Università di Pisa | Università degli Studi di Siena | Università degli Studi di Perugia | Università degli Studi di Camerino | CNR Area della Ricerca di Pisa

Corso di formazione in materia di protezione degli animali utilizzati a fini scientifici

Accreditato dal Ministero della Salute secondo D.M. 5 agosto 2021 e D.D. 18 Marzo 2022

16 giugno 2023

Modulo 10-10bis

Etica, benessere degli animali e Tre R (livello 2)

Centro 3R

Docente: Arti Ahluwalia. Universita' di Pisa e Centro 3R

Replacement.

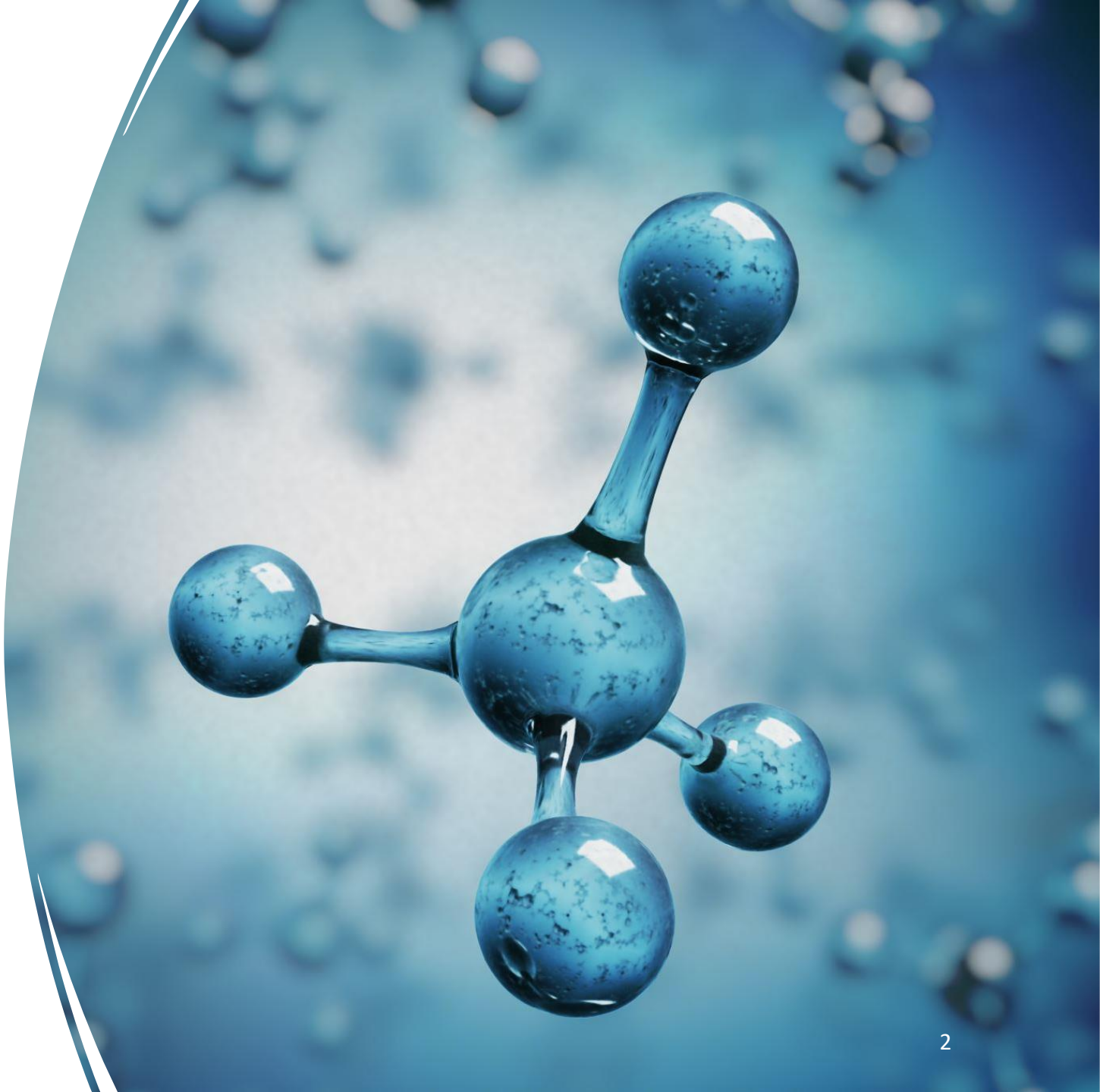
Methods which permit a given purpose to be achieved without conducting experiments or other scientific procedures on animals

In chemico

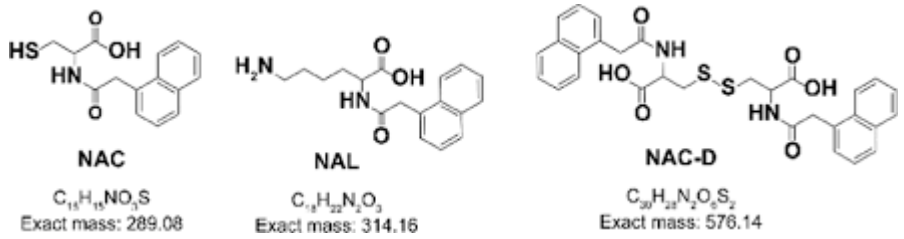
In vitro

In silico

Integrated



What is *in chemico?*



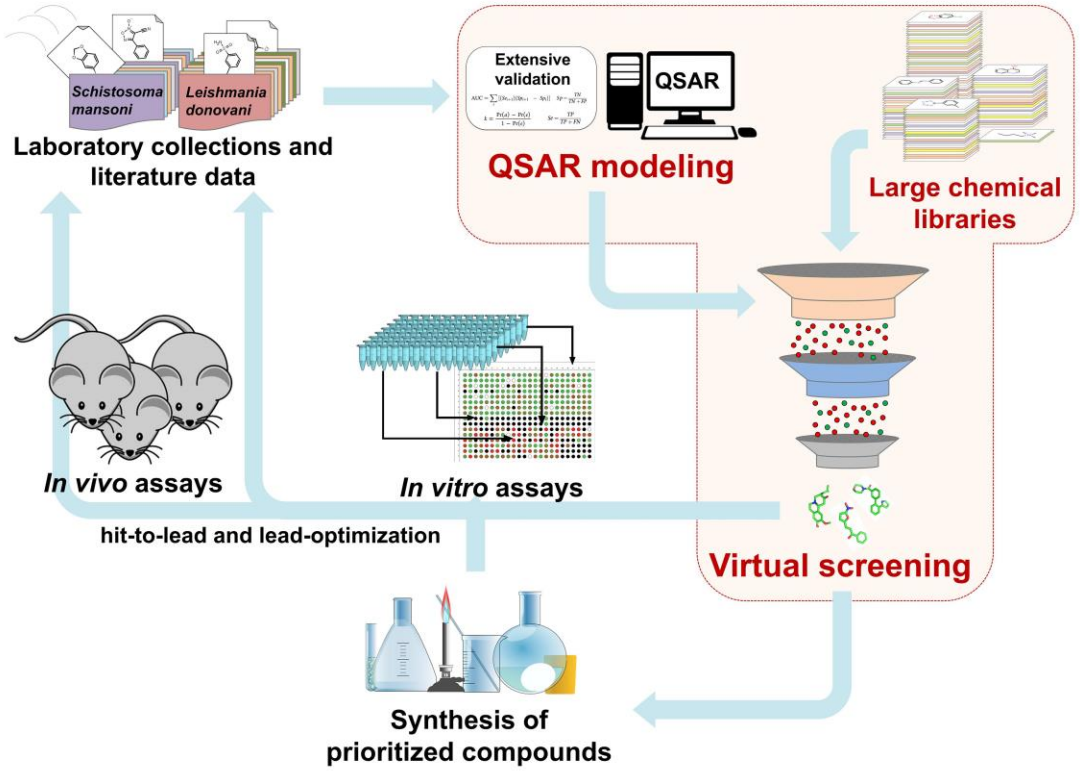
- Chemical reactivity
- Reagents
- Direct peptide reactivity Assay
-

KEYWORDS: chemicals, proteins, peptides

What is *in silico?*

- Numbers
- Data
- Computers
- Equations

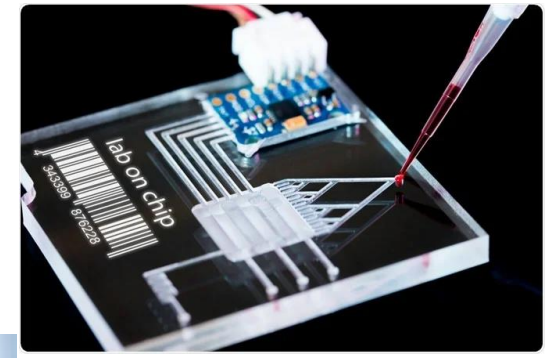
KEYWORD: QUANTITATIVE



What is *in vitro?*

- Organelles (eg mitochondria)
- Cells
- Tissues & organoids
- Whole organs
- From animals or humans

KEYWORDS: in the lab, not whole organisms



Replacement

- Most progress in regulatory science and testing
- EPA (US environmental protection agency) has approved several Non Animal tests
- FDA recently passed legislation (FDA modernization act Dec 2022)

Regulatory testing

- OECD guidelines on how to conduct tests on chemicals
- Reach (EC) No 1907/2006 = Registration, Evaluation, Authorisation and Restriction of Chemicals
- EMA, FDA (pharmacological products and ATMP)
- Cosmetics

- Most progress in regulatory science and testing
- EPA (US environmental protection agency) has approved several Non Animal tests
- FDA recently passed legislation (FDA modernization act Dec 2022)

For a complete list of OECD Test guidelines for Health effects visit https://www.oecd-ilibrary.org/environme for-the-testing-of-chemicals-section-4-health-effects_20745788/datedesc#collectionsort.

Alternative non-animal test guidelines

Test No. 428: Skin Absorption: In Vitro Methods

Test No. 430: In Vitro Skin Corrosion: Transcutaneous Electrical Resistance Test Method (TER)

Test No. 431: In Vitro Skin Corrosion: Reconstructed Human Epidermis (RHE) Test Method

Test No. 432: In Vitro 3T3 NRU Phototoxicity Test

Test No. 435: In Vitro Membrane Barrier Test Method for Skin Corrosion

Test No. 439: In Vitro Skin Irritation: Reconstructed Human Epidermis Test Method

Test No. 442C: In Chemico Skin Sensitization

Test No. 455: Performance-Based Test Guideline for Stably Transfected Transactivation In Vitro Assays to Receptor Agonists and Antagonists; accompanying software is also available

Test No. 467: Defined Approaches for Serious Eye Damage and Eye Irritation

Test No. 473: In Vitro Mammalian Chromosomal Aberration Test

Test No. 476: In Vitro Mammalian Cell Gene Mutation Tests Using the Hprt and xprt Genes

Test No. 479: Genetic Toxicology: In Vitro Sister Chromatid Exchange Assay in Mammalian Cells

Test No. 482: Genetic Toxicology: DNA Damage and Repair, Unscheduled DNA Synthesis in Mammalian

Test No. 487: In Vitro Mammalian Cell Micronucleus Test

Test No. 490: In Vitro Mammalian Cell Gene Mutation Test Using the Thymidine Kinase Gene

Test No. 492B: Reconstructed Human Cornea-like Epithelium (RHCE) Test Method for Eye Hazard Identif

Test No. 493: Performance-Based Test Guideline for Human Recombinant Estrogen Receptor (rrER)In Vit Chemicals with ER Binding Affinity

Test No. 497: Defined Approaches on Skin Sensitisation

REACH is a regulation of the European Union, adopted to improve the protection of human health and the environment from the risks that can be posed by chemicals, while enhancing the competitiveness of the EU chemicals industry. It also promotes alternative methods for the hazard assessment of substances in order to reduce the number of tests on animals.

In principle, REACH applies to all chemical substances; not only those used in industrial processes but also in our day-to-day lives, for example in cleaning products, paints as well as in articles such as clothes, furniture and electrical appliances. Therefore, the regulation has an impact on most companies across the EU.

REACH places the burden of proof on companies. To comply with the regulation, companies must identify and manage the risks linked to the substances they manufacture and market in the EU. They have to demonstrate to ECHA how the substance can be safely used, and they must communicate the risk management measures to the users.

If the risks cannot be managed, authorities can restrict the use of substances in different ways. In the long run, the most hazardous substances should be substituted with less dangerous ones.

