MACRAMÉ

Advanced Characterisation Methodologies to assess and predict the Health and Environmental Risks of Advanced Materials

MACRAMÉ´s Approach towards the Harmonisation of *in vitro*/ *ex vivo* Models for Inhalation Toxicology

WORKSHOP ON "*HARMONISATION AND STANDARDISATION OF TEST METHODS FOR NANO AND ADVANCED MATERIALS*"

22ND – 23RD NOVEMBER 2023 - ONLINE

The MACRAMÉ project has received funding from the European Union's Horizon Europe Research and Innovation programme under grant agreement No. 101092686.

MACRAMÉ's central Objectives

- detect, characterise and quantify Advanced Materials (AdMas) during handling and processing along the product life-cycle,
- assess potential impacts on (human) health and the environment in intended or unintended exposure situations (i.e. 'Exposure Points') in the product value-chain,
- advance the wide-spread applicability of the developed test and characterisation methods, by demonstrating their effectiveness and efficiency in the context of existing, market-relevant industrial AdMas containing products, and
- prepare and initiate standardisation, harmonisation and technological & regulatory validation of test- and characterisation-methods.

The MACRAMÉ R&I Approach

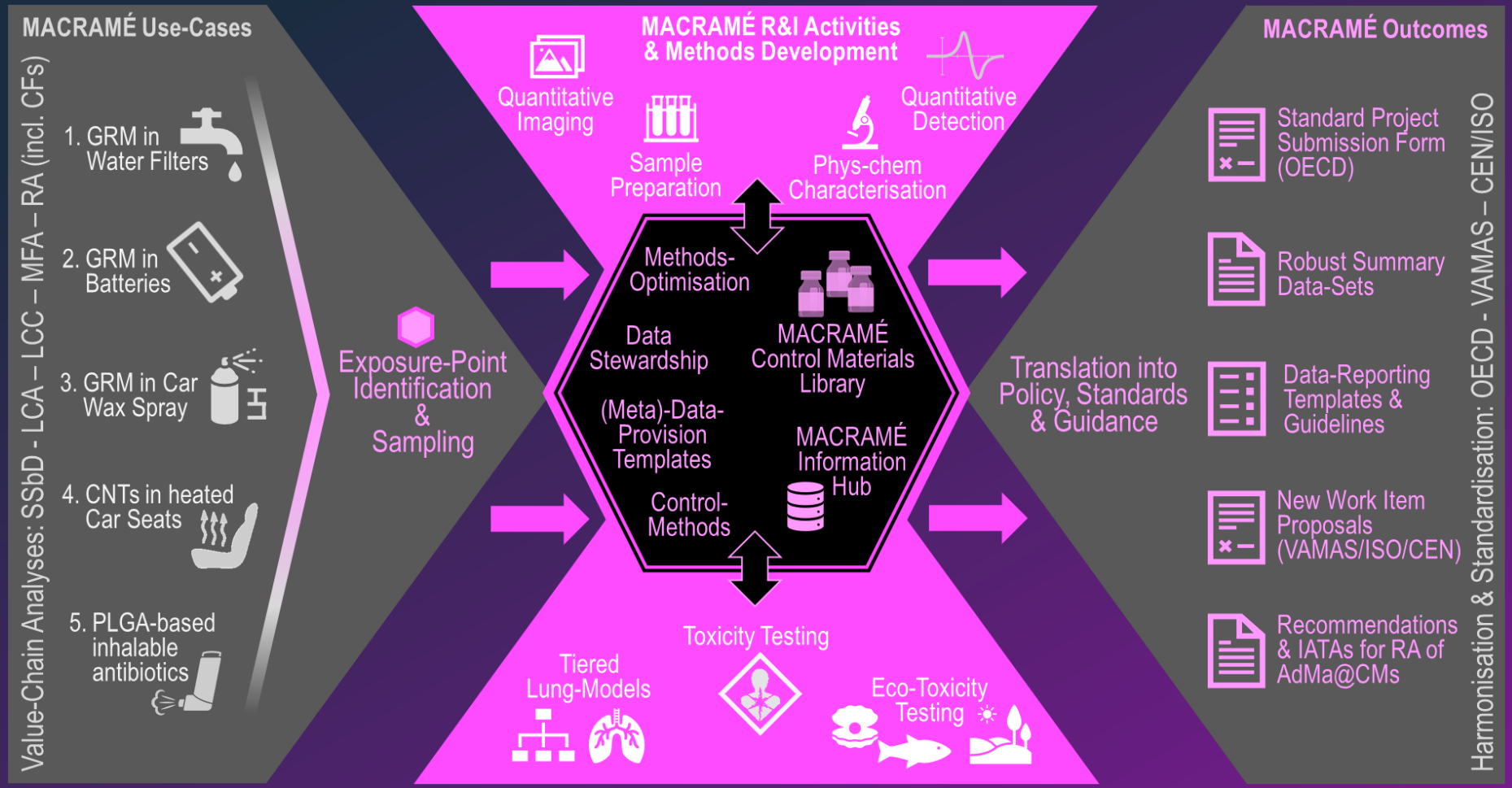


Illustration of the MACRAMÉ R&I Approach (AdMa@CMs: Advanced Materials in complex matrices; CF: Characterisation Factor; GRM: graphene-related material; IATA: integrated approaches to testing and assessment; LCA: Life-Cycle Assessment; LCC: Life-Cycle-Costing; MFA: Material-Flow Analysis; RA: Risk-Assessment; SSbD: Safe-&-Sustainable-by-Design).

