic acid	25 ng/ml
alpha-tocopherol phosphate	3 ng/ml
para-Aminobenzoic acid	12 ng/ml
Ascorbic acid	12 ng/ml
Cholesterol	50 ng/ml
Choline chloride	3.5 μg/ml
Ergocalciferol	25 ng/ml
17-beta Estradiol	0.5 pg/ml
Folic acid	0.33 μg/ml
Glutamine	2 mM
Glutathione	12 ng/ml
Hydrocortisone	0.25 ng/ml
Hypoxanthine Na	1.75 μg/ml
I-inositol	4.5 μg/ml
Linoleic acid	1 μg/ml
Lipoic acid	50 ng/ml
Non-essential amino acids[b]	0.1 mM
O-Phosphoryl ethanolamine	5 μg/ml
Pyruvate Na	1 mM
Ribose	125 ng/ml
Selenous acid	8 ng/ml
Thiamine HCl	80 ng/ml
Triiodothyronine	0.2 pg/ml
Uracil	75 ng/ml
Vitamin B12	0.35 μg/ml
Xanthine	85 ng/ml
Proteins (human)	
Basic fibroblast growth factor	1 ng/ml
Collagen	100 ng/ml
Epidermal growth factor	10 ng/ml
Fetuin	40 ng/ml
Fibronectin	1.33 μg/ml
Insulin	2 μg/ml
Insulin-like growth factor 1	5 ng/ml
Laminin	20 ng/ml
Platelet-derived growth factor AA	2 ng/ml
Transferrin	50 μg/ml
Vitronectin	100 ng/ml
Human serum albumin	1.25 mg/ml

[a] The sources and catalogue numbers are found in Supplemental information Table S1.
[b] L-Glycine, L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, L-proline, and L-serine.

Aim of work

➢ 3D Bioprinting of Humanized Xeno-free Liver Models for toxicity evaluation

Challenges:

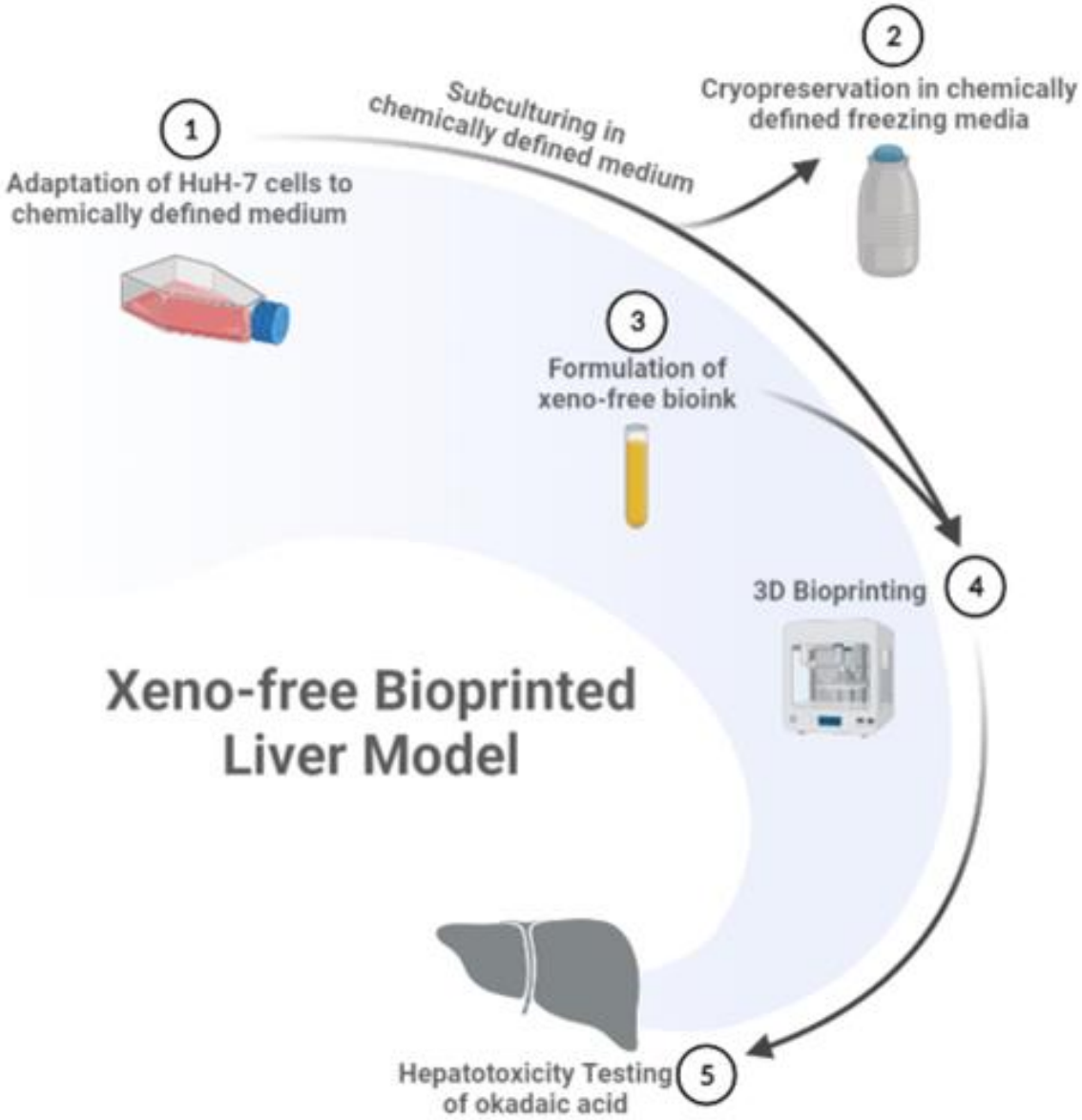
Chemically defined medium

- The limited availability of commercial CDM
- Most of reported literature use animal-derived products in cell culture e.g. trypsin, freezing medium,. etc

Animal-free bioink

- Majority of reported literature use animal-derived components in their bioink e.g. gelatine, Matrigel, ECM, …etc
- Other non-animal alternatives do not have a binding domain for cell attachment e.g. alginate, chitosan, gellan gum, agarose, …etc
- Commercial xeno-free bioink are extremely expensive

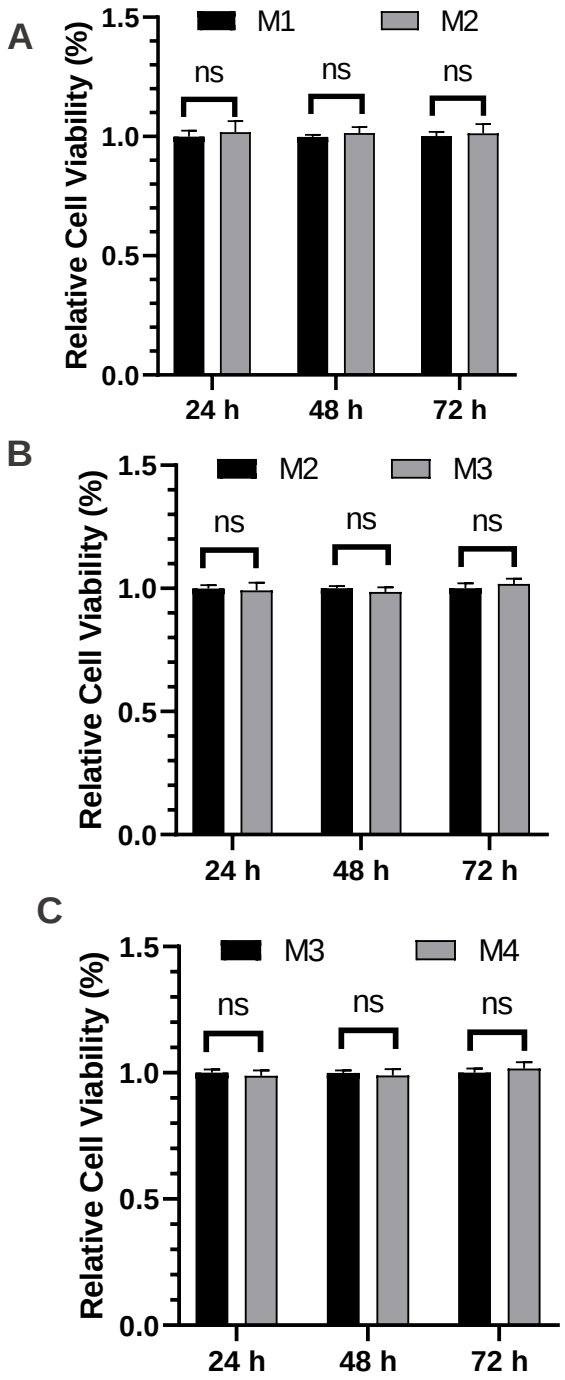
Work plan


1
Adaptation of HuH-7 cells to chemically defined medium
Subculturing in chemically defined medium
2
Cryopreservation in chemically defined freezing media
3
Formulation of xeno-free bioink
4
3D Bioprinting
Xeno-free Bioprinted Liver Model
Hepatotoxicity Testing of okadaic acid
5