REACH stands for Registration, Evaluation, Authorisation and Restriction of Chemicals. It entered into force on 1 June 2007.

How does REACH work?

REACH establishes procedures for collecting and assessing information on the properties and hazards of substances.

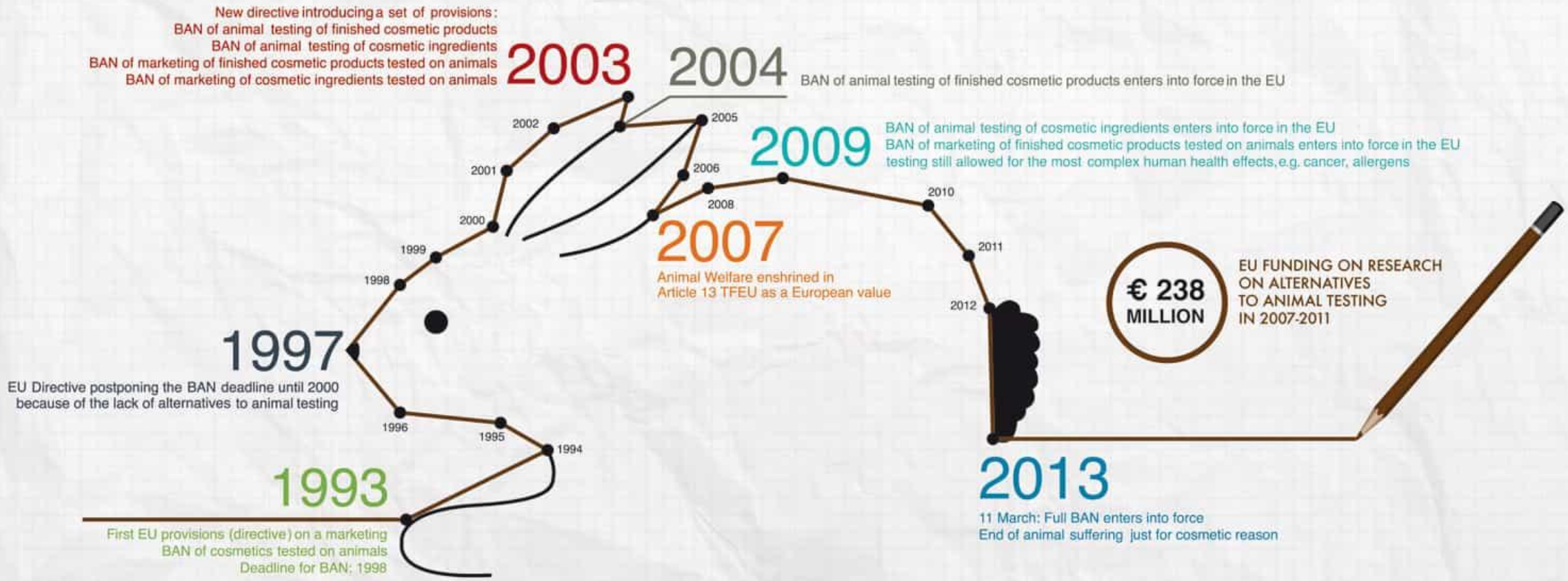
Companies need to register their substances and to do this they need to work together with other companies who are registering the same substance.

ECHA receives and evaluates individual registrations for their compliance, and the EU Member States evaluate selected substances to clarify initial concerns for human health or for the environment. Authorities and ECHA's scientific committees assess whether the risks of substances can be managed.

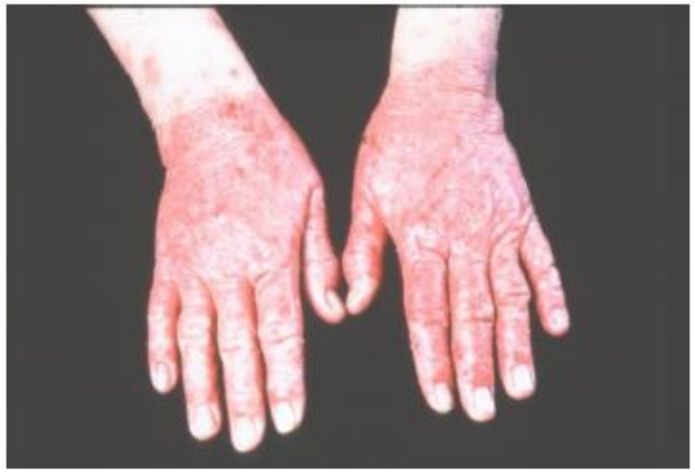
Authorities can ban hazardous substances if their risks are unmanageable. They can also decide to restrict a use or make it subject to a prior authorisation.

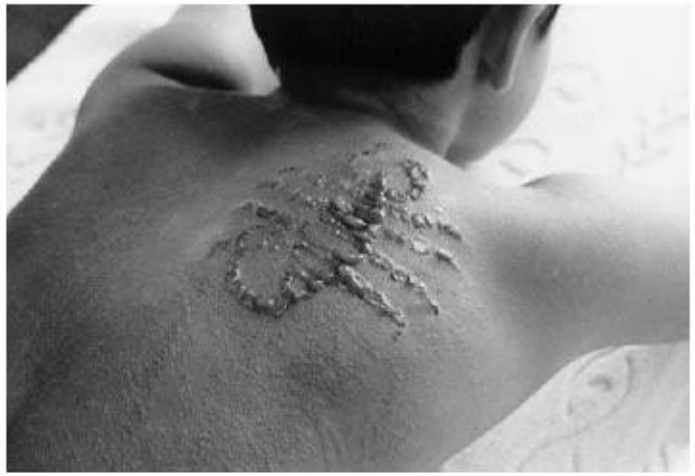
CONNECTING THE DOTS FOR ANIMALS:

HISTORY OF THE EU BAN ON ANIMAL TESTING FOR COSMETICS



In chemico testing: skin senstisation



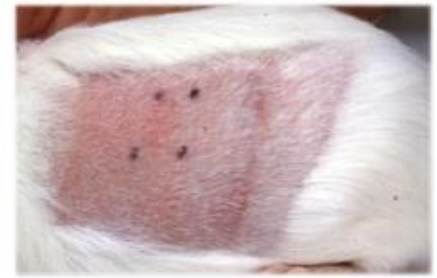


Common allergens and sources of exposure

Allergens	*Source*
Epoxy resin system(ERS)	Adhesives, paints
Formaldehyde	Pesticides, biocides
Fragrance mix	Toiletries, cosmetics
Neomycinsulfate	Creams, deodorants
Nickel sulfate	Costume jewelry, tools

In Vivo

- Guinea Pig Maximization Test (GPMT) and Buehler Test

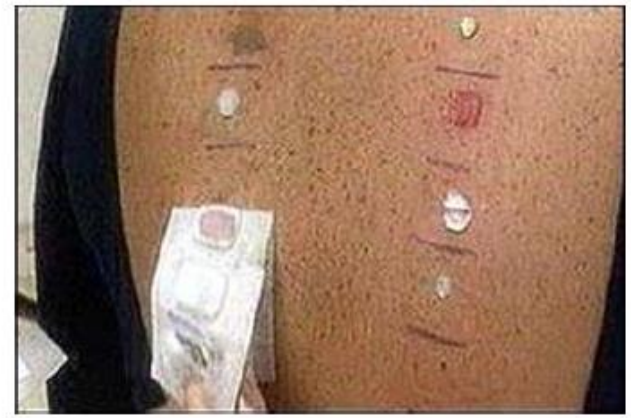


Guinea Pig Maximization Test

- Local Lymph Node Assay (LLNA)

