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

MACRAMÉ's Approach towards the Harmonisation of *in vitrol ex vivo* Models for Inhalation Toxicology

WORKSHOP ON "HARMONISATION AND STANDARDISATION OF TEST METHODS FOR NANO AND ADVANCED MATERIALS" 22ND – 23RD NOVEMBER 2023 - ONLINE