Adaptation of HuH-7 to CDM

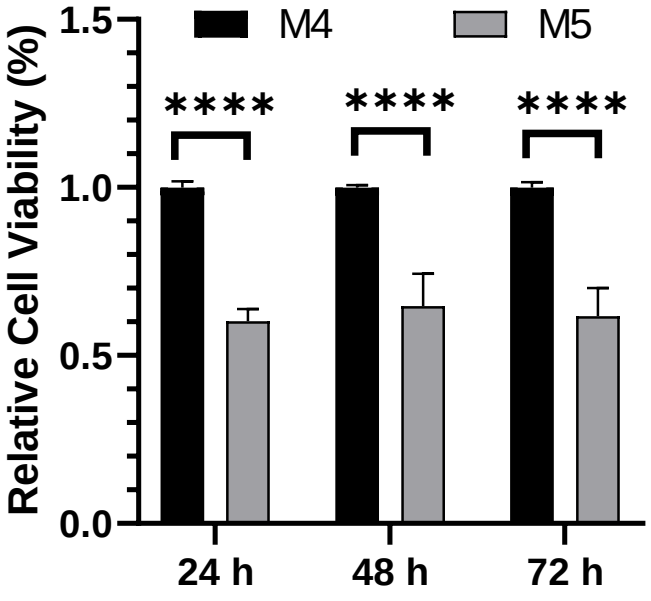
Component	M1	M2	M3	M4	M5	M6
Basal Media	DMEM /Low glucose	DMEM /F-12	DMEM /F-12	DMEM /F-12	DMEM /F-12	DMEM /F-12
L-glutamine	2 mM	2 mM	N/A	N/A	N/A	N/A
HEPES	N/A	10 mM	10 mM	10 mM	10 mM	10 mM
D-(+)-Glucose	4.5 g/l	4.5 g/l	4.5 g/l	4.5 g/l	4.5 g/l	4.5 g/l
Glutamax	N/A	N/A	2 mM	2 mM	2 mM	2 mM
Penicillin/Streptomycin	N/A	N/A	N/A	1X	1X	1X
Fetal Bovine Serum	10%	10%	10%	10%	N/A	N/A
Non-essential amino acids	N/A	N/A	N/A	N/A	1X	1X
Insulin-Transferrin-Selenium	N/A	N/A	N/A	N/A	1X	1X
Hepatocyte Growth Factor	N/A	N/A	N/A	N/A	N/A	10 nM
Epidermal growth factor	N/A	N/A	N/A	N/A	N/A	10 nM

Step 1: Switch from DMEM with FBS to DMEM/F12 with FBS
Step 2: Include GlutaMAX
Step 3: Include Pen/Strep

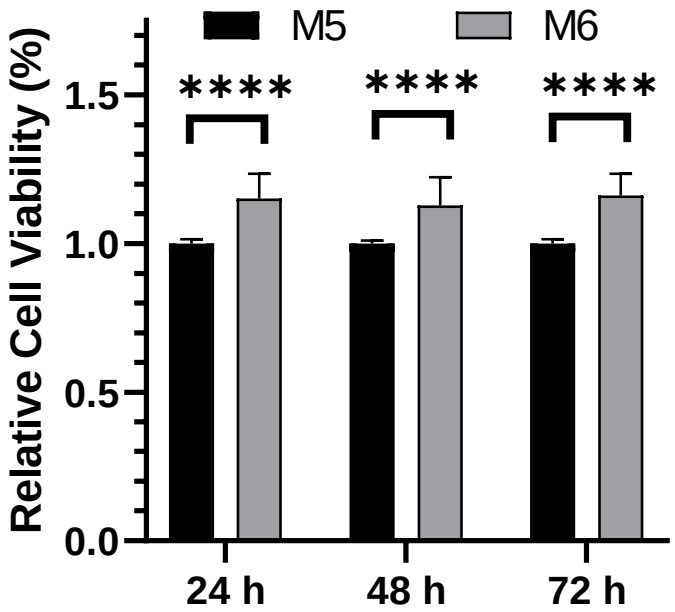


Adaptation of HuH-7 to CDM

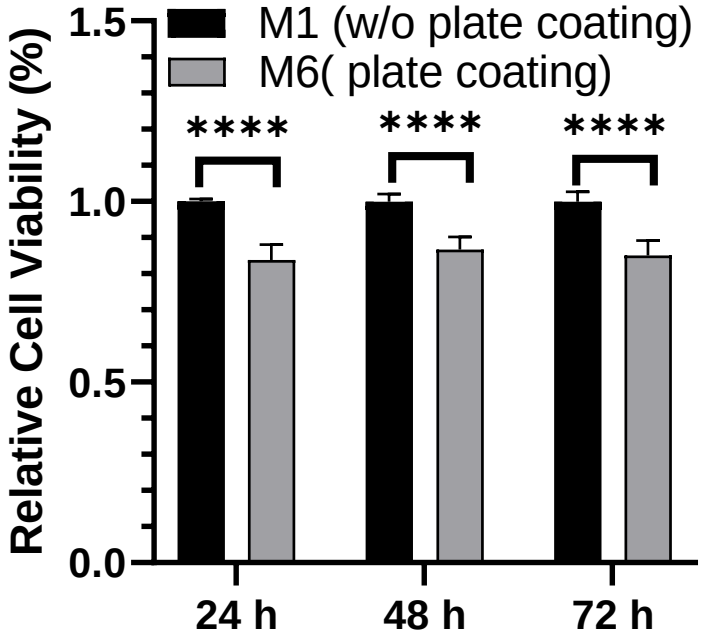
Step 4: Omit FBS



Step 5 : Addition of two growth factors (HGF/EGF)

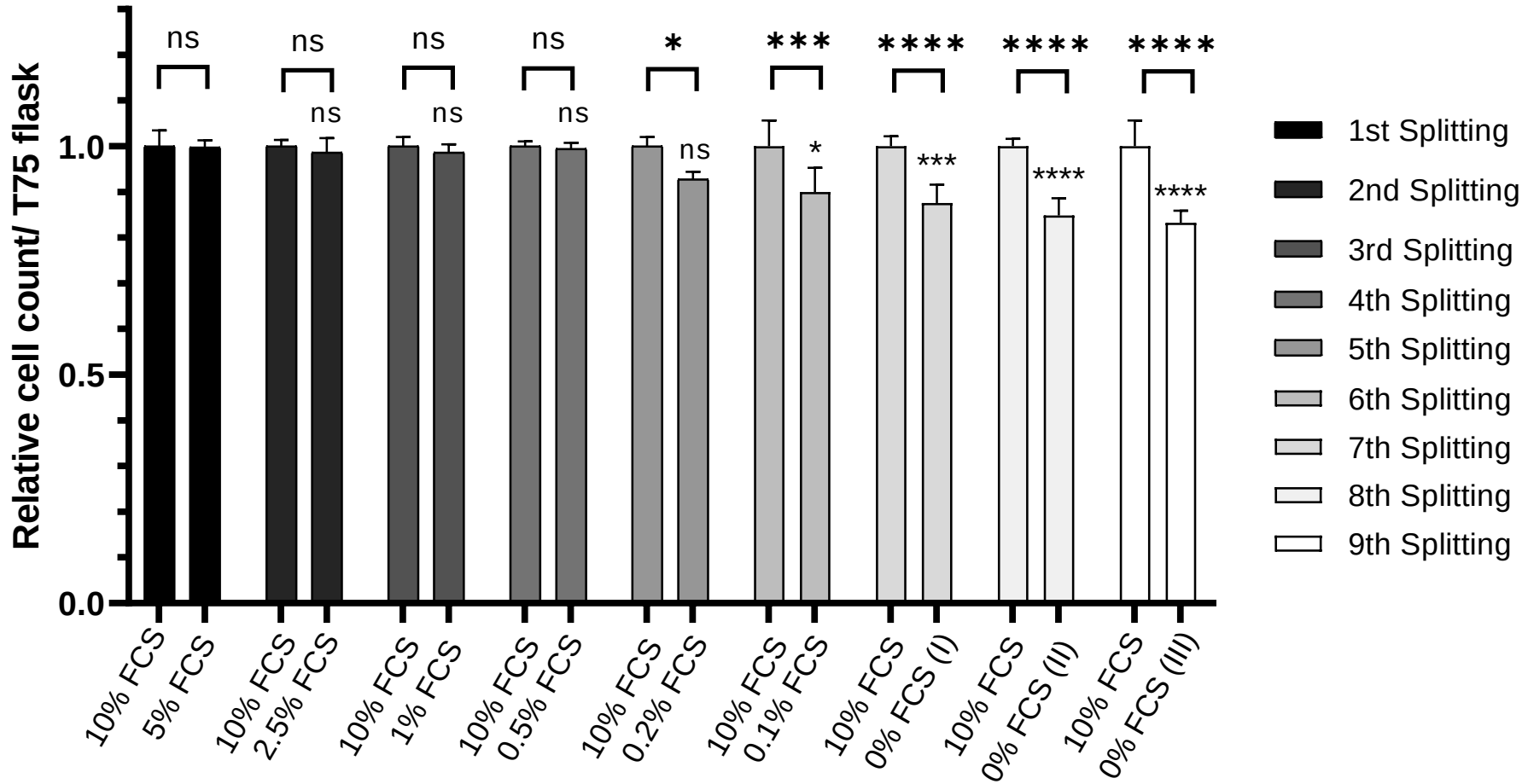


Step 6: Plate coating of the human collagen: 80-95% cell viability



Sequential adaptation

- In an alternative experiment, cells were sequentially adapted to lower FCS concentrations.
- The result was similar (~80% growth rate).