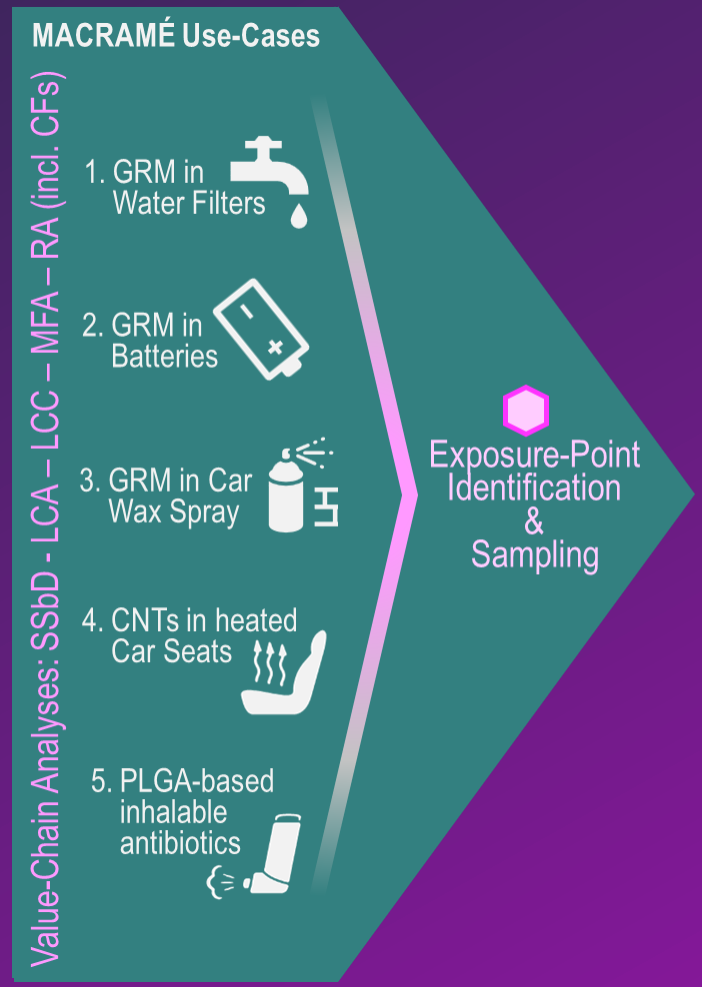
… building on real, market-relevant Products

5 market-relevant industrial MACRAMÉ Use-Cases:

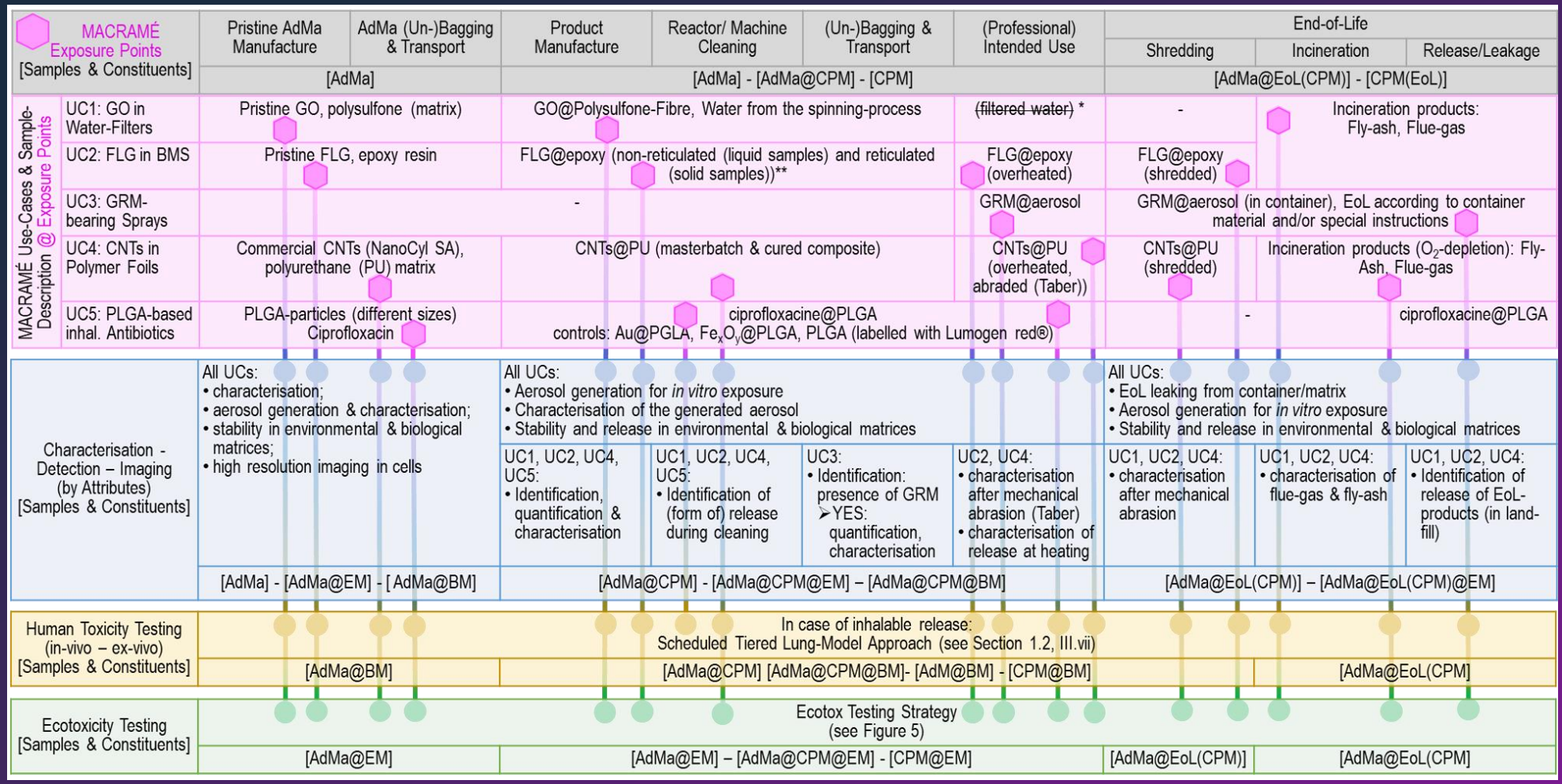
- Graphene oxide (GO) flakes in drinking-water filters;
- Few-layer graphene (FLG) in battery management systems (BMS);
- Graphene-related materials (GRM) in car polish consumer sprays;
- Carbon nanotubes (i.e. CNTs) in car-seats; and
- Poly lactic-co-glycolic acid (PLGA) for inhalable antibiotics.