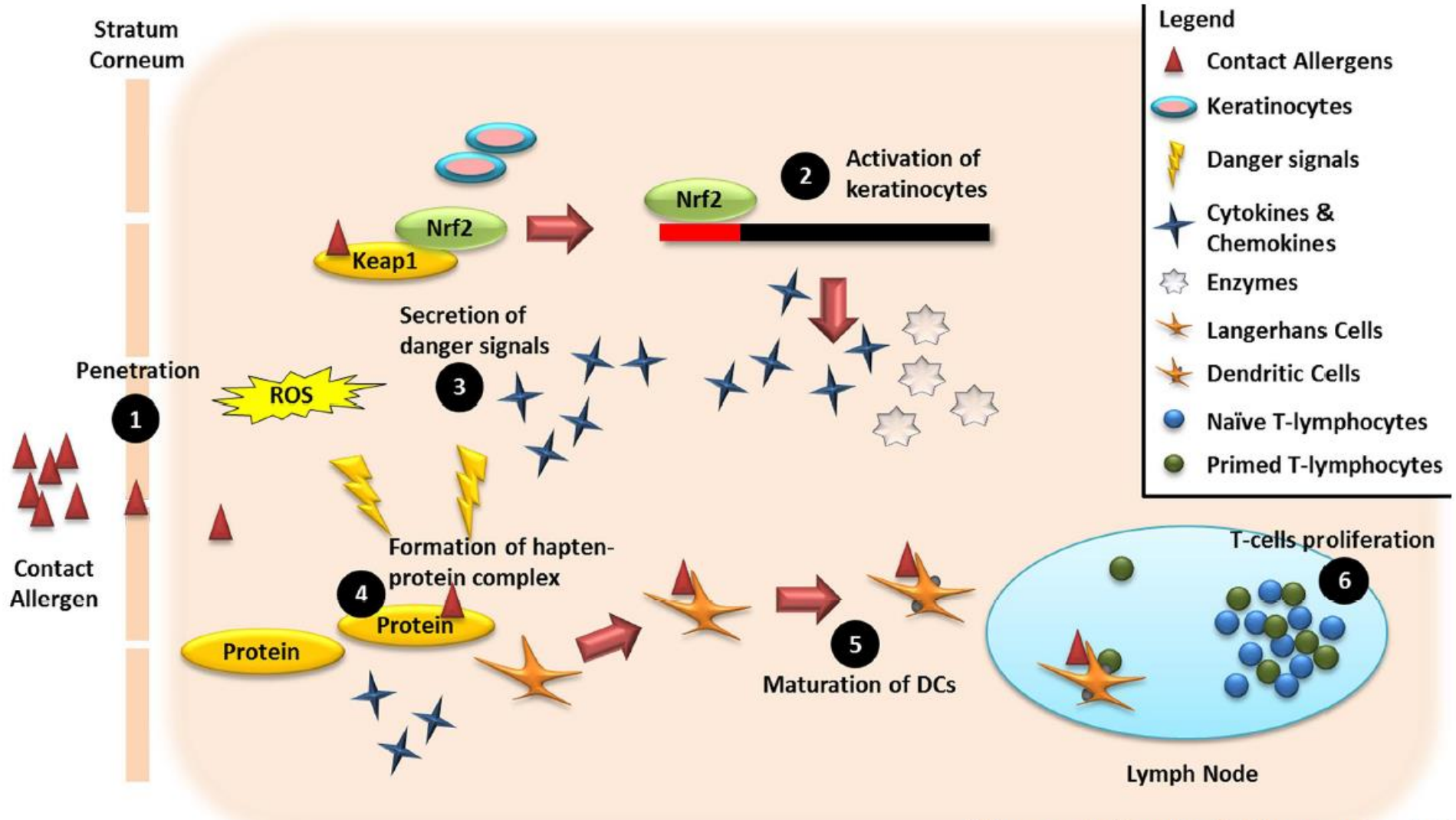
Local Lymph Node Assay

- Human Patch Testing



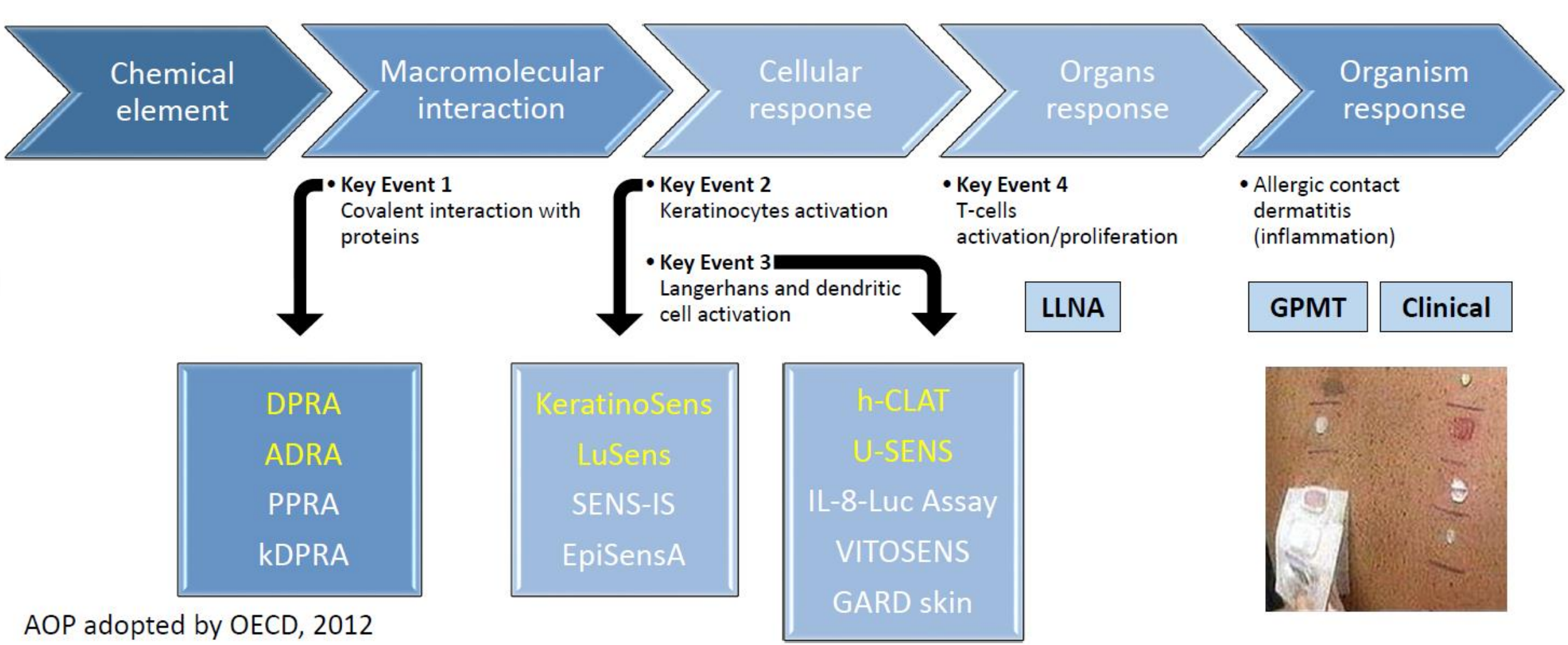
Human Patch Testing

Mechanistic overview supporting endpoint development



Wong et al,. *Frontiers in Pharmacology* 2015 ,(6) 94 1-13

Skin sensitization Adverse Outcome Pathway (AOP)



AOP adopted by OECD, 2012

Direct Peptide Reactivity Assay (DPRA) (OECD TG 442C)
Key event 1

TOXICOLOGICAL SCIENCES **81**, 332–343 (2004)
doi:10.1093/toxsci/kfh213
Advance Access publication July 14, 2004

Development of a Peptide Reactivity Assay for Screening Contact Allergens

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Received April 26, 2004; accepted June 22, 2004

Allergic contact dermatitis resulting from skin sensitization is a common occupational and environmental health problem. In recent years, the local lymph node assay (LLNA) has emerged as a practical option for assessing the skin sensitization potential of chemicals. In addition to accurate identification of skin sensitizers, the LLNA can also provide a reliable measure of relative sensitization potency; information that is pivotal in successful management of human health risks. However, even with the significant animal welfare

subsequent elicitation of an allergic hypersensitivity reaction in the skin, are processes dependent upon recognition of chemical allergens in the skin by Langerhans cells (LC) and the induction of specific T lymphocyte responses (Kimber *et al.*, 2000, 2002). For many years guinea pigs were the species of choice for the hazard identification of skin sensitizing chemicals. More recently, however, the local lymph node assay (LLNA) has been developed as an alternative approach based upon charac-

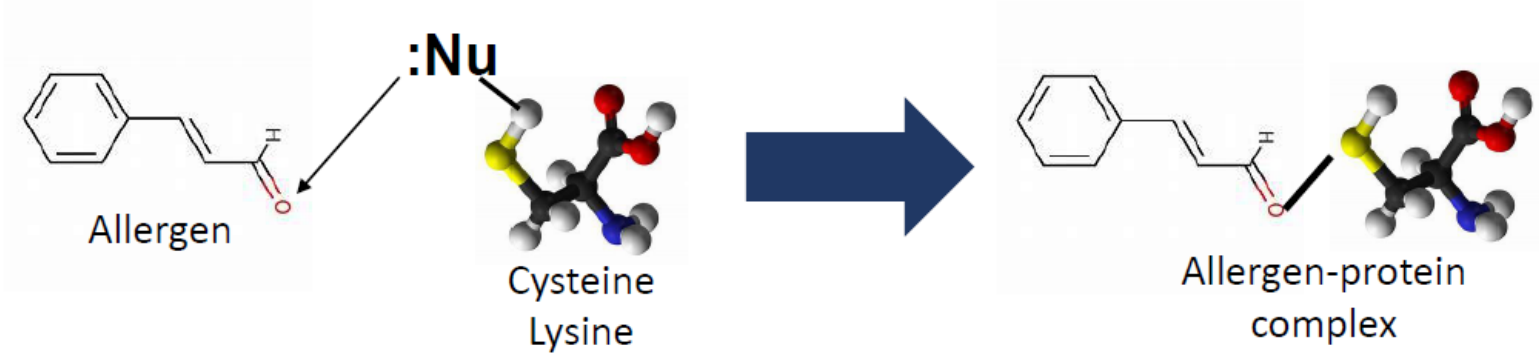
Direct Peptide Reactivity Assay (DPRA) (OECD TG 442C)

Key event 1

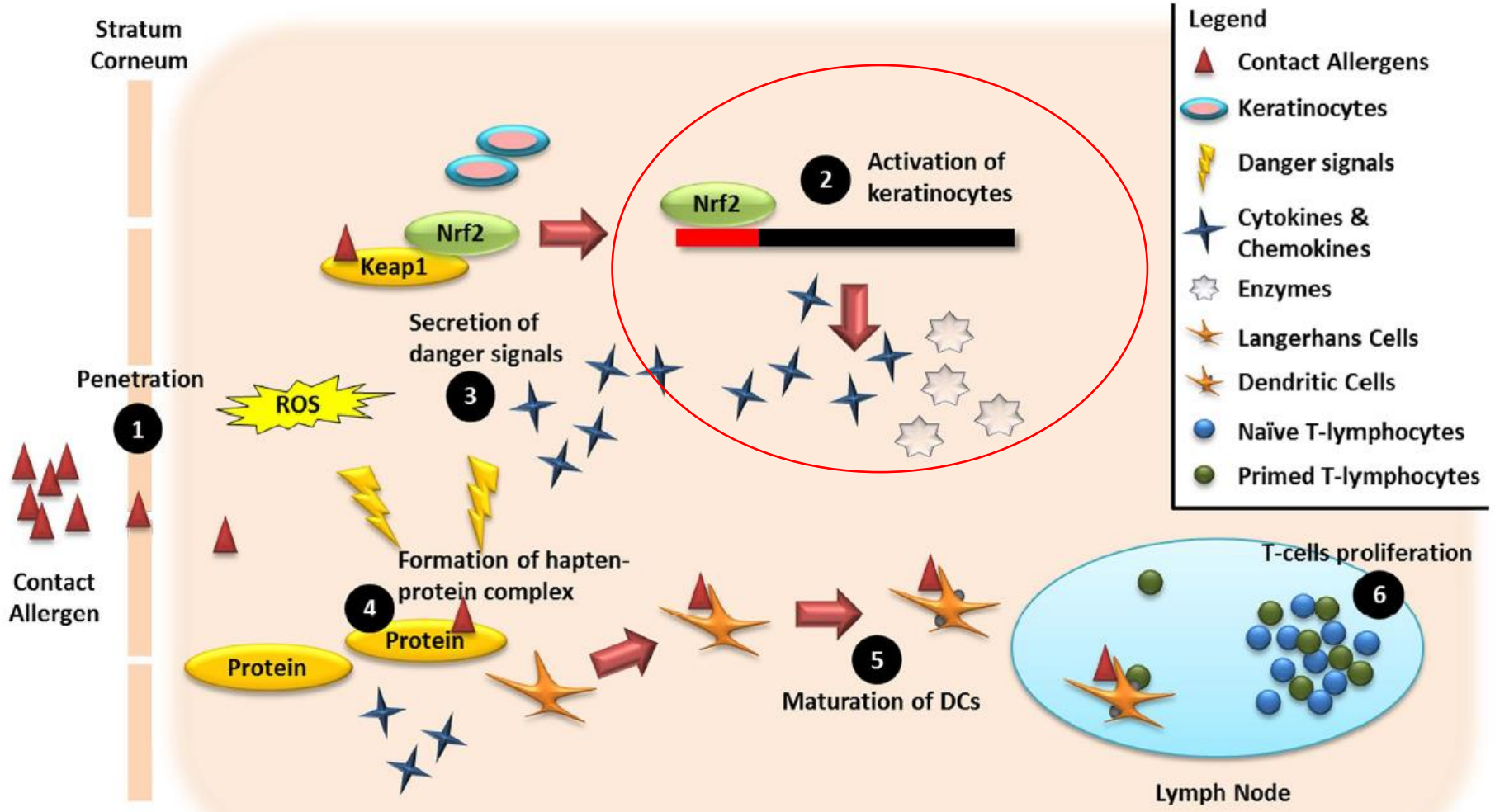
Addresses the process of haptenation (covalent binding of low-molecular weight substances (haptens) to skin proteins)

Molecular Initiating Event (MIE)

Measures peptide reactivity of test chemicals by quantifying the depletion of synthetic peptides containing either *lysine* or *cysteine*



In vitro – skin, OECD tiered testing strategy

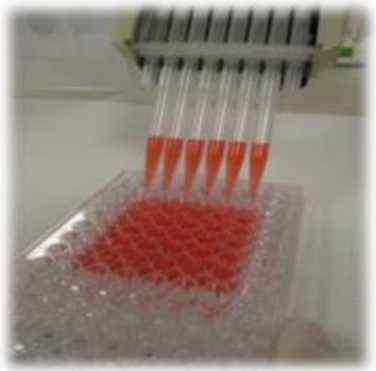


Wong et al,. *Frontiers in Pharmacology* 2015 ,(6) 94 1-13

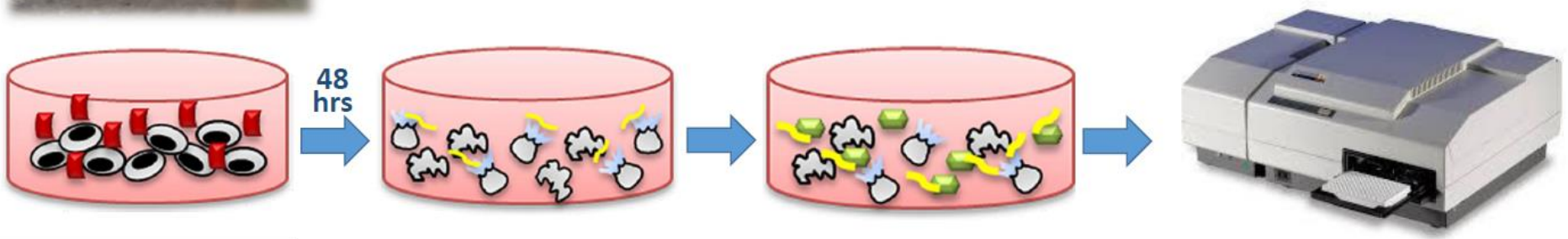
KeratinoSensTMAssay, (OECD TG442D)
Key event 2

Addresses keratinocyte responses by activation of antioxidant/electrophile response element dependent pathway (Keap1-Nrf2-ARE)

- The repressor protein Keap1 reacts with electrophiles, allowing dissociation of the transcription factor Nrf2 to translocate to the nucleus and induce the antioxidant response element (ARE)
- Reporter construct with a copy of the ARE-element of the human AKRIC2 gene upstream of a luciferase gene