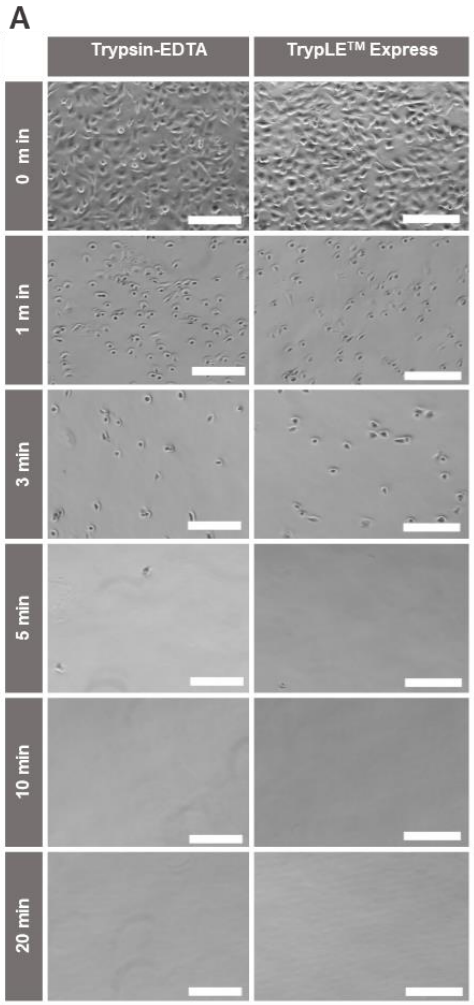
Cell-Detaching

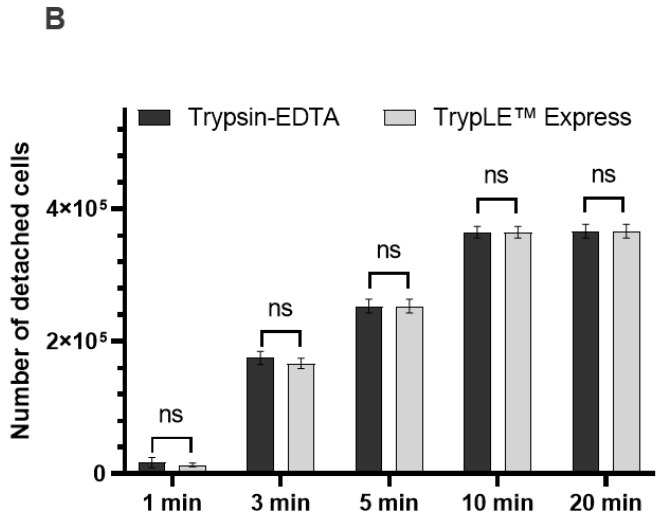
TRYPSIN-EDTA 1X

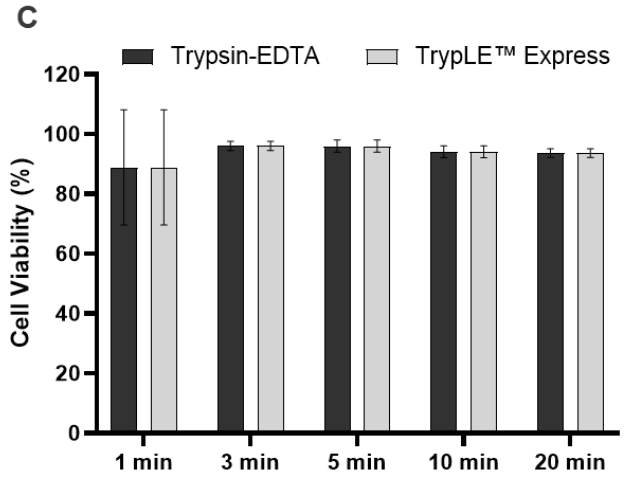
Porcine-derived

TrypLE™ Express

Xeno-free

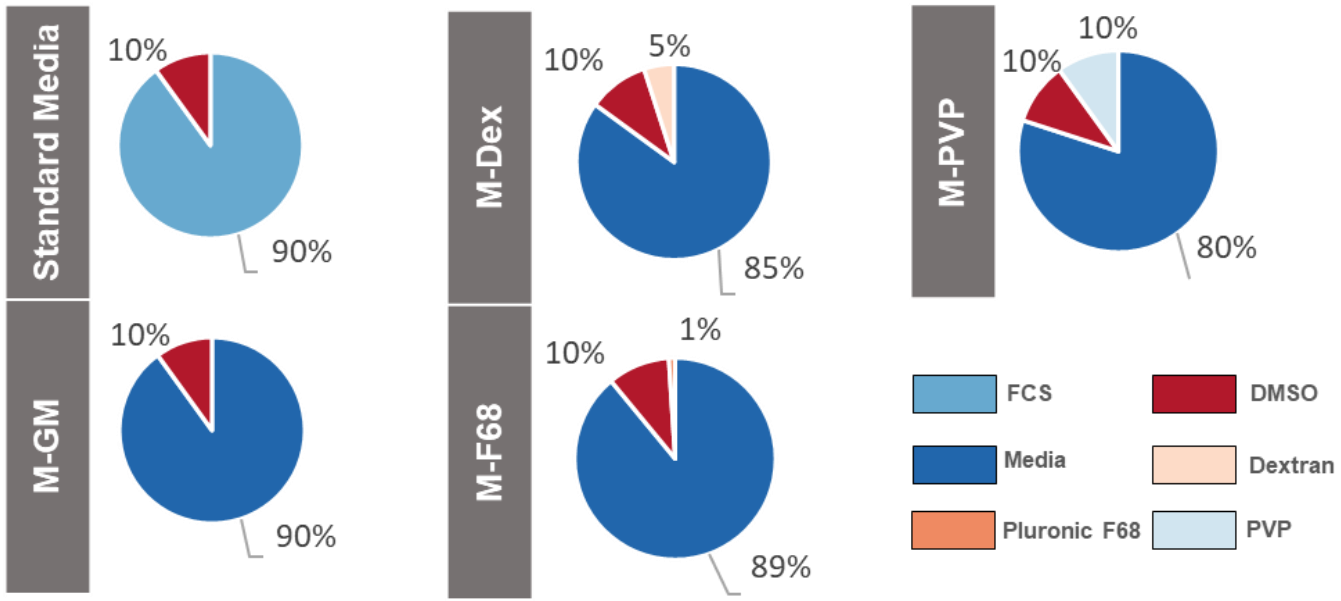


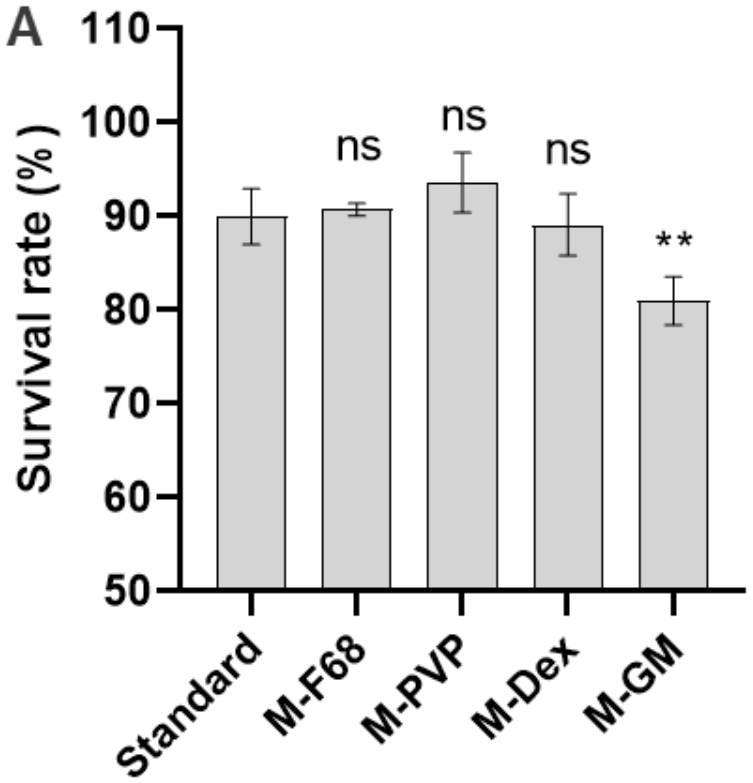




Cryopreservation

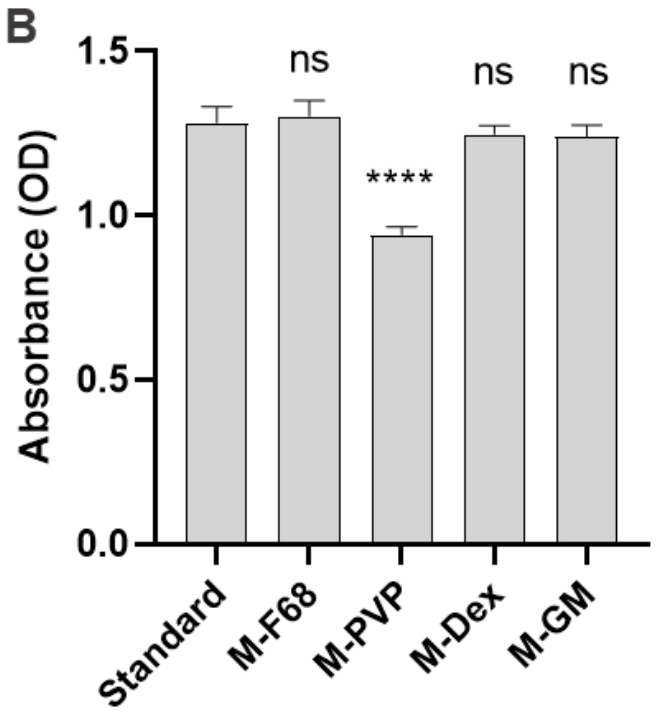
- Cell are normally frozen in 90% FBS and 10% DMSO.
- We tested various alternatives, s. pie chart.
- Medium with dextran and Pluronic F68 performed as good as the standard medium and is a suitable animal-free alternative.