… selected to conduct:

- SSbD: Safe-&-Sustainable-by-Design
- LCA: Life-Cycle Assessment
- LCC: Life-Cycle-Costing
- MFA: Material-Flow Analysis
- RA: Risk-Assessment



The MACRAMÉ's Demonstrator
– Analyses, Assessments & Validations in industrial Value-Chains –

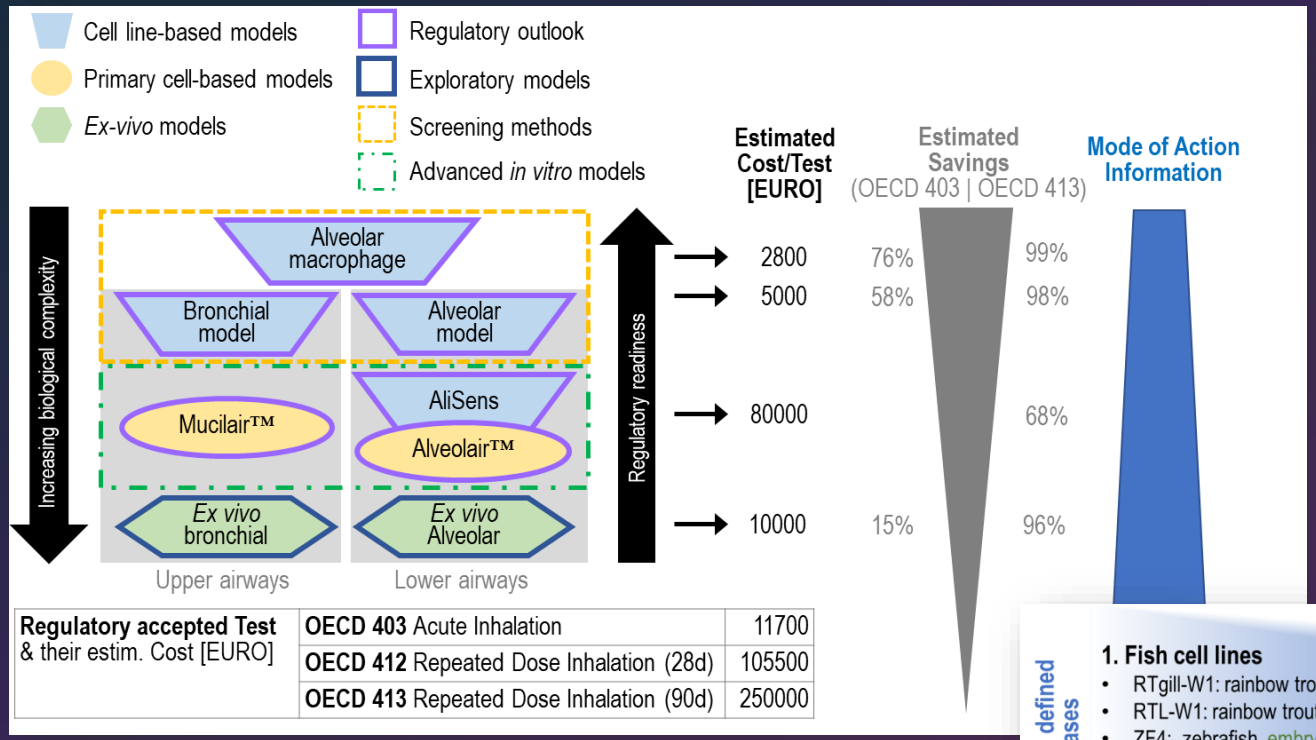


Legend:
AdMa = Advanced Material
CM = Complex Matrix
- EM = Environmental Matrix
- BM = Biological Matrix
- CPM = Complex Product Matrix

EoL = End-of-Life

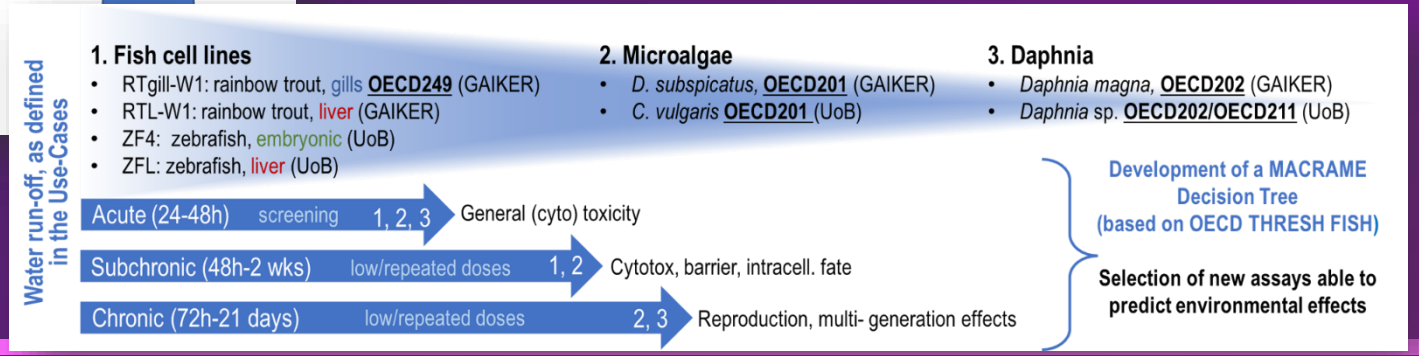
AdMa@CMs
- AdMa@EM
- AdMa@BM
- AdMa@EoL(CPM)
- AdMa@EoL(CPM)@EM

The MACRAMÉ Toxicology Laboratory
– Characterisation & Testing of AdMas in complex Matrices –