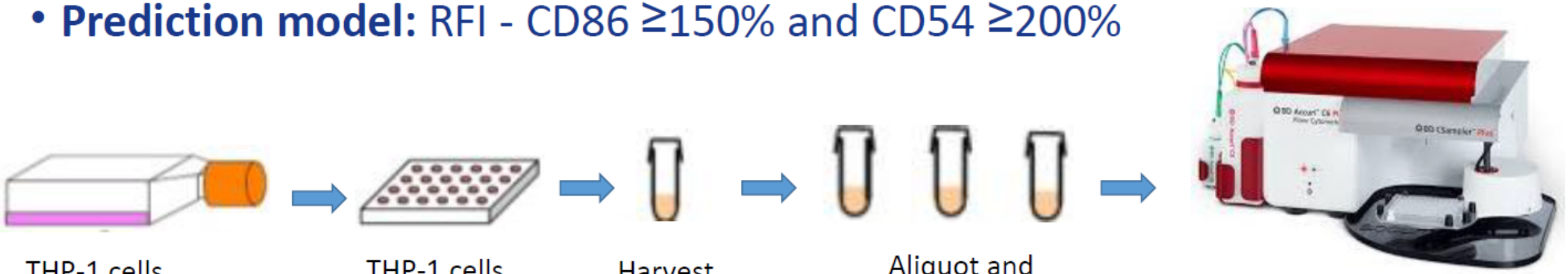
- HaCaT (immortalized keratinocyte cell line)
- 48 hour incubation with test material (12 concentrations)
- Addition of Promega lysis buffer and luciferase substrate
- Quantitative gene induction by luciferase activity



Wong et al., 2015

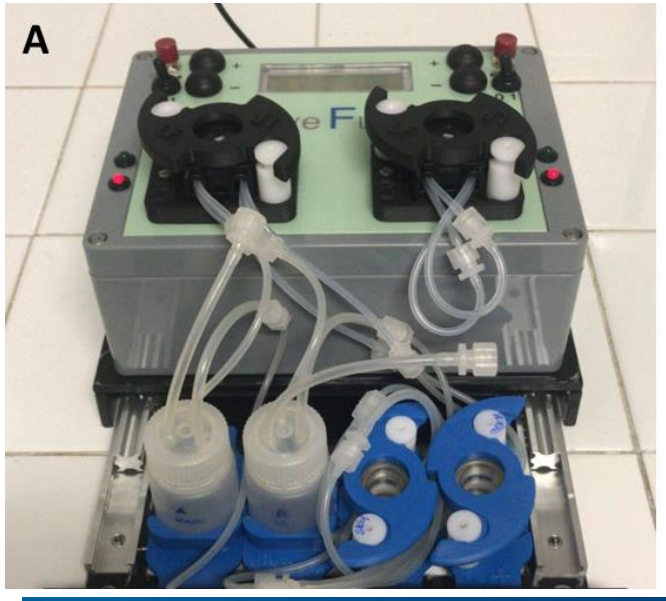
Human Cell Line Activation Test (h-CLAT) (OECD TG 442E), Key event 3

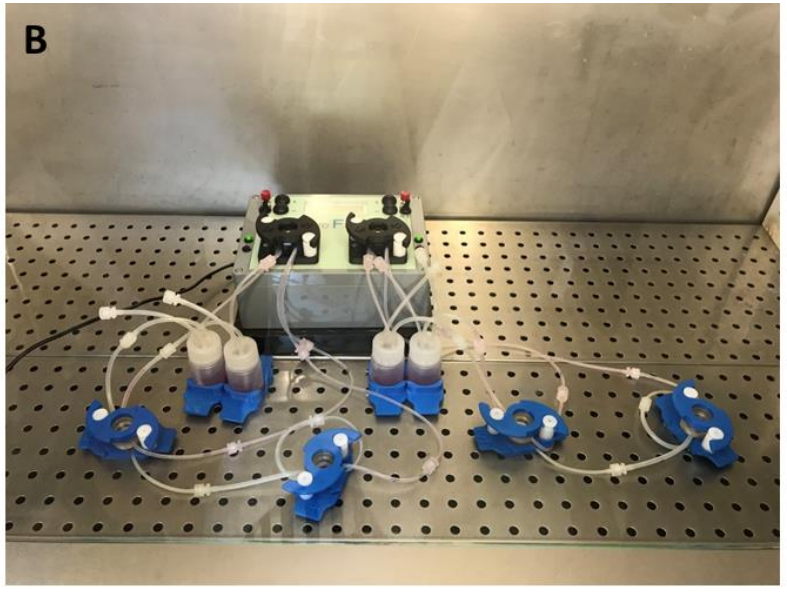
- **Test system:** THP-1 cells: an immortalized human monocytic leukemia cell line, used as a surrogate for DC
- Measures modulation of the expression of dendritic cell surface phenotypic biomarkers (CD86 and CD54) by flow cytometry
- **Prediction model:** RFI - CD86 ≥150% and CD54 ≥200%

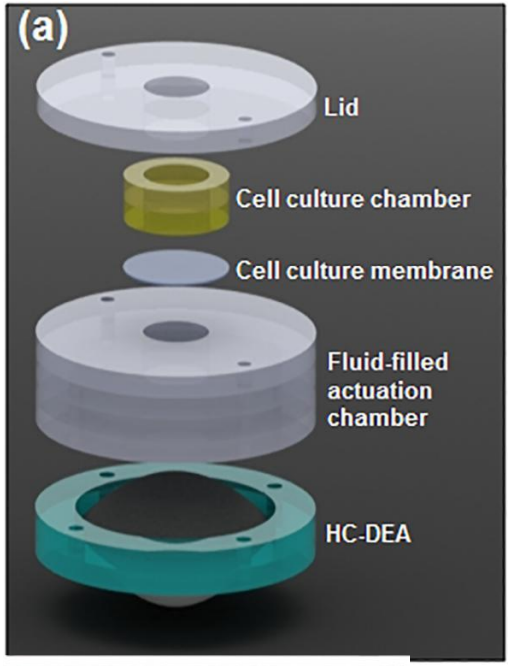


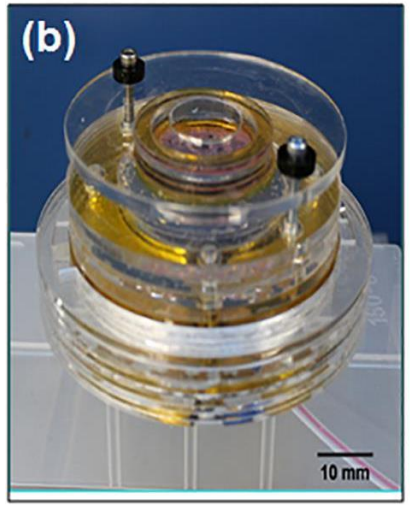
Can we go further with *in vitro*?

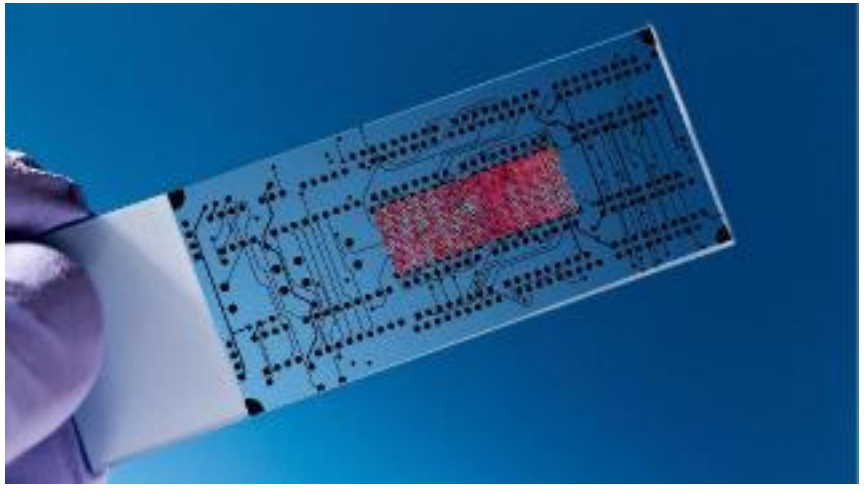
Organs on plates, organs on chips, organoids

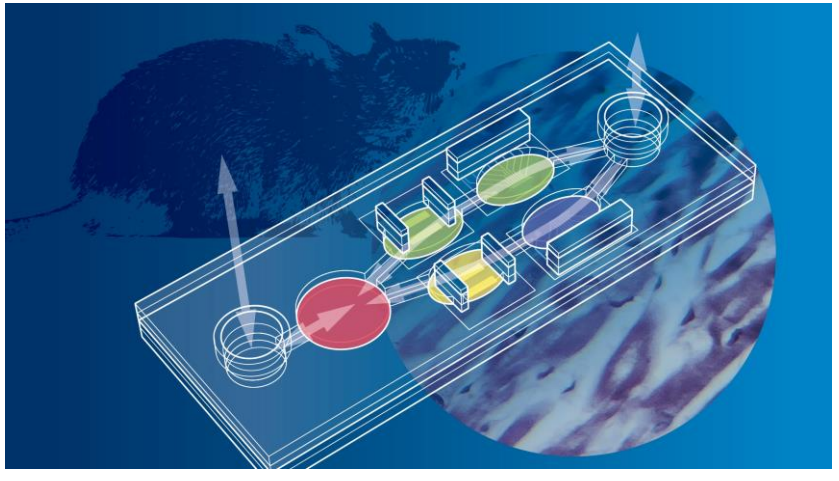












Organoids and 3D cultures

› Nat Med. 2017 Dec;23(12):1424-1435. doi: 10.1038/nm.4438. Epub 2017 Nov 13.

Human primary liver cancer–derived organoid cultures for disease modeling and drug screening

› Cell Metab. 2019 Aug 6;30(2):374-384.e6. doi: 10.1016/j.cmet.2019.05.007. Epub 2019 May 30.

Modeling Steatohepatitis in Humans with Pluripotent Stem Cell–Derived Organoids

› PLoS One. 2018 Feb 13;13(2):e0192824. doi: 10.1371/journal.pone.0192824. eCollection 2018.

Systemic and vascular inflammation in an in–vitro model of central obesity

› Sci Rep. 2019 Aug 15;9(1):11890. doi: 10.1038/s41598-019-48347-2.

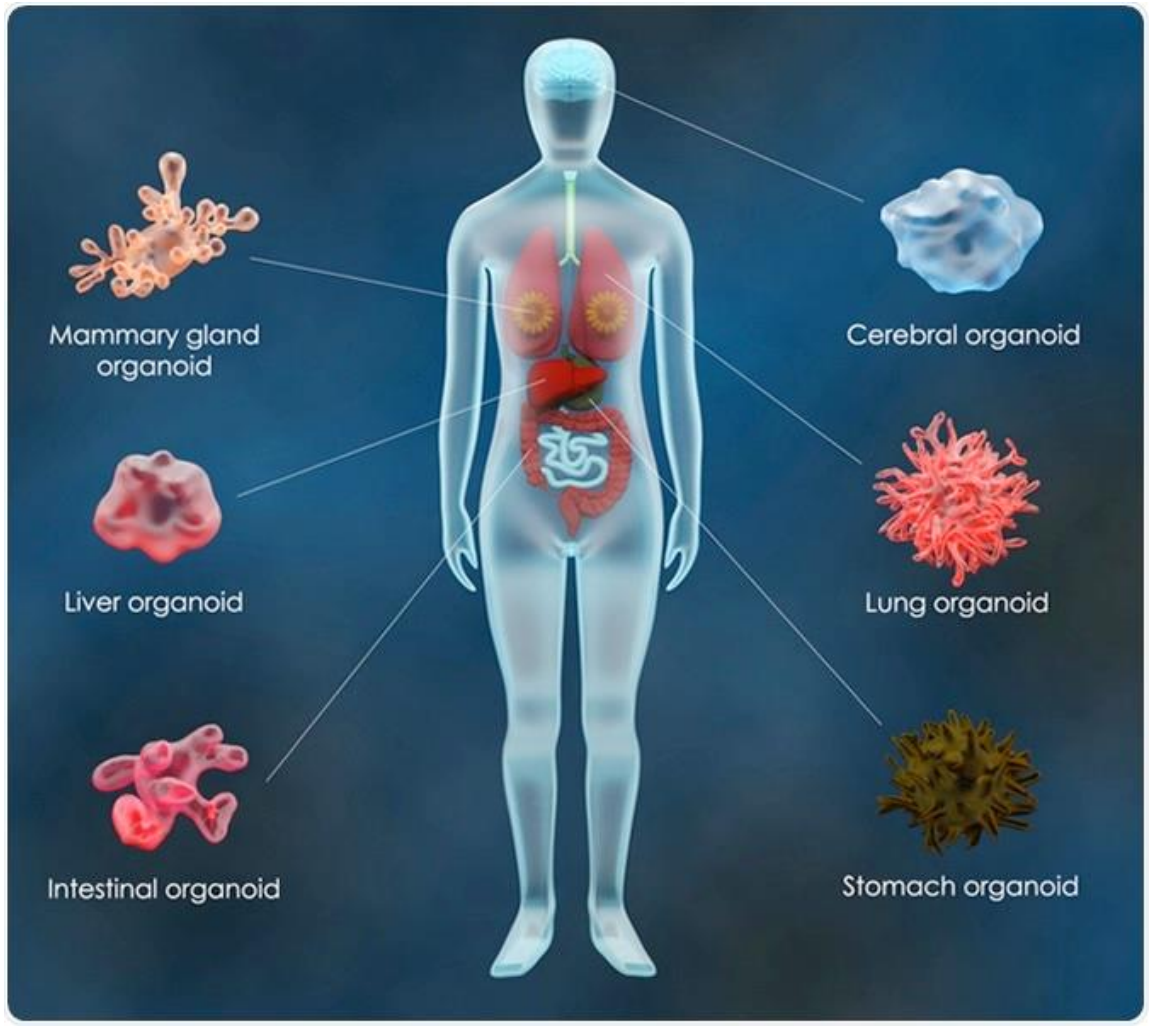
Allometric Scaling of physiologically–relevant organoids

› Nature. 2016 Jun 9;534(7606):267-71. doi: 10.1038/nature18296. Epub 2016 May 11.