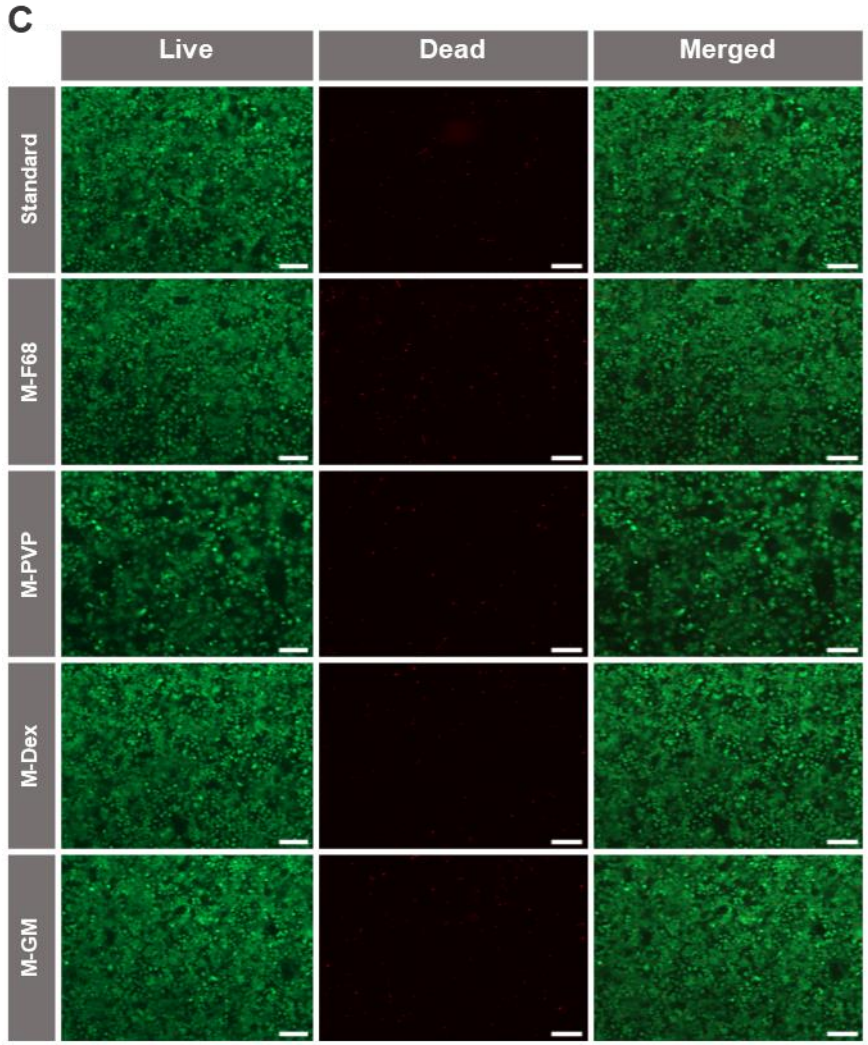


Cryopreservation

- Further characterization after cell seeding



XTT assay



Live/Dead staining

Bioink Formulation

Table S4. Composition of bioinks

Component (each 1 mL)	Xeno-free bioink	Matrigel based bioink
RPMI	200 µL	100 µL
Sod. alginate (10%)	250 µL	250 µL
Human collagen I (3.2 mg/mL)	100 µL	N/A
$CaSO_4$ (1.22 M)	50 µL	50 µL
Matrigel	N/A	200 µL
Supplement mix (I)	N/A	400 µL
Supplement mix (II)	400 µL	N/A

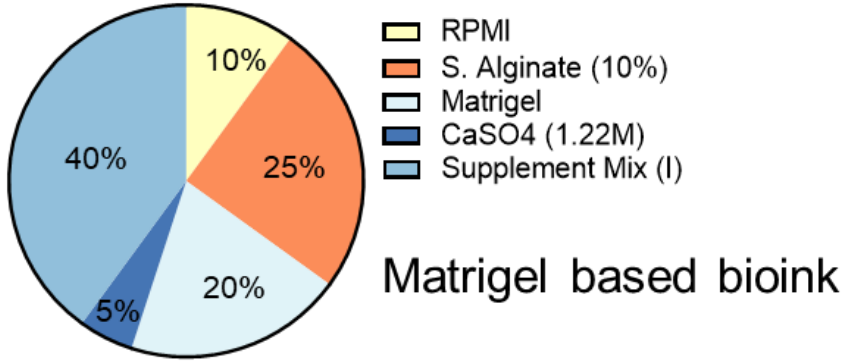
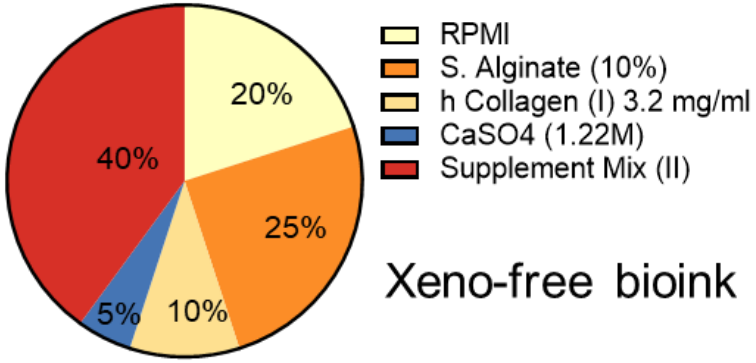
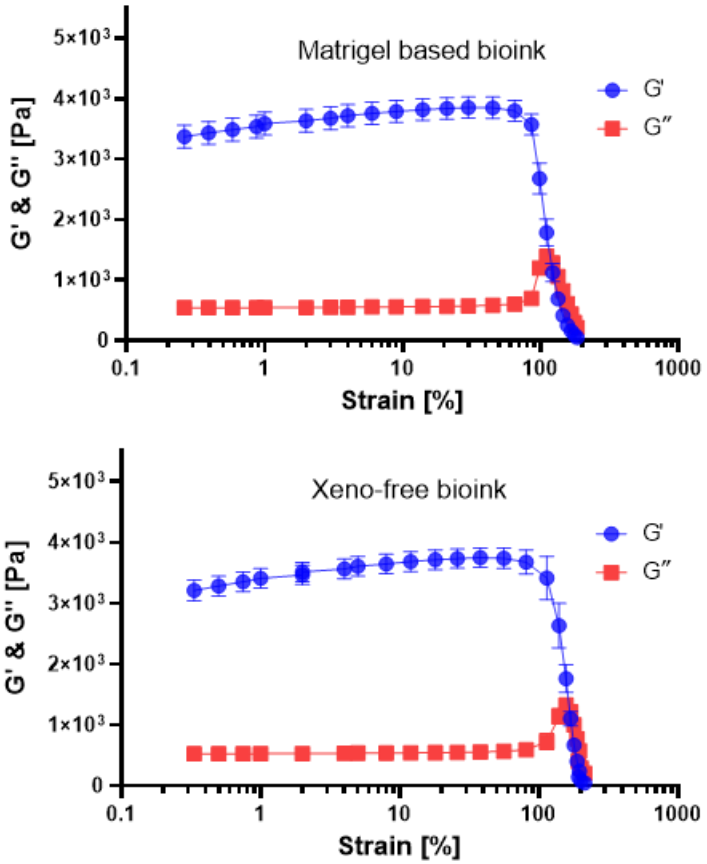