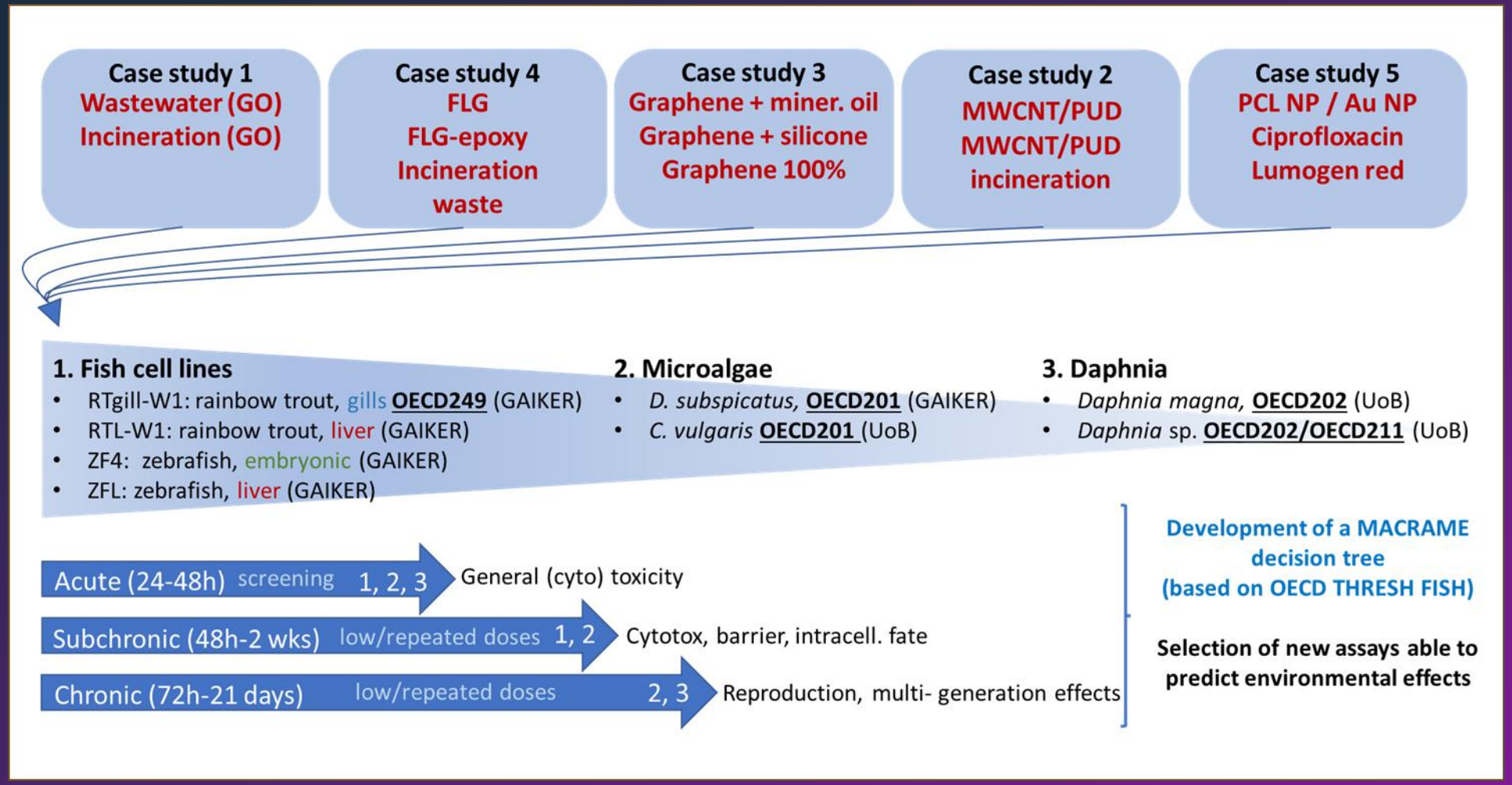
Regulatory accepted Test & their estim. Cost [EURO]		
	OECD 403 Acute Inhalation	11700
	OECD 412 Repeated Dose Inhalation (28d)	105500
	OECD 413 Repeated Dose Inhalation (90d)	250000

In vitro and *ex vivo* Models that will be assessed within the Project, indicating the estimated cost that can be saved and MoA- Information that can be obtained at the different tiers.



Schematic illustration of the MACRAMÉ ecotoxicity testing approach and outcomes.

The MACRAME's tiered eco-toxicological approach



Roadmap towards harmonized methods for *in vitro* lung inhalation toxicology

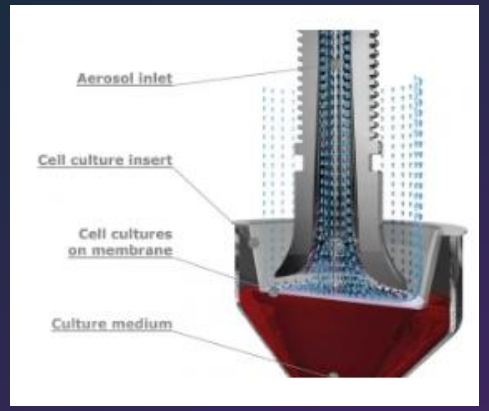
- How to generate reproducible and realistic aerosol?
- How to generate appropriate suspension for submerged test (e.g. the AMA assay)

How predictive are the *in vitro/ex vivo* biological systems for regulatory relevant endpoints?

How to administer reproducibly aerosols to *in vitro* biological systems and determine the delivered dose?

How do the *in vitro* system cope with real life materials/products?