The Brazilian Zika virus strain causes birth defects in experimental models

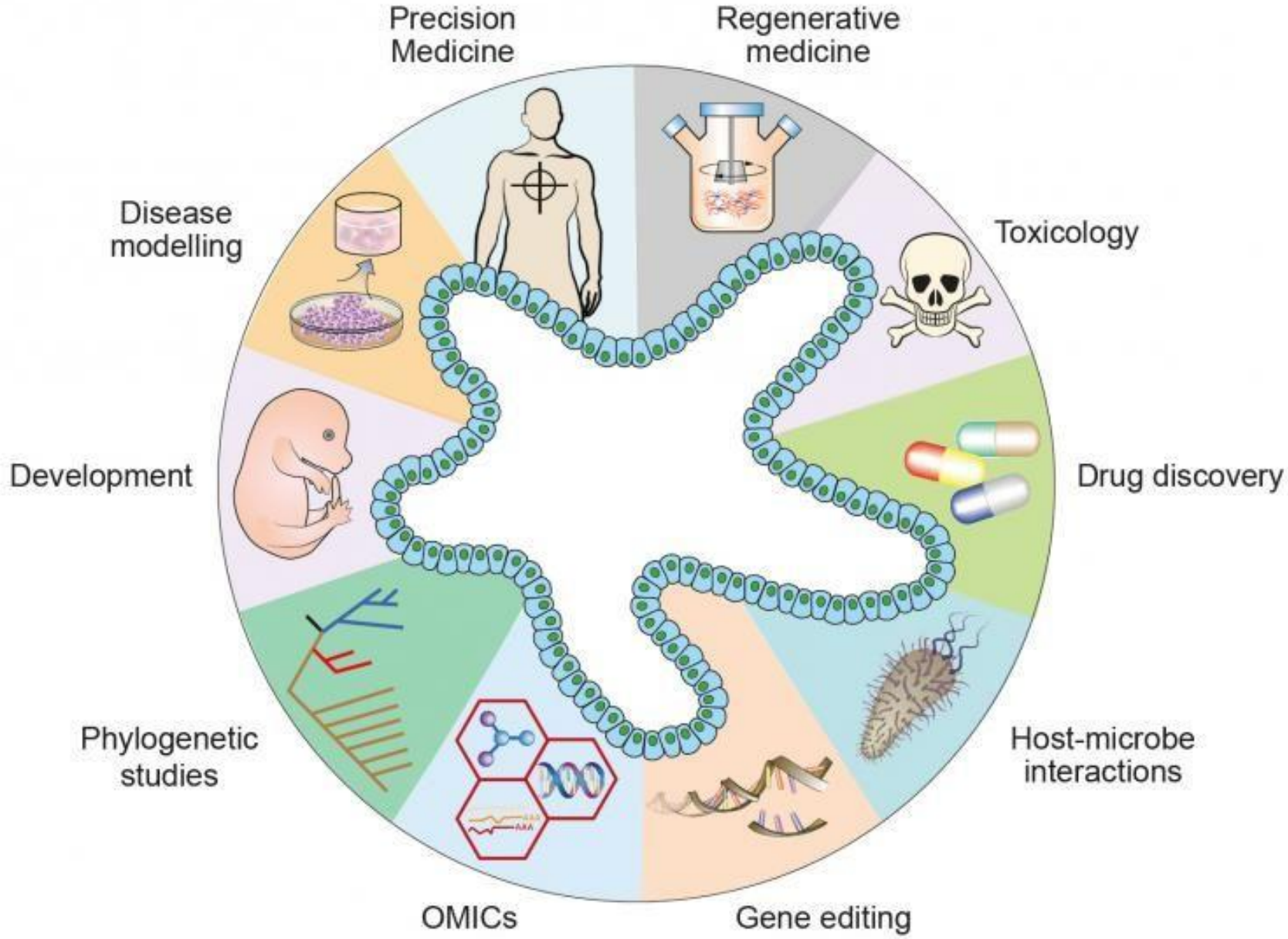
ORGANOIDS

An organoid is a three-dimensional cellular structure that closely resembles and functions similarly to a specific organ in the body. Organoids are typically generated from stem cells or tissue samples and are cultured in vitro (in a laboratory setting). They can mimic the architecture, cell types, and physiological functions of the organ they represent, making them valuable models for studying organ development, disease mechanisms, and drug testing. Organoids have been created for various organs, including the brain, liver, kidney, intestine, and many others.

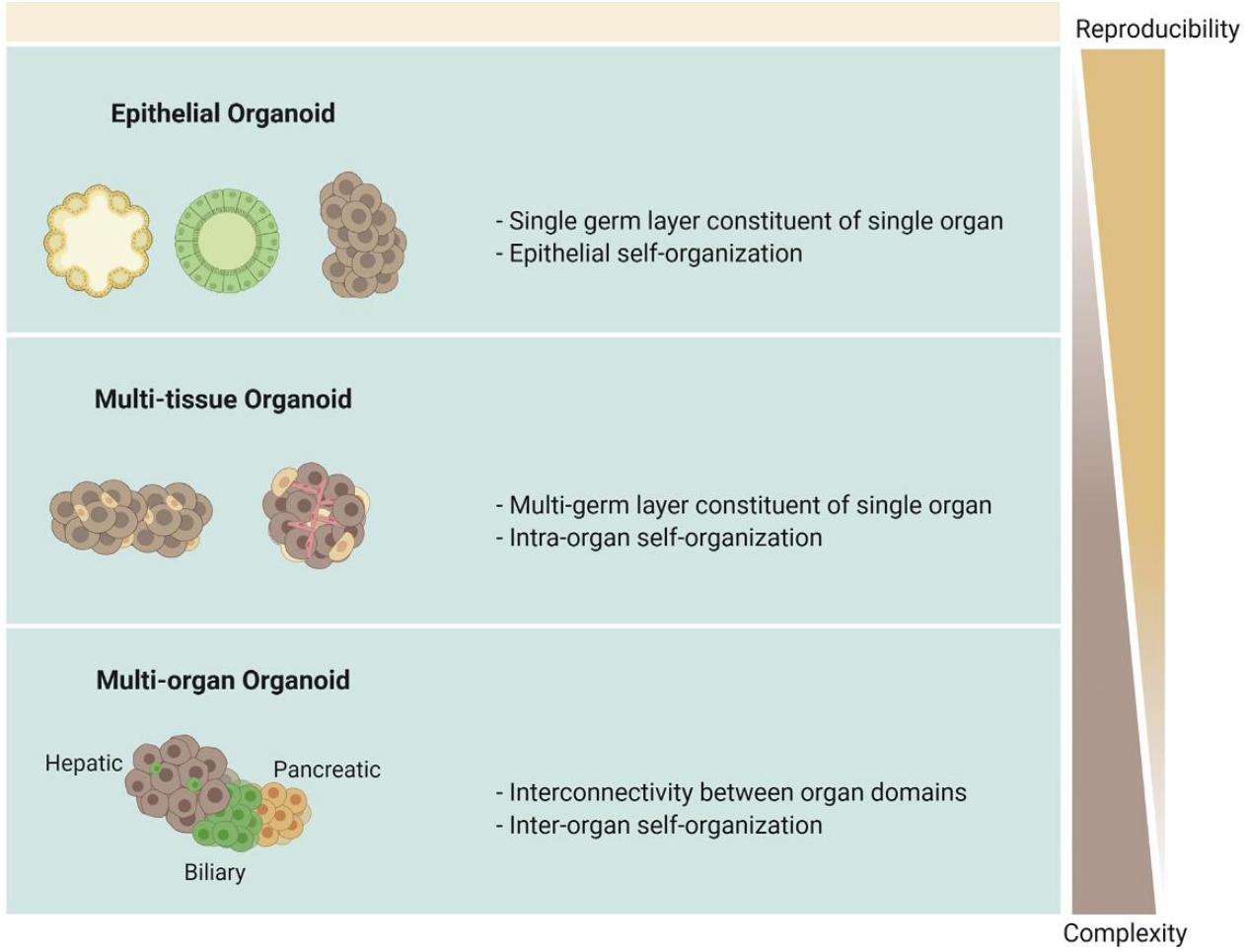


- **Physiological cell density** ($5.14*10^{14}$ cells/m^3)
- Often no **vascular network**
- Many **different protocols**