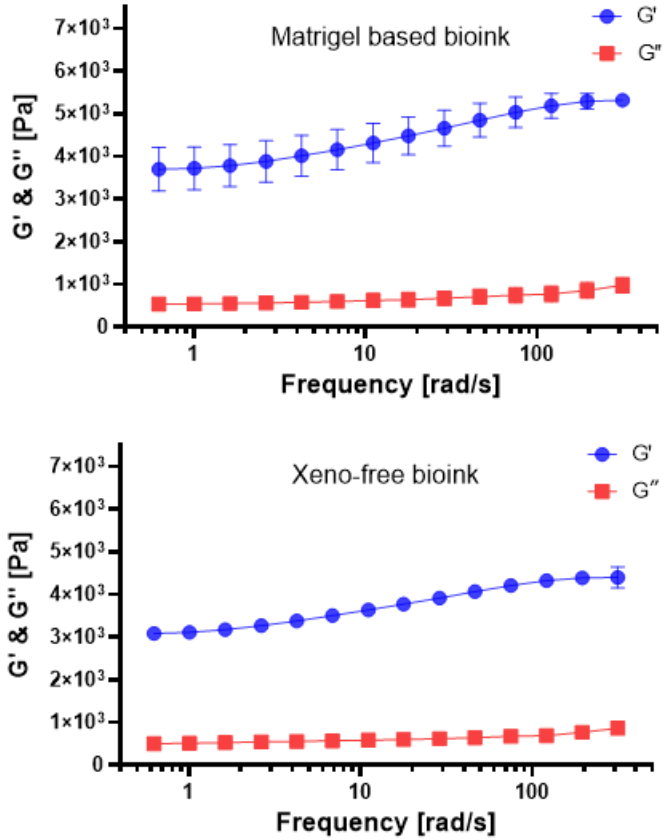
Table S5. Composition of the supplement mixtures

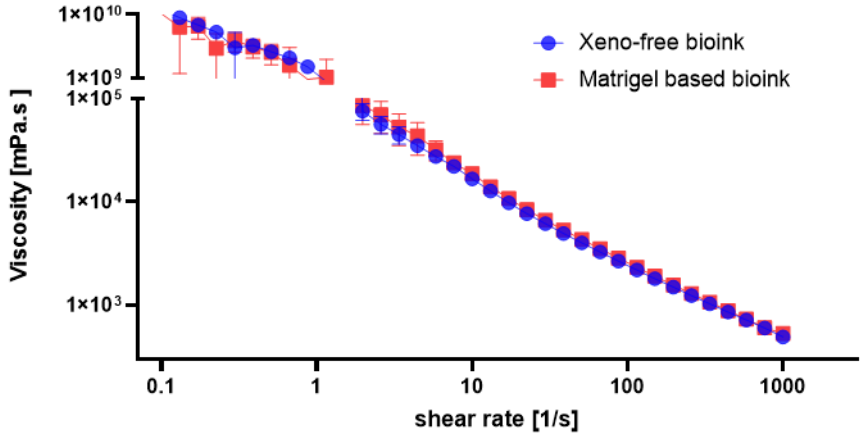
Component (each 1 mL)	Supplement Mix (I) 2,5X	Supplement Mix (II) 2,5X
RPMI	850 µL	600 µL
Nonessential amino acids (NEAA)	25 µL	25 µL
HEPES (1M)	75 µL	75 µL
Penicillin – Streptomycin (10 000 000 U/L)	25 µL	25 µL
GlutaMAX	25 µL	25 µL
Human serum	0	250 µL