Many different exposure systems … many variables



- Venturi nozzle
- Rotating drum
- Thermal precipitation

Cyto-TP
Continuous flow

Vibrating mesh
Cloud System

High pressure nebulization

Chemical "printing"

Pro and cons of each aerosolization and exposure method

	Pro	Cons
Submerged exposure	• Well established dispersion protocols • Deposition can be performed prior of addition of cells	• Often are necessary additives (e.g. EtOH, BSA, PVP) • Loss of soluble compounds
Continuous flow	• Very realistic aerosols Can work with dry or wet materials • Compatible with water-or organic-solvents-based solutions	• Requires large amounts of materials • Low deposition efficiency • No assumptions can be done on dose • Long handling and cleaning time
Cloud System	• Very short handling and cleaning time • Dose can be estimated based on nominal dose	• Requires water-based solutions • The nebulization of long/large materials needs to be assessed • Cannot work with dry materials
High pressure nebulization	• Very short handling and cleaning time • Requires small amounts of material • Compatible with dry material • Dose can be estimated based on nominal dose	Still under testing
Printing	• Very short handling and cleaning time • Compatible with water-based or organic-solvents-based solutions • Absolute dosing	• Cannot work with dry materials • Only small/short materials can be used

Experimental approach for aerosolization

Test materials

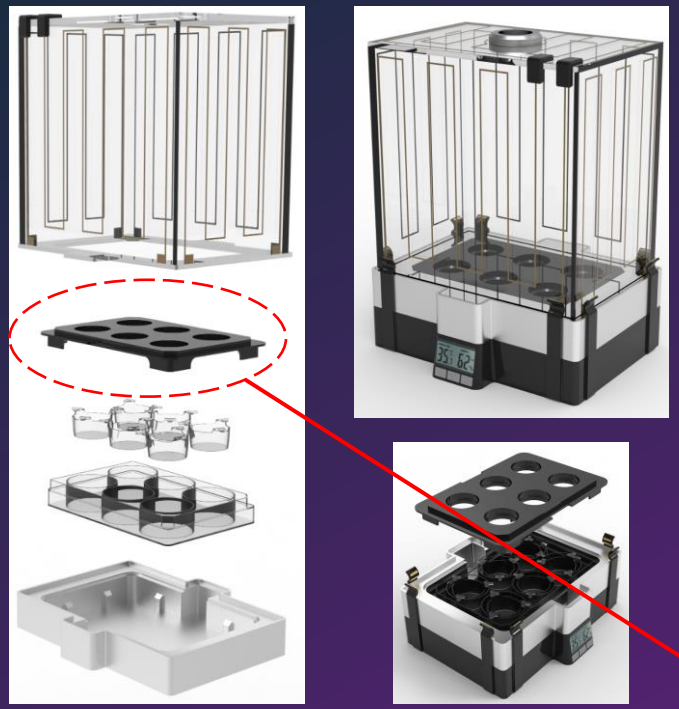
Material	Reference / Supplier	Concentrations	Note
Corundum particles	JRC	10-40 μg / cm^2	Negative control particles
Quartz DQ12	IBE	10-40 μg / cm^2	Positive control particles
JRC NM401	JRC	TBD	Fiber, Multi-walled Carbon Nanotubes
Clean Air Control			

Characterization of material deposited after aerosolization

- SEM pictures of grids deposited in transwell
- Microbalances measure the deposited doses (whenever applicable)
- Measurement of necessary deposition time
- Optimization of handling time (set-up, exposure, cleaning)

Limited exchange of equipment is foreseen.

Further testing of the NebuLIST



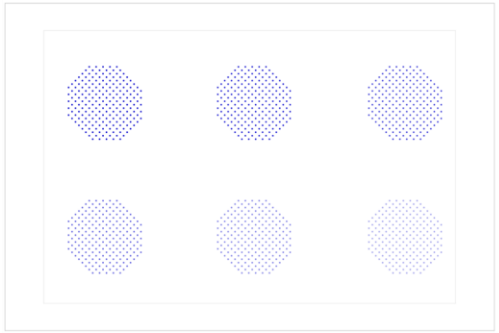
IP developed by LIST and VitroCell within the HEU Project PHOENIX offering the following advantages:

- higher deposition efficiency
- faster set-up and cleaning operations
- increased stability and reproducibility
- complete separation of the apical side from the basolater side
- reduced risk of biological contamination
- reduced risk of chemical carry over

- useful for centering and keeping still the inserts when exposing with the TECAN digital dispenser

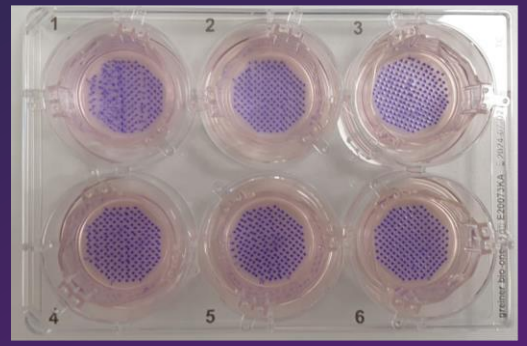
TECAN D300e digital dispenser

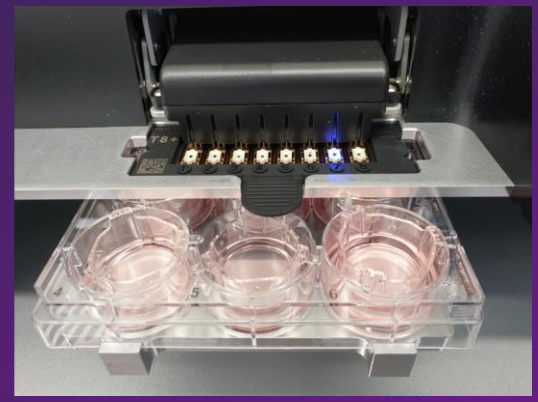
Name	Grid	Spacing (mm)	Size (mm)	Spots	nL per sports *1 nL mini*	total (nL)	Pattern
200 spots - 22 x 0,65 mm	22 x 22	0,9	18,9	200	30,0	6000	



Titration:

DMSO nL/spot	DMSO nL total	Chemical nL/spot	Chemical nL total
25	5000	5	1000
20	4000	10	2000
15	3000	15	3000
10	2000	20	4000
5	1000	25	5000
0	0	30	6000





DMSO dispensing

Selected dispensehead cassette specifications for DMSO dispensing:

- 70-100% DMSO.
- Nanoparticles < 1 micron in diameter in stable suspension at concentrations ≤ 0.5%.