APPLICATIONS



CLASSIFICATION



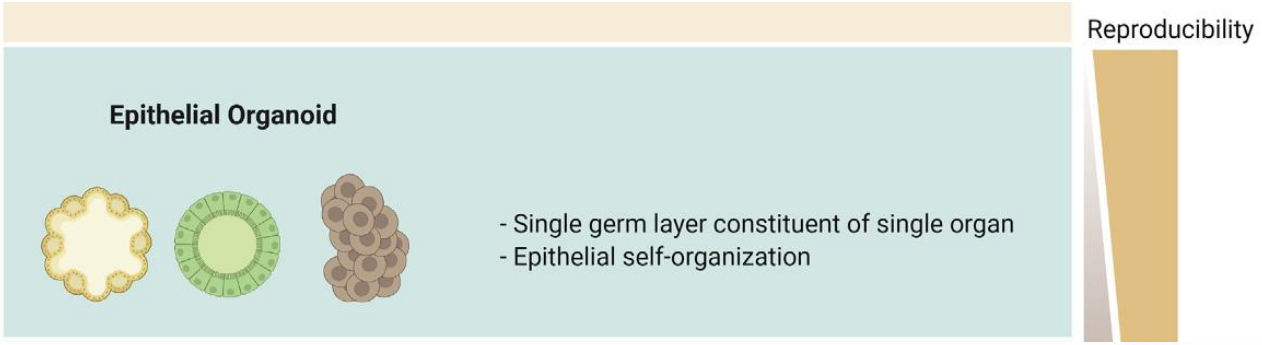
Marsee et al., Cell. 2021

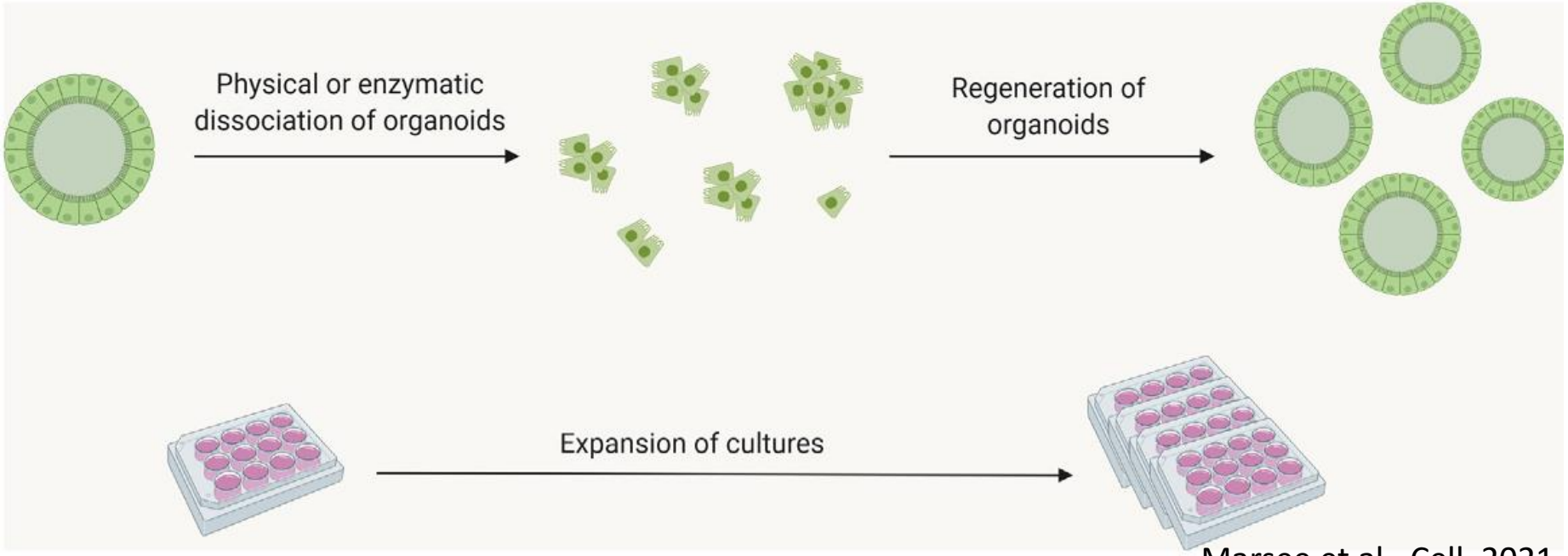
EPITHELIAL ORGANOIDS

Cells arising from a single germ layer. Under specific and controlled culture conditions (physical, enzymatic or chemical and/or by chemical dissociation), organoids can fragment into single cells or groups of cells with the ability to re-organize and expand, in turn forming other organoids.

Useful for developmental studies

(**SELF-RENEWA**L).

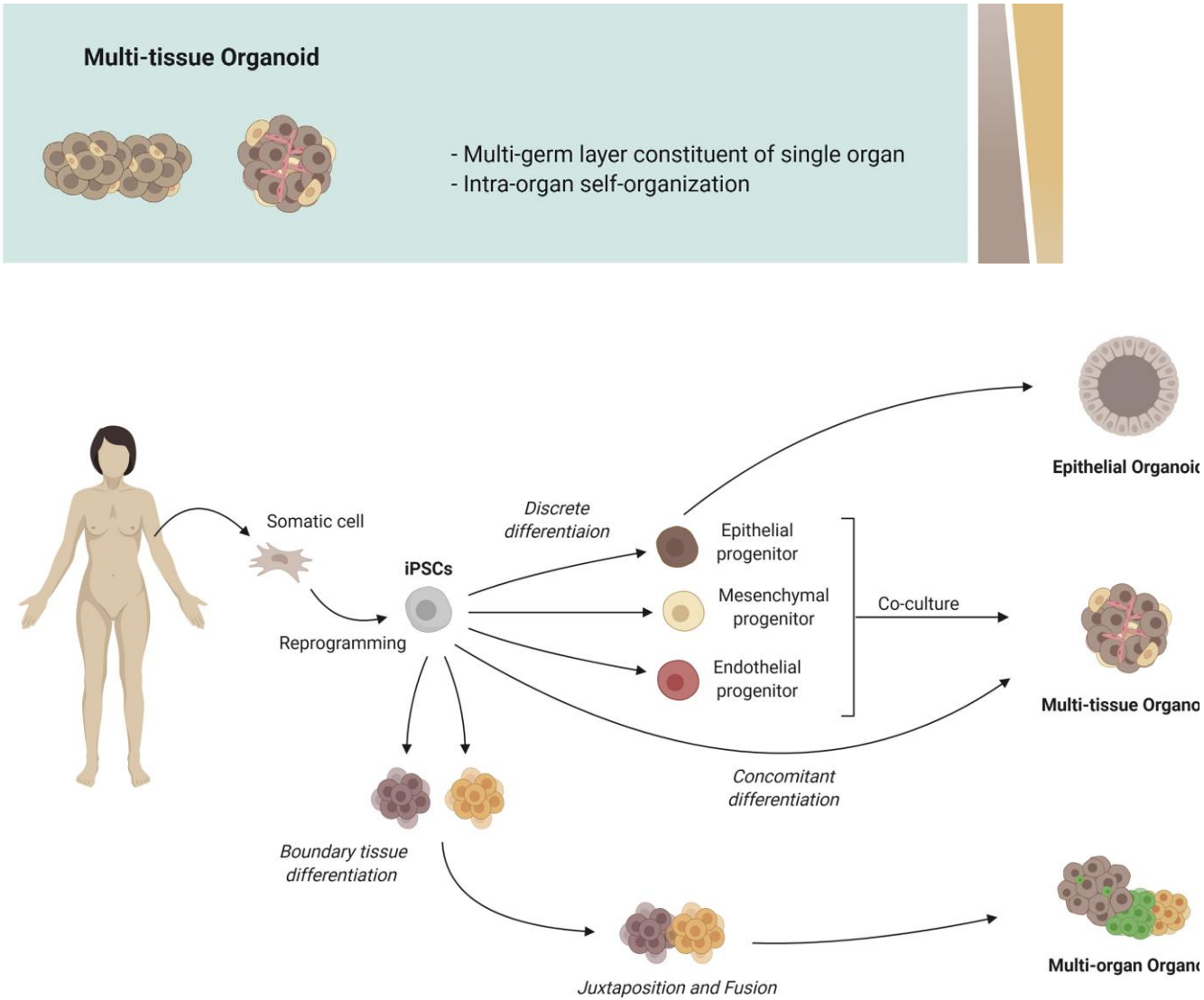




Marsee et al., Cell. 2021

MULTI-TISSUE ORGANOIDS

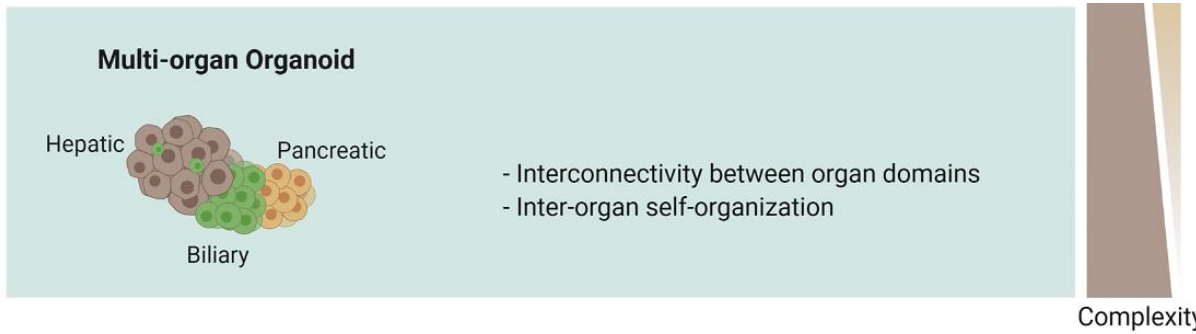
Multi-tissue organoids are established through the co-culture of cells derived from at least two germ layers. Unlike epithelial organoids, current protocols do not support the self-renewal of multi-tissue organoids, which would require the coordinated expansion of parenchymal and supporting cell types. **Instead, cells interact to attain a stable level of maturity and function An advantage of multi-tissue organoids is their tissue-like, hetero-cellular composition. Multi-tissue organoid systems are well placed for studying the heterotypic cell-cell interactions of multiple cell types normally present in the native tissue**. Importantly, these cultures show intra-organ self-organization between epithelial and supporting cell types, similar to that of the native tissue



MULTI-ORGAN ORGANOIDS (ASSEMBLOIDS)

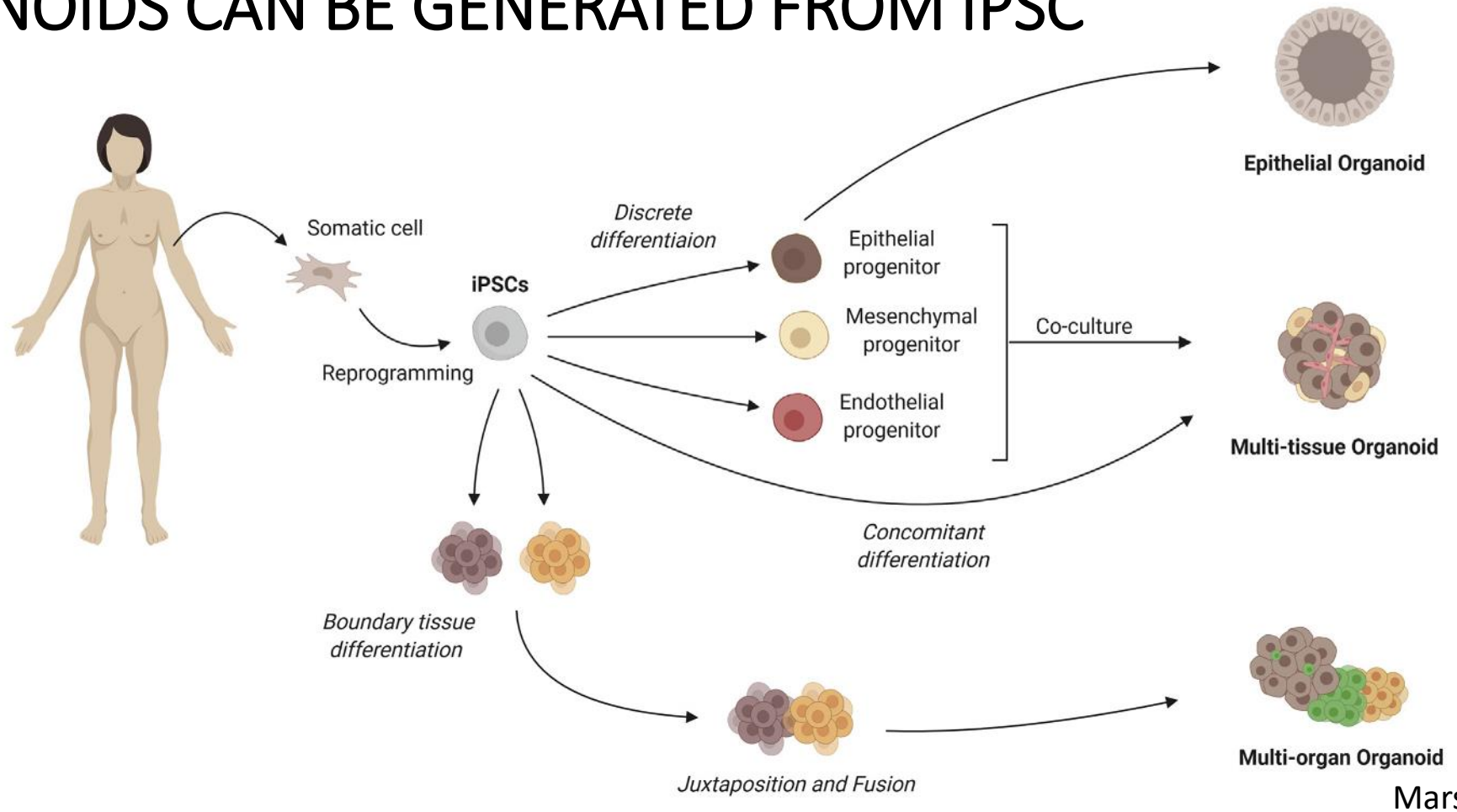
They are more complex to generate, but the cells themselves are capable of organizing themselves to replicate even inter-organ connections over time.