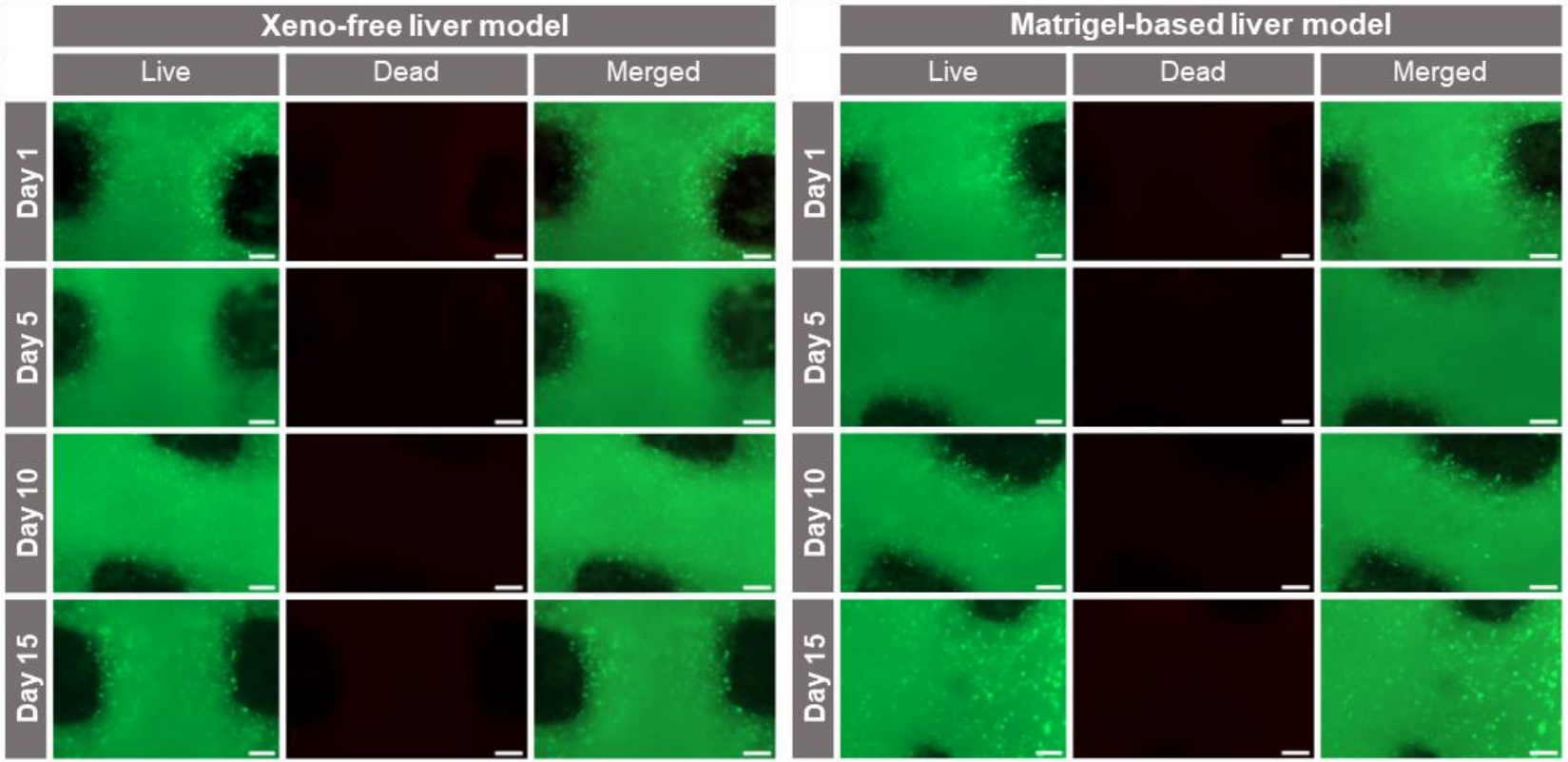
Bioink Formulation

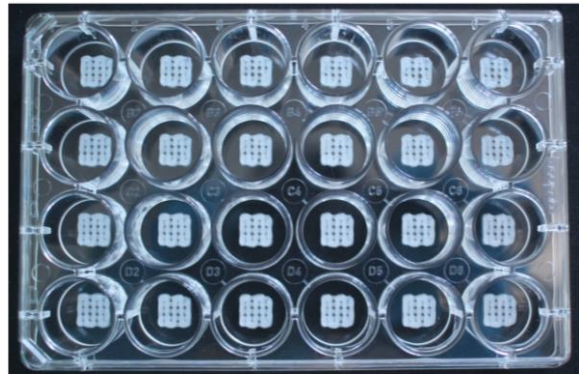


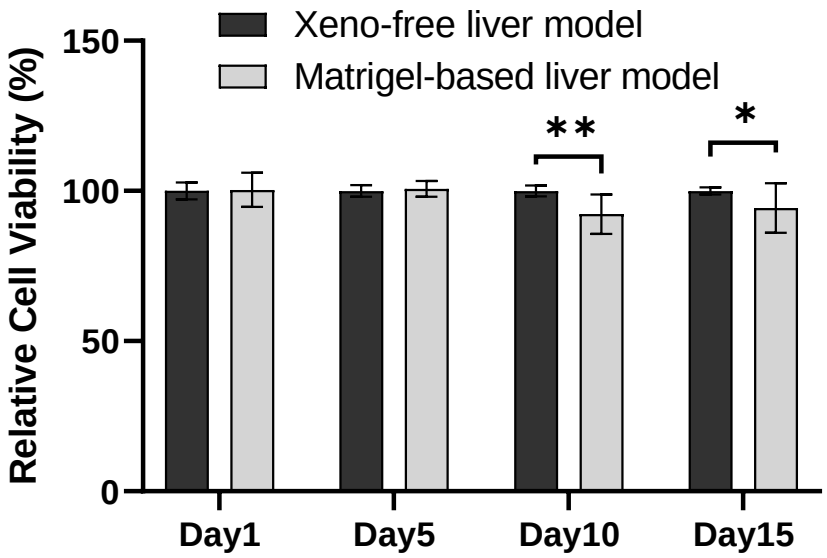




Matrigel-based vs xeno-free model

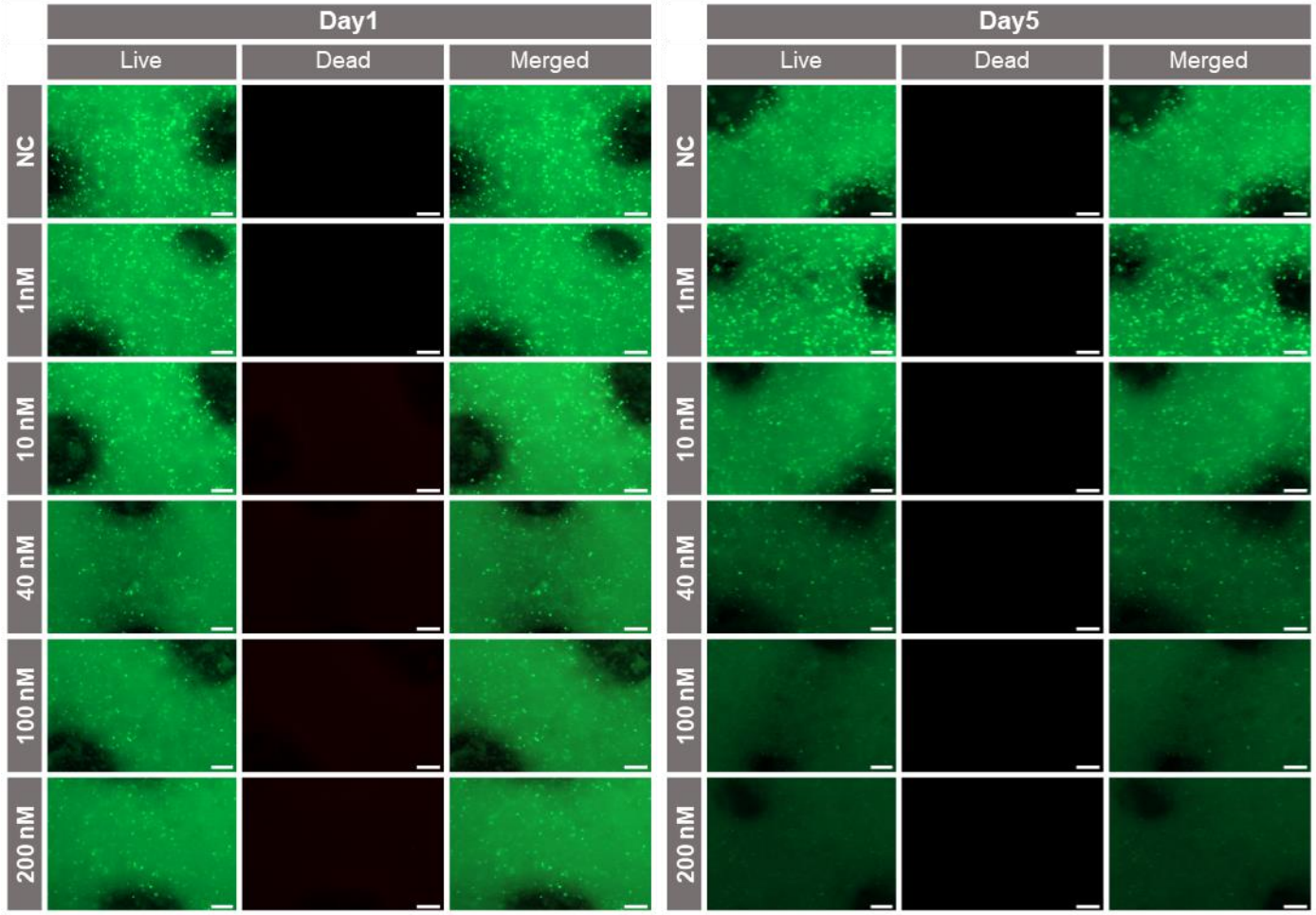


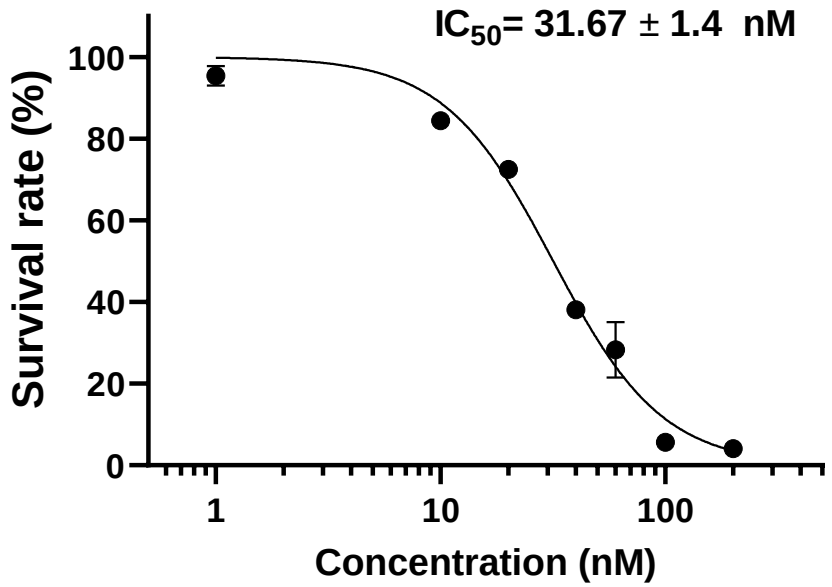




Printability, cell viability (life/dead staining) and metabolic activity (XTT assay) were comparable to that of a Matrigel-based bioink.