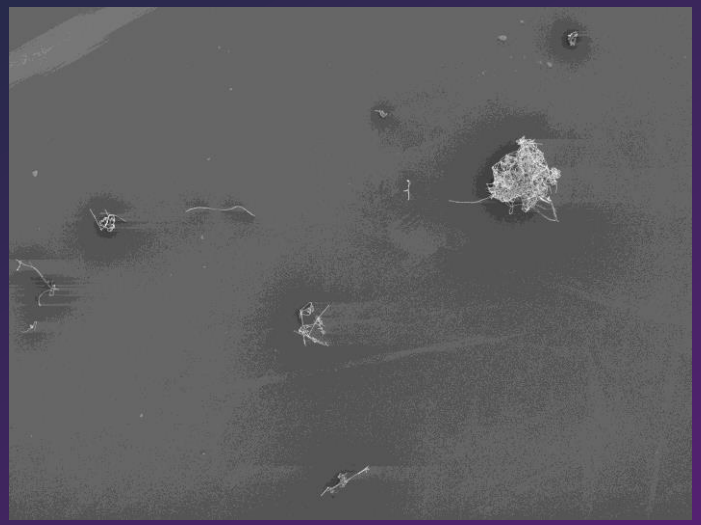
Surfactant	Surfactant Concentration	Glycerol
Brij 35	0.1 %	0 - <5%
Brij 35	0.1 %	5 - 20%
Triton X100	0.1 %	0 - <5%
Triton X100	0.1 %	5 - 20%
Tween 20	0.3%	0 - <5%
Tween 20	0.3%	5 - 20%

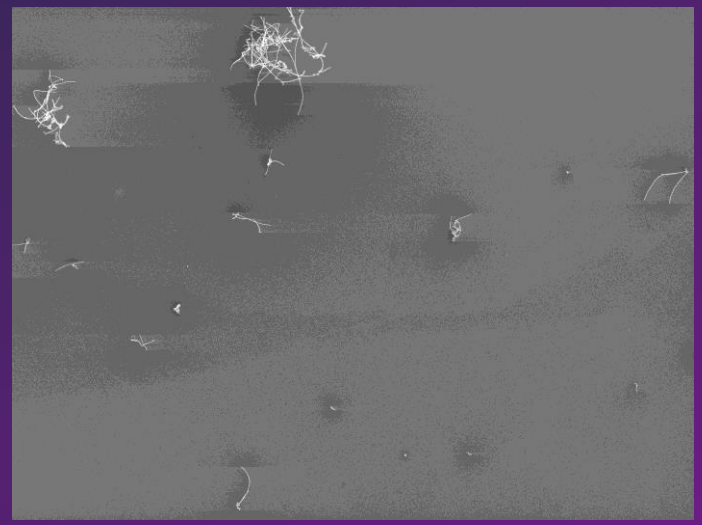
Some very preliminary results for the PowderX

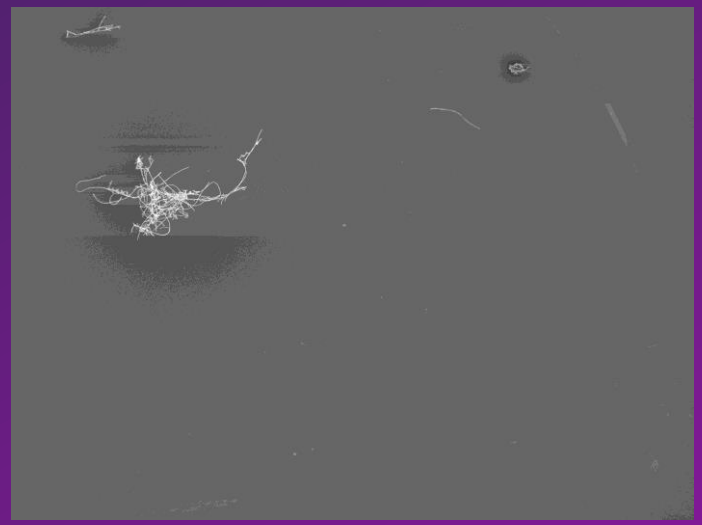
NM-401

Preliminary results (type of deposited objects):

- 25-50% single fibres
- 15-25% high aspect ratio fibrous agglomerates
- 20-40% low aspect ratio fibrous agglomerates







Materials' control library

- Quartz DQ12 – IBE
- Corundum ERM-FD066 – JRC
- ZnO (NM-111) – JRC
- TiO2 (NM-105, NM-101) – JRC
- SiO_2 NM-201, NM-203) – JRC
- CeO2 (NM-212) – JRC
- BaSO4 (NM220) – JRC
- Mn_2O_3 - ACS Materials
- Au NPs (20 nm) – MyBiotec
- Au NPs (50 nm) – MyBiotec
- SiC nanowires – ACS Materials
- MWCNTs (NM401) – JRC
- Bentonite (NM-600) – JRC
- WC – (1 of 2 still to be selected) nanoarmour

Materials selected for the validation of AMA (Tier 1)

A subset will be used for higher Tiers testing

- PLGA (empty, lumogen dye, cyprofloxacine, lumogen+cyprogloxazine) – MyBiotec
- PLGA-Au (50 nm) – MyBiotec
- PCL (empty, lumogen dye, cyprofloxacine, lumogen+cyprogloxazine) – MyBiotec
- PCL-Au (50nm) – MyBiotec

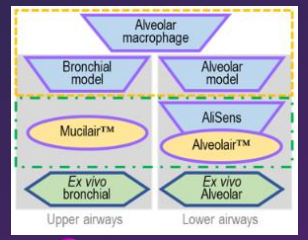
Dispersed UC5 materials, partly modified for bio-imaging studies