Heterogeneous cellular composition
Useful for studying the interactions between cells of different phenotypes, which coexist in an organ in vivo



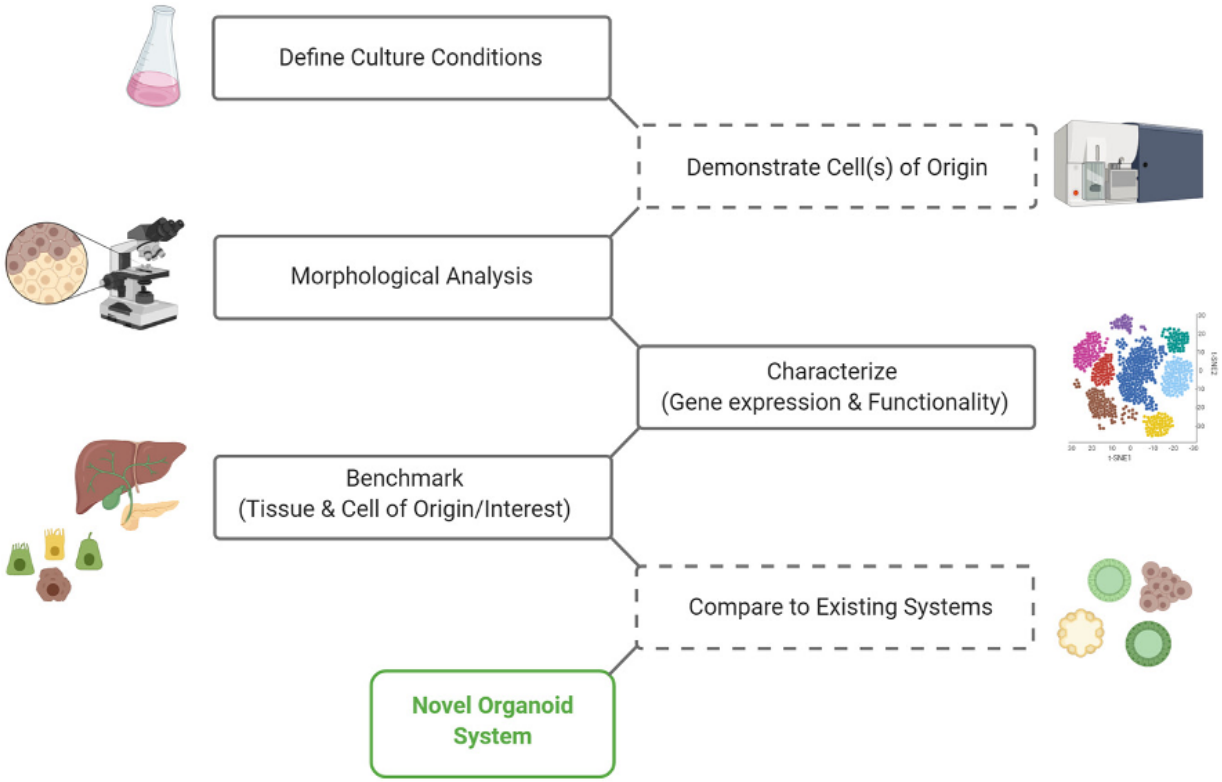
Marsee et al., Cell. 2021

ORGANOIDS CAN BE GENERATED FROM IPSC



Marsee et al., Cell. 2021

NEED FOR A STANDARD FRAMEWORK

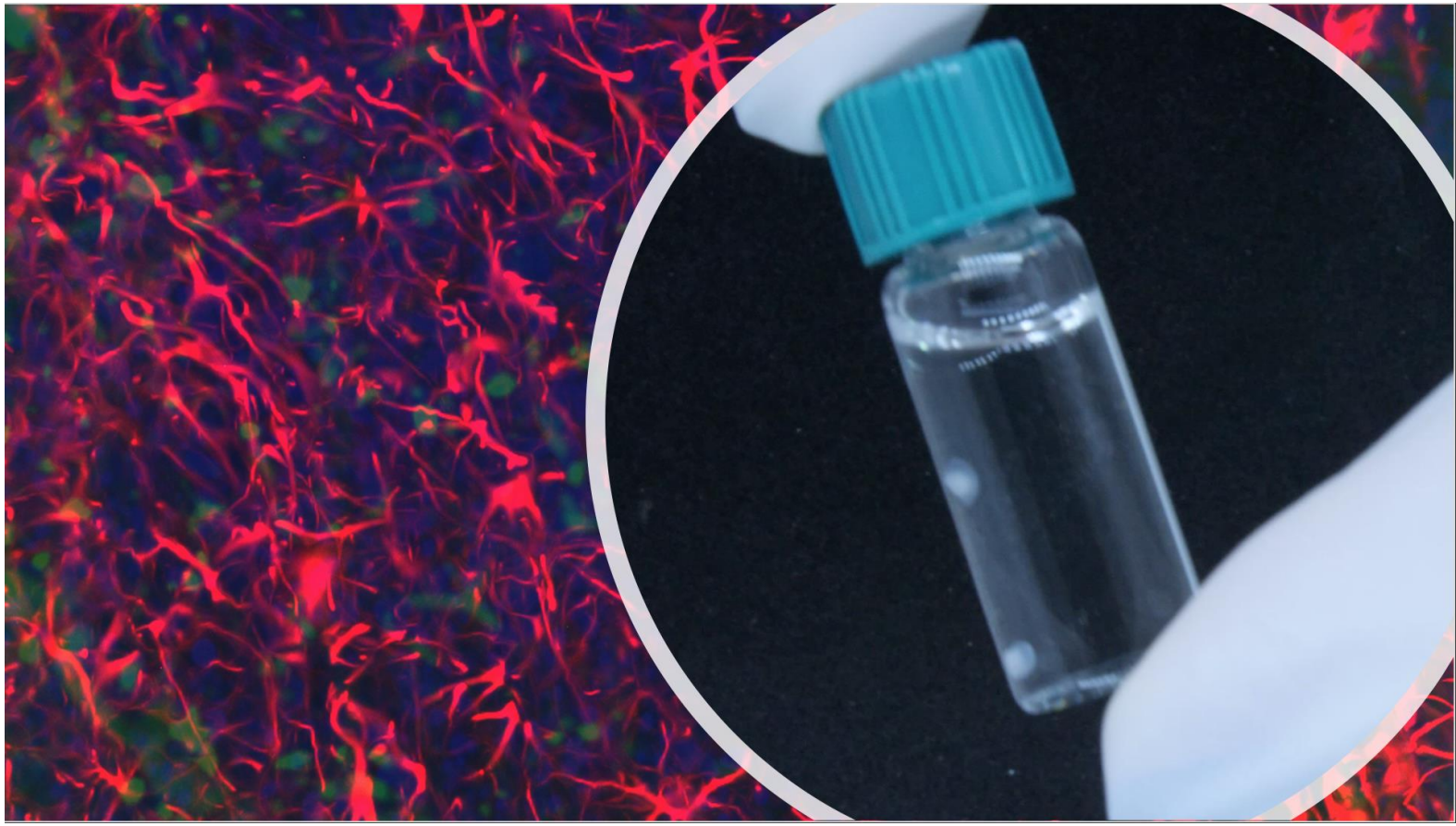


Marsee et al., Cell. 2021

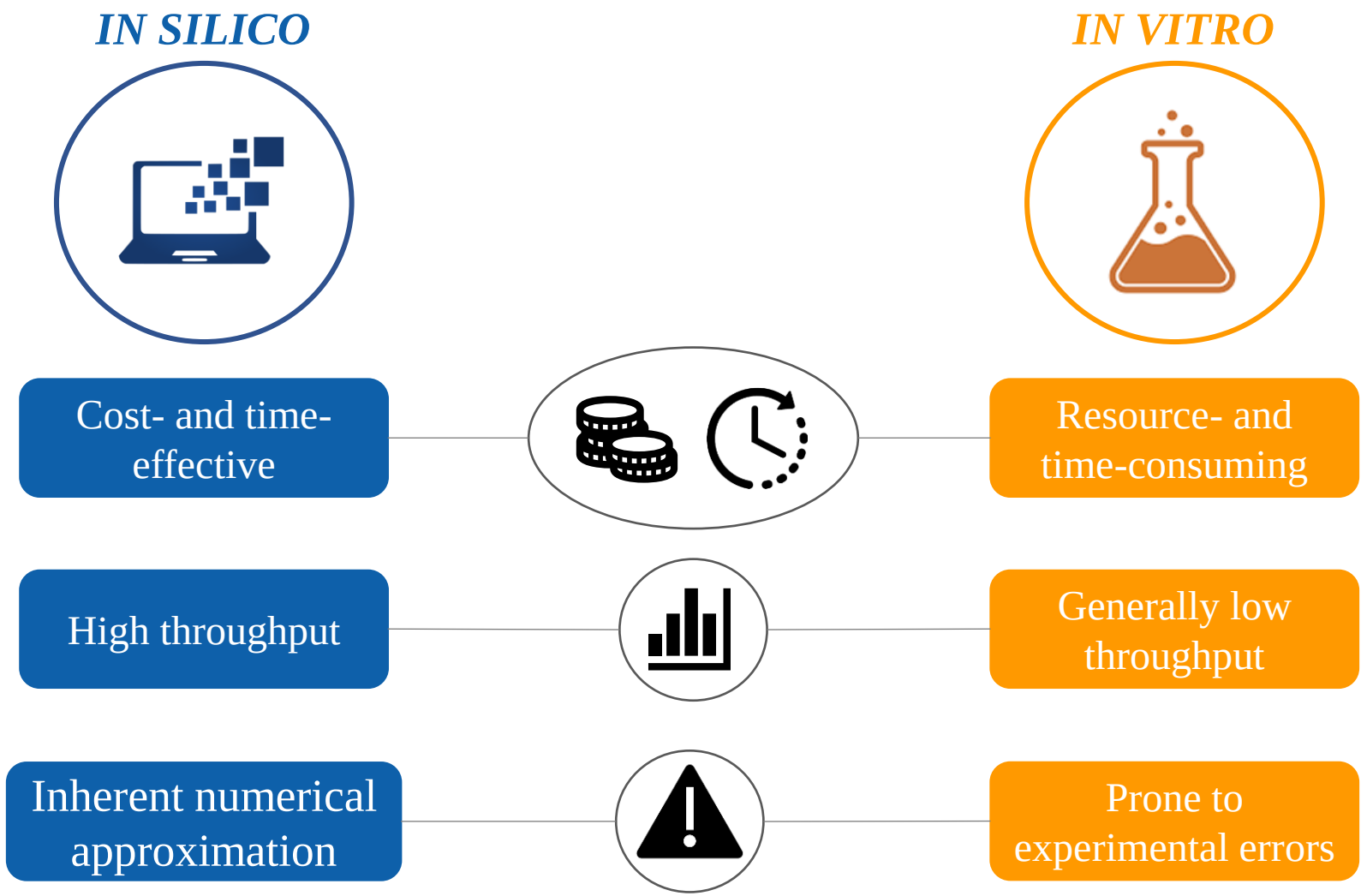
OPEN QUESTIONS

- STANDARDISATION
- VASCULARISATION & NUTRIENT SUPPLY

- **ESTABLISH AND QUANTIFY VALIDITY AND PHYSIOLOGICAL RELEVANCE**



In silico and in vitro



Why *in silico*?

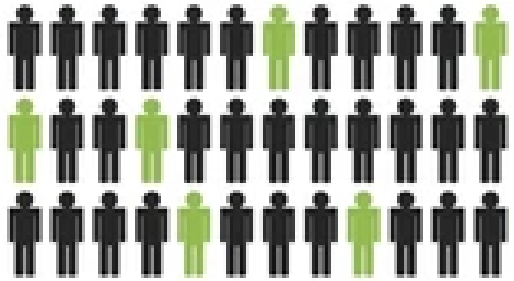
TO MAKE UNDERSTANDING EASIER

- Support **hypothesis testing**
- Elucidate **complex** pathophysiological **mechanisms**

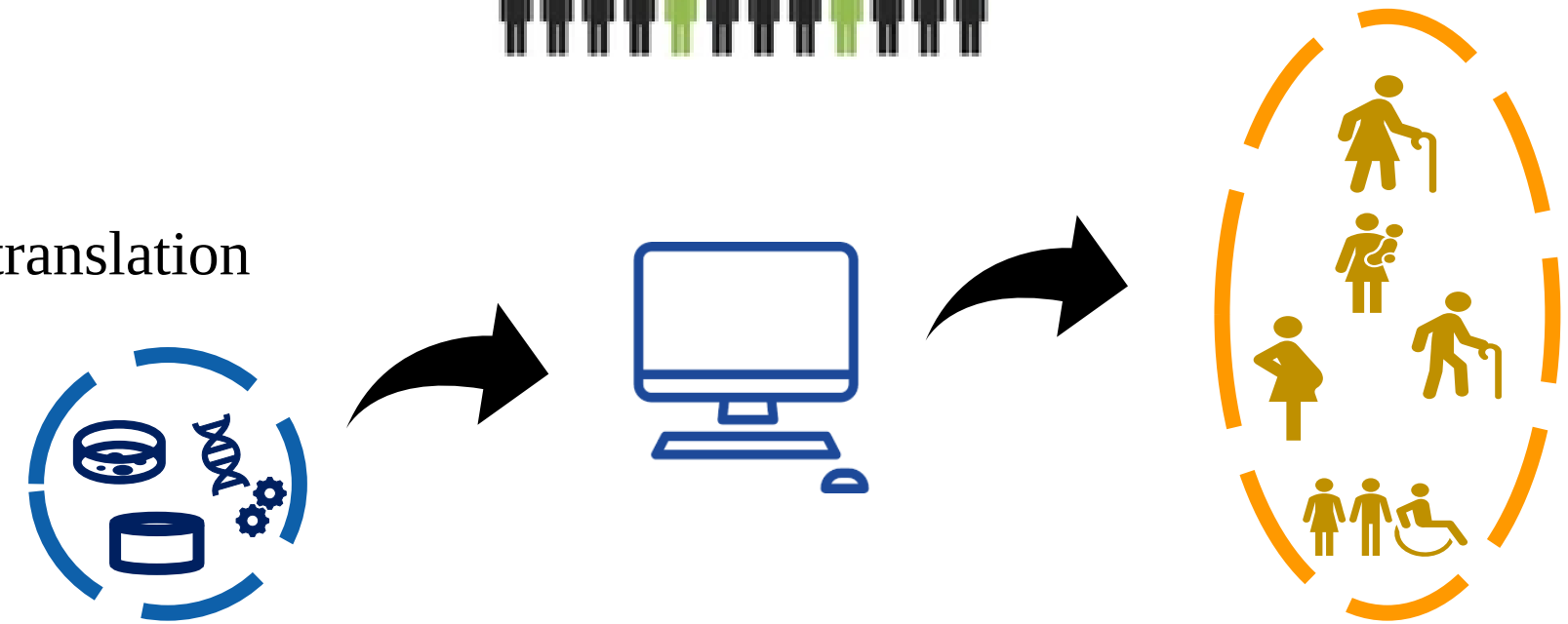
Why *in silico*?

TO EXTRAPOLATE RESULTS & MAKE PREDICTIONS

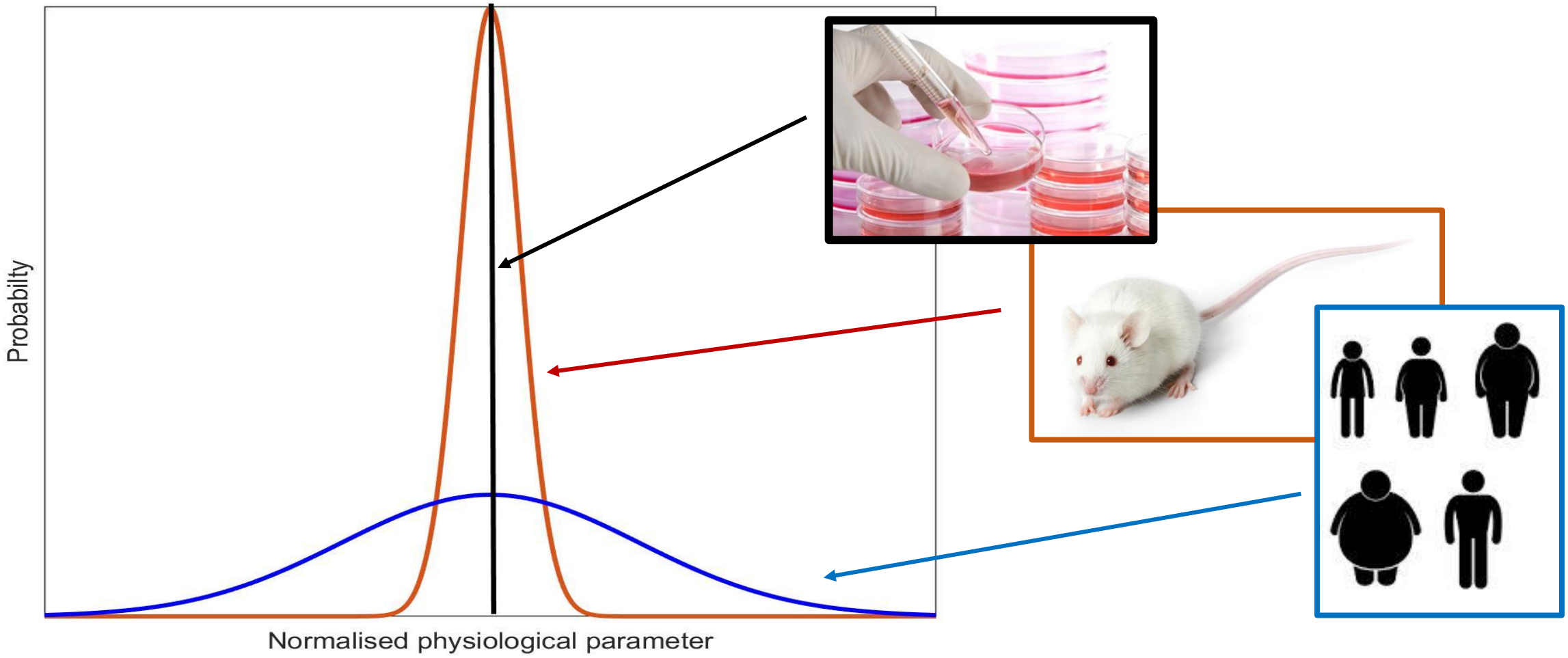
- From samples to **whole populations**



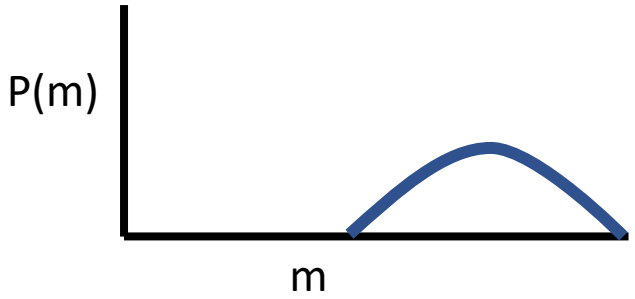
- ***In vitro*-to-*in vivo*** translation

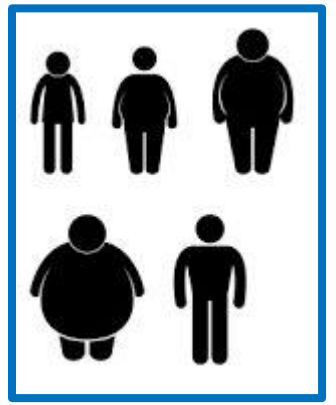


VARIABILITY vs STANDARDISATION



VARIABILITY AND SCALABILITY

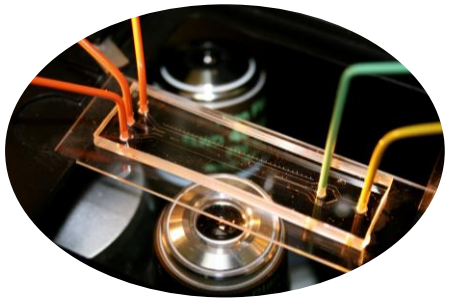




Botte … Ahluwalia *PNAS*, 2021

Why *in silico*?

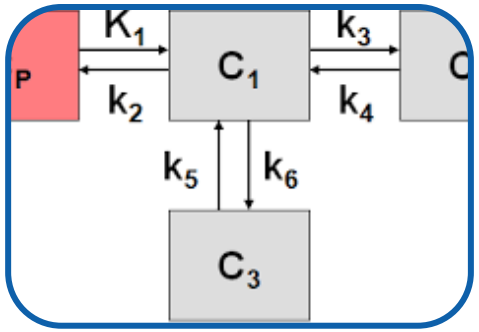
TO DESIGN EXPERIMENTS



- **Optimizing** experimental **conditions**
- **Minimizing** the **number** of tests needed

Approaches empowering 3Rs

REPLICATING THE BIOPHYSICS OF THE SYSTEM

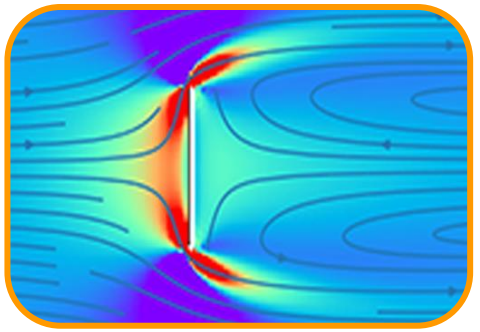


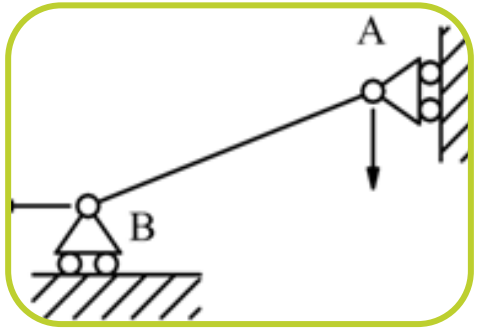
PBPK

- Pharmacokinetics
- Chemical, drug, nanoparticle distribution

Transport

- Mass
- Energy
- Momentum



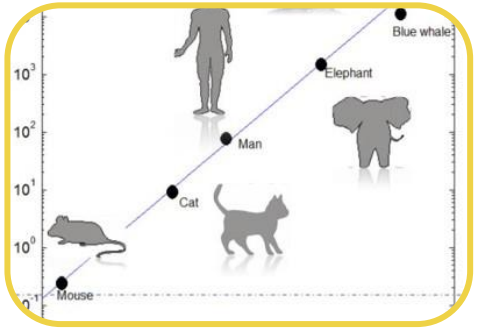


Structural mechanics

- Mechano-transduction
- Cell motility
- Ligand-receptor mechanisms

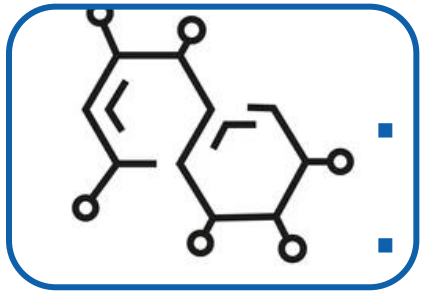
Scaling

- Size-related metabolism
- Bioinspired design



Approaches empowering 3Rs

DECISION-MAKING BASED ON A PRIORI KNOWLEDGE

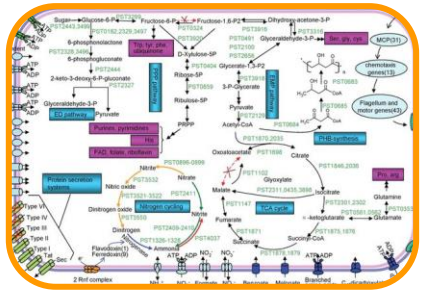


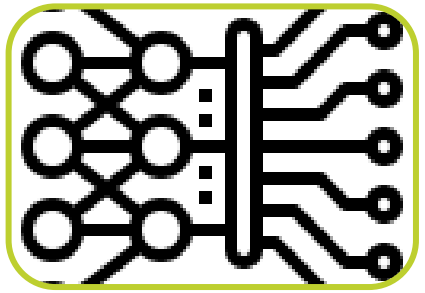
QSAR

- Structure-effect extrapolation
- Toxicity assessment
- Drug design

AOP

- Prediction of causality pathways
- Risk assessment



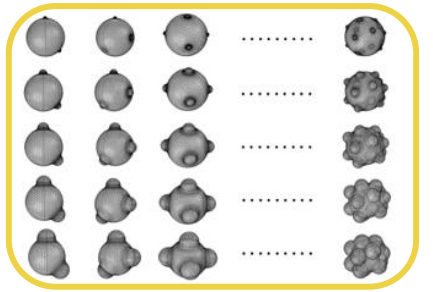


Machine learning

- Data-based
- Classification
- Clustering

Genetic/Evolutionary algorithms

- Global design optimization
- Iterative methods



Limitations

- ***In silico*** methods **yet** to be **fully exploited**
- Need to **bridge the gap** between **computational** modelling and **experimental** testing

Integrated approaches require more overlap, more interaction, and a mutual understanding of methods, constraints and limitations

The progress towards ‘human based’ is part of the process of scientific advancement

Discovered in dogs, 1889

First used in humans 1922

Industrial production in cows 1923

Synthetic human insulin in recombinant bacteria 1978

Grazie per l’attenzione