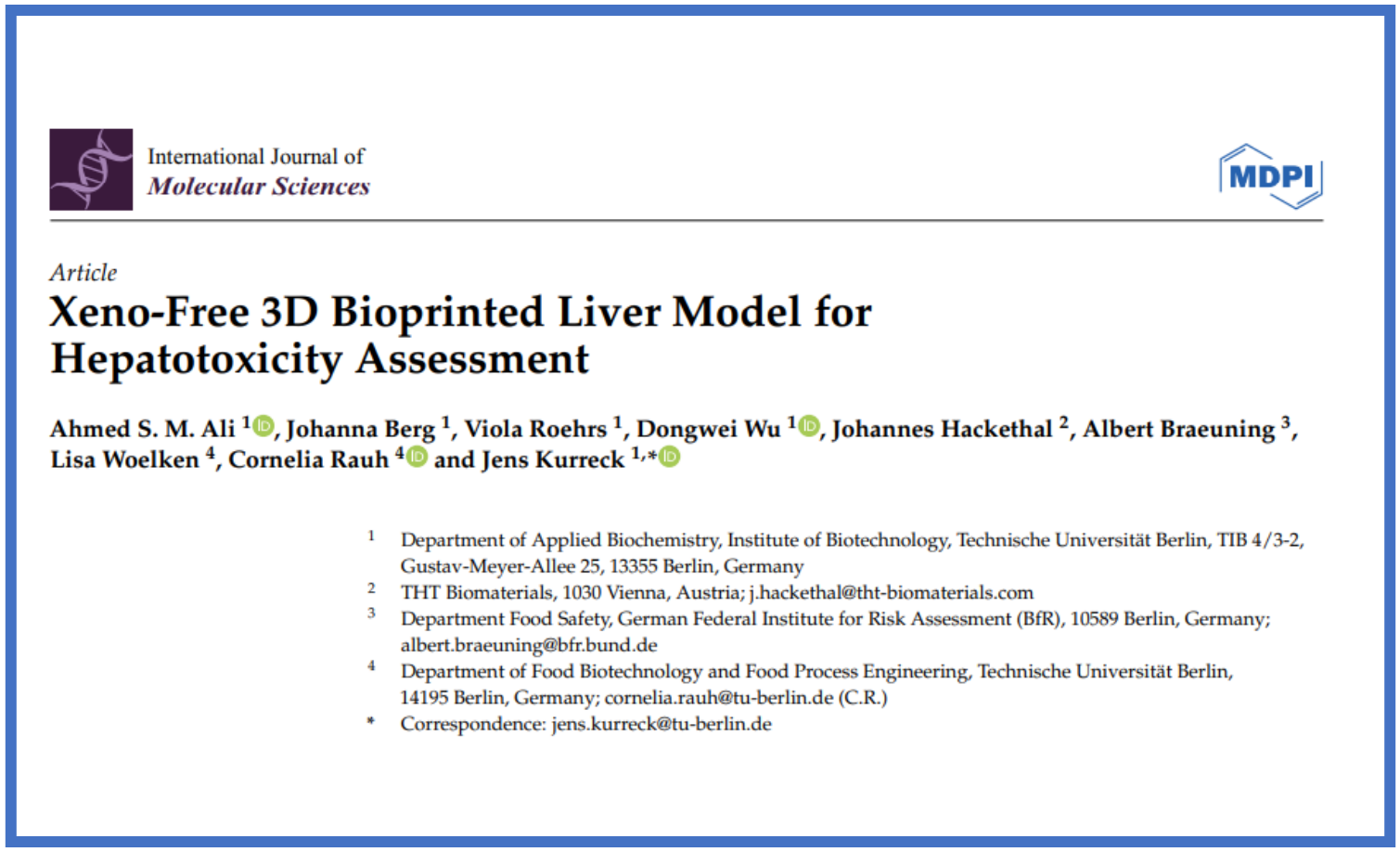
Toxicity testing of Okadaic in 3D





- Toxicity of okadaic acid was investigated in bioprinted xeno-free liver model.
- A concentration-dependency was observed.

Publication and knowledge transfer



International Journal of
Molecular Sciences

MDPI

Article

Xeno-Free 3D Bioprinted Liver Model for Hepatotoxicity Assessment

Ahmed S. M. Ali [1], Johanna Berg [1], Viola Roehrs [1], Dongwei Wu [1], Johannes Hackethal [2], Albert Braeuning [3], Lisa Woelken [4], Cornelia Rauh [4] and Jens Kurreck [1,*]

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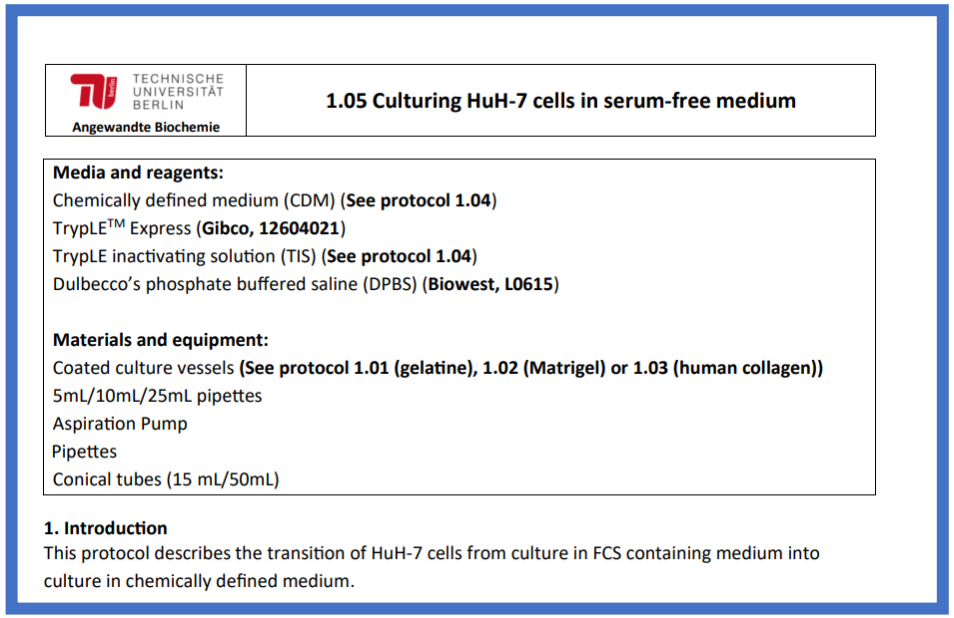
Open Access publication



TECHNISCHE UNIVERSITÄT BERLIN
Angewandte Biochemie

1.05 Culturing HuH-7 cells in serum-free medium

Media and reagents:
Chemically defined medium (CDM) (**See protocol 1.04**)
TrypLE™ Express (**Gibco, 12604021**)
TrypLE inactivating solution (TIS) (**See protocol 1.04**)
Dulbecco's phosphate buffered saline (DPBS) (**Biowest, L0615**)

Materials and equipment:
Coated culture vessels **(See protocol 1.01 (gelatine), 1.02 (Matrigel) or 1.03 (human collagen))**
5mL/10mL/25mL pipettes
Aspiration Pump
Pipettes
Conical tubes (15 mL/50mL)

1. Introduction
This protocol describes the transition of HuH-7 cells from culture in FCS containing medium into culture in chemically defined medium.

Detailed protocol

Highlights

- Simple xeno-free 3D bioprinted liver model
- HuH-7 cells adapted to chemically defined media
- Chemically defined freezing medium was formulated
- Xeno-free bioink was formulated
- Monoculture system, HuH-7 cells
- It is based on Not the best option for high throughput