The MACRAME's tiered lung approach

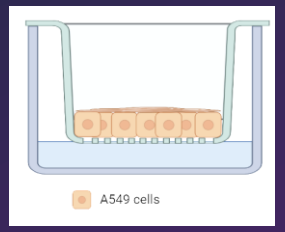
MOA information

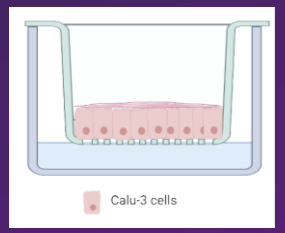
Cost saving

Tier - 1

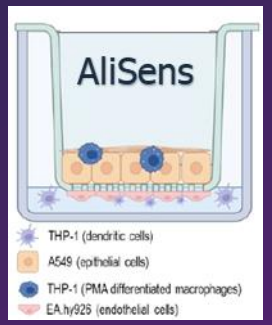


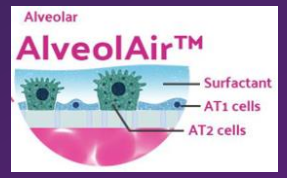
Tier - 2

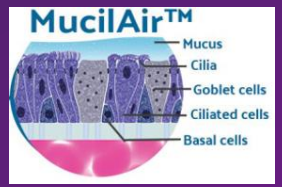




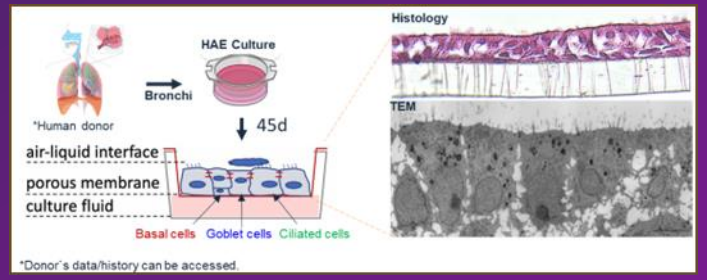
Tier - 3







Tier - 4



* Alveolar Macrophage by Dennis Kunkel Microscopy/science Photo Library

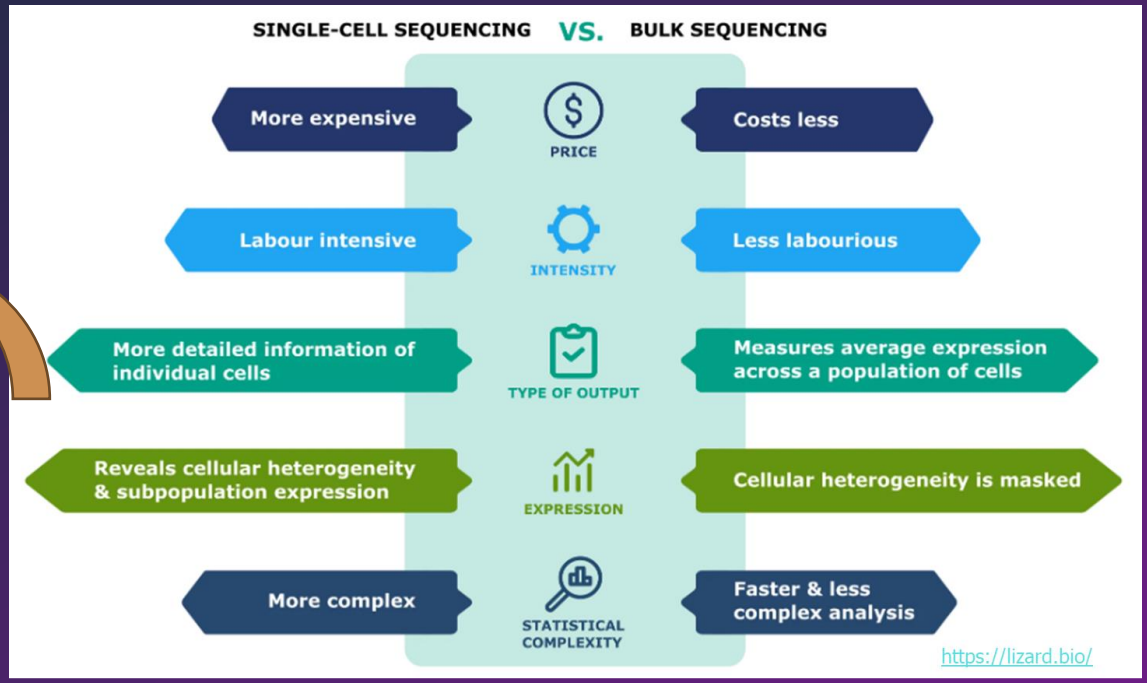
Qualification of the biological systems using reference materials

- Cell viability (e.g. Alamar Blue)
- Cytotoxicity detection by FACS (Sytox Blue, Thermo)
- Oxidative stress detection by FACS (e.g. CellROX™ Deep Red Reagent, Thermo)
- Cytokines synthesis/release (in supernatants or cell lysates)
- Cell morphology and particle uptake (special preparation e.g. for enhanced dark field, fluorescence microscopy, SEM, TEM)
- Genotoxicity (e.g. micronuclei using a FACS approach or in vitro COMET)
- Optimization of the protocol for dispersion of cells from 3D models for FACS analysis.