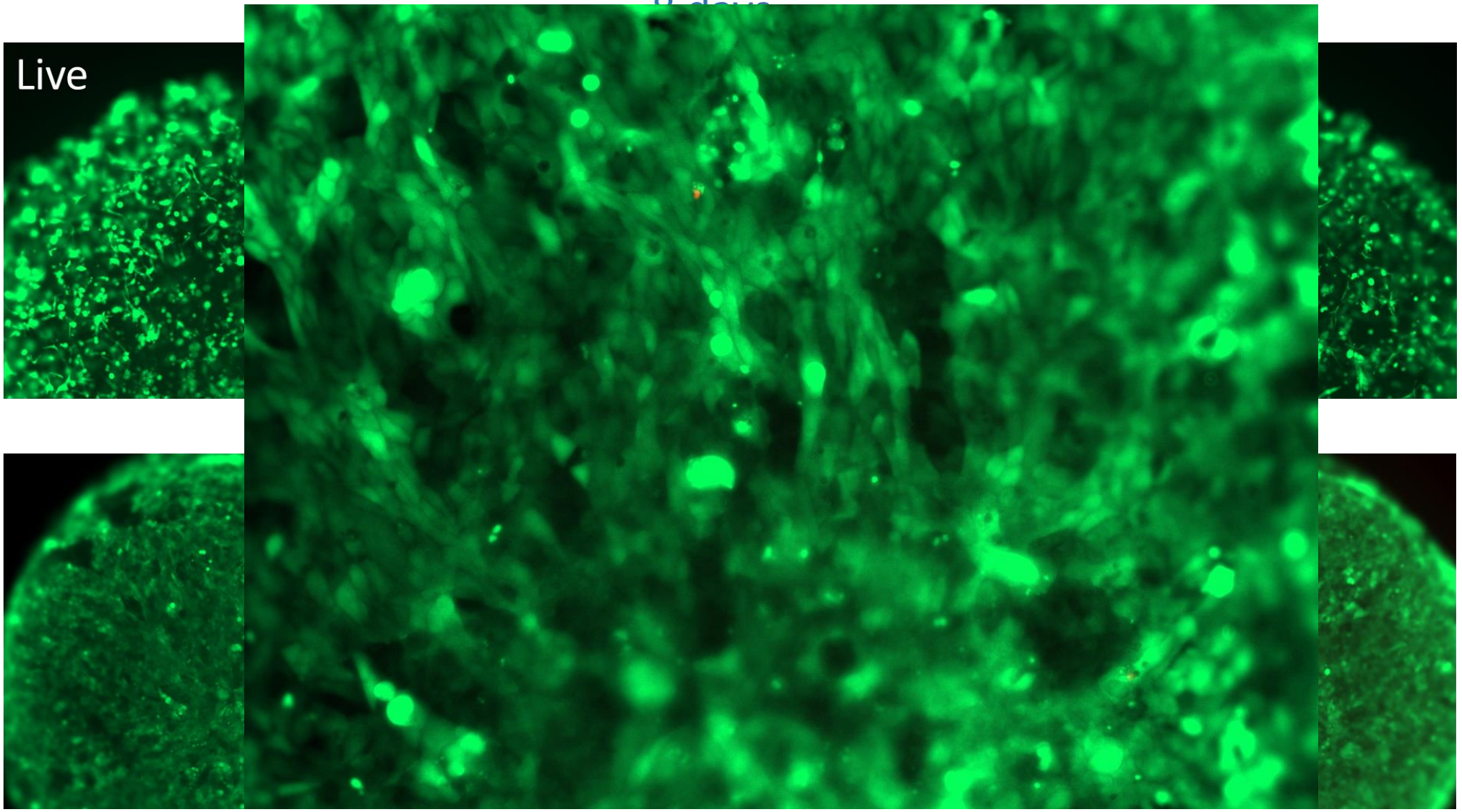
High throughput system

Assessing the biocompatibility

Cells: HepaRG

72 hours

High throughput system



High throughput system

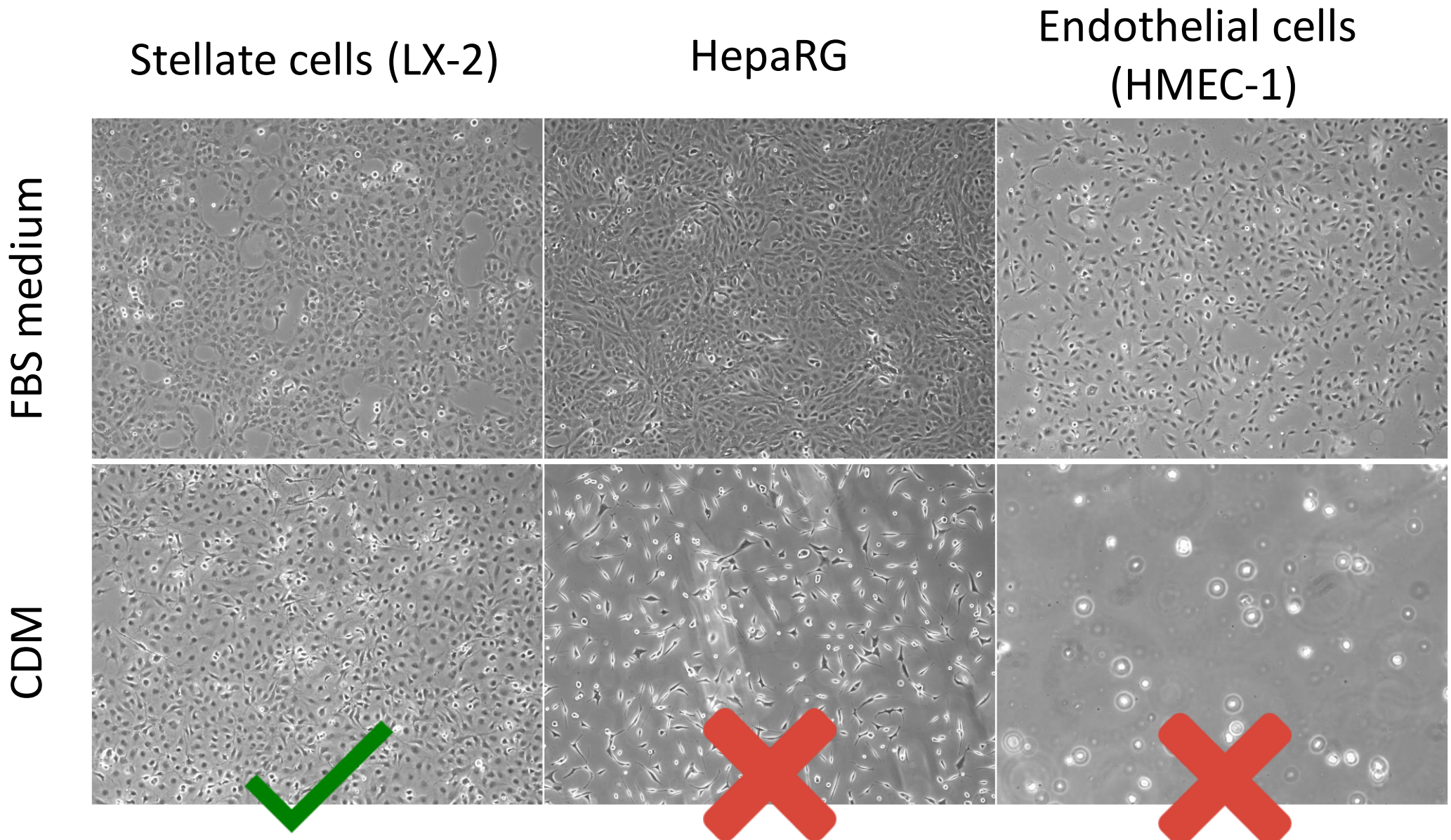
Xeno-free multicellular liver model?

HepaRG

LX-2 stellate cells

HMEC-1

CDM for multiple cell lines

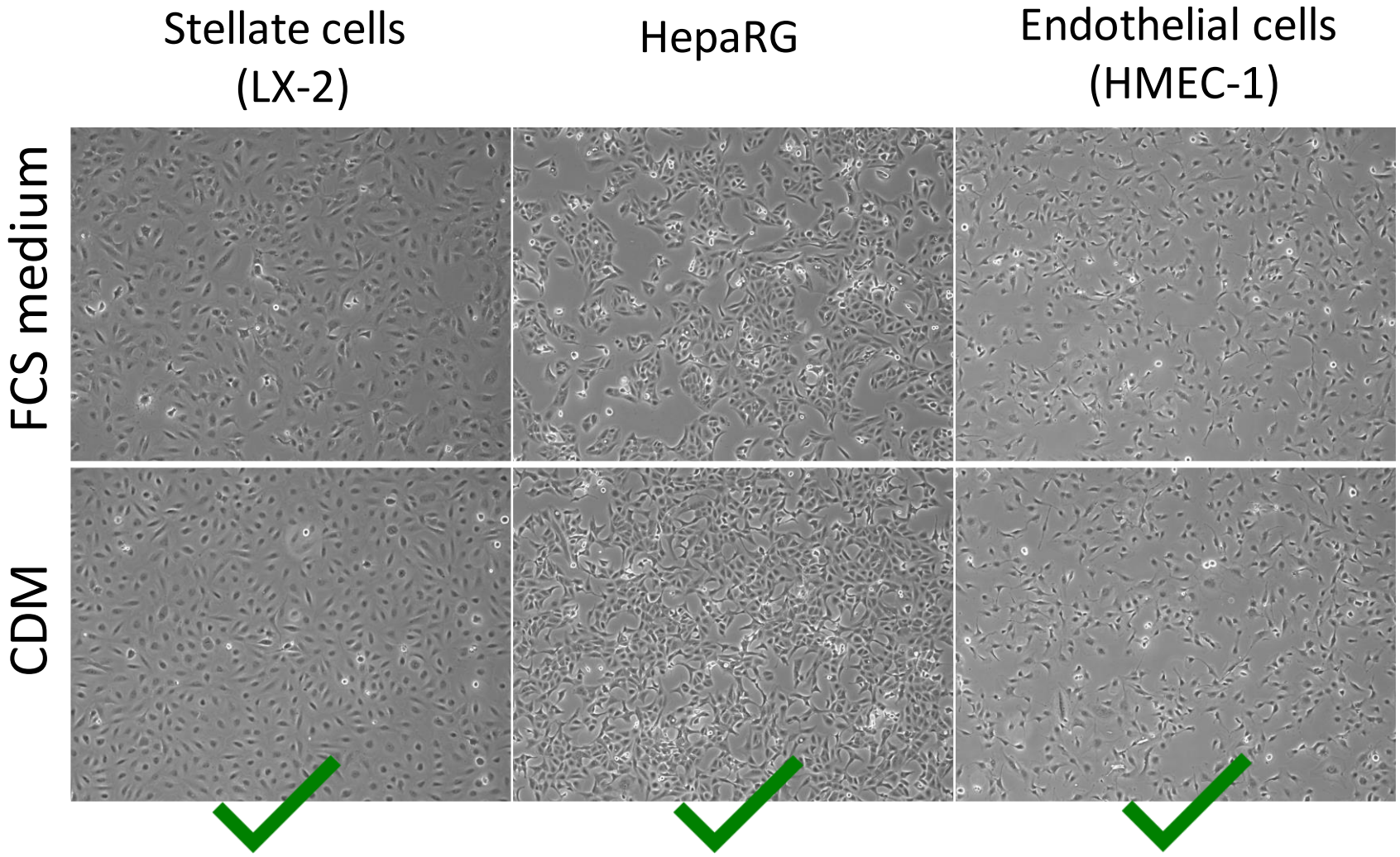


CDM for multiple cell lines

CDM Ver 2.3

- Coated plated vs non-coated plates ?
- Different basal media?
- Different FCS replacement?
- Different attachment factors?
- Growth factors?
- Hormones?
- Fatty acids?
- Carrier proteins?

CDM Ver 4.6



Outlook

- Characterize the morphology, proliferation and functionality of cells with CDM
- Test the xeno-free bioink for co-culture
- Evaluating the efficacy of the model for predicting the hepatotoxicity

рахмат
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謝謝
ngiyabonga
xiexie
spas
teşekkür ederim
Баярлалаа
mersi
kia ora
barka
welalin
tack
mahalo
tapadh leat
спасибо
faafetai lava
vinaka
blagodaram
dank je
misaotra
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grazzi
matondo
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dankie
thank you
gracias
хвала
nanni
nandri
dhanyavad
mauruuru
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akun
asante
manana
obrigada
tenki
bedankt
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감사합니다
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merci