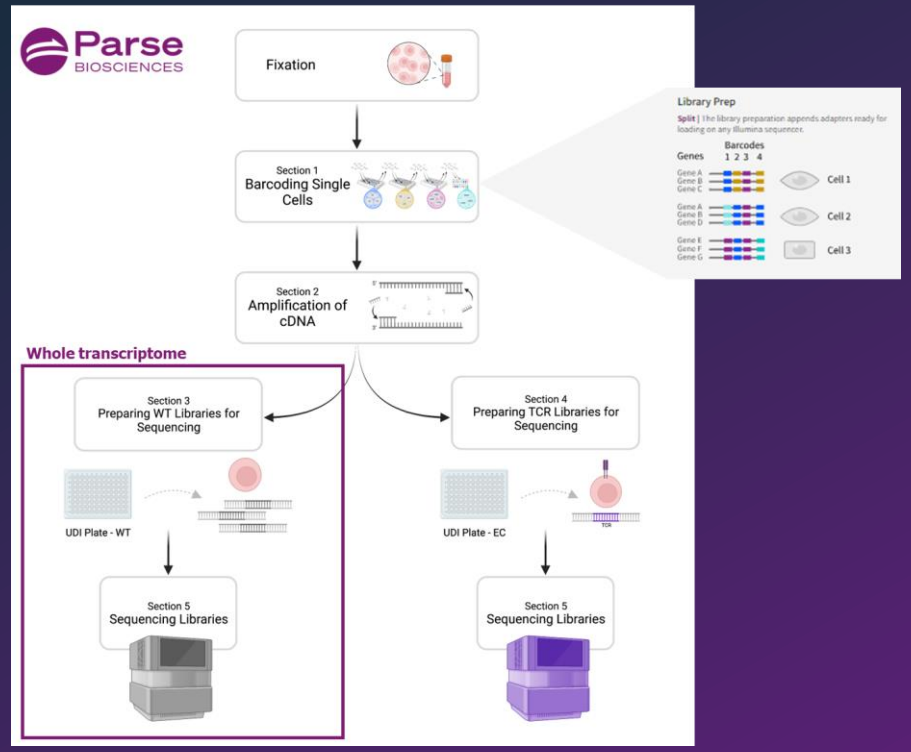
Advanced characterization of the biological systems and molecular techniques – scRNA-seq applied to NAMs

Single cell transcriptomics vs bulk transcriptomics

Better inform decision-making via improved biomarker identification



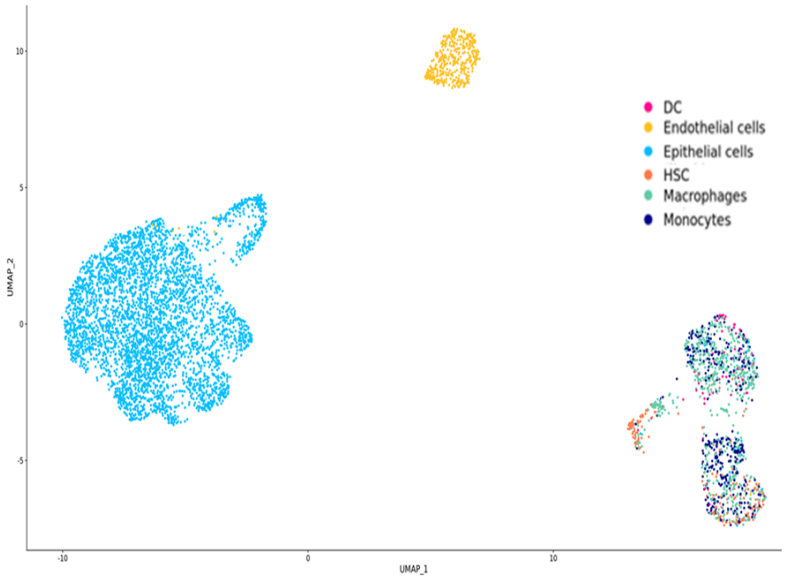
Experimental comparison sc-RNA-Seq vs Bulk RNA-Seq

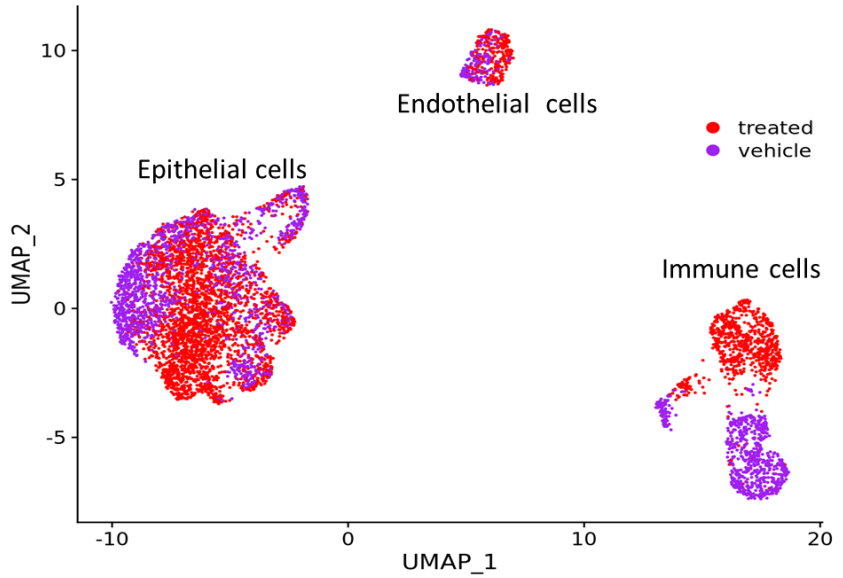


		Sc-RNA-Seq				Bulk RNA-Seq			
Chemical	Biol. Resp	Biol Rep.	N° conc.	Times	N° of lib.	Biol Rep.	N° conc.	Times	N° of lib.
LPS	Immune resp.	2	2	2	8	3	2	2	12
Acrolein	Irritant	2	2	2	12	3	3	2	18
Salicylic Acid	Irritant	2	2	2	4	3	1	2	6
Trimellitic anhydride	Resp. Sens.	2	1	1	4	3	2	1	6
Ethylenediamine	Resp. Sens.	2	1	1	4	3	2	1	6
Mercaptobenzothiazole	Skin Sens.	2	1	1	4	3	2	1	6
Diphencyprone	Skin Sens.	2	1	1	4	3	2	1	6
DMSO	Vehicle	2	1	1	4	3	2	1	6

Some (very) preliminary results of single cell trascriptomics

Use of SingleR algorithm to match the clusters to known cell types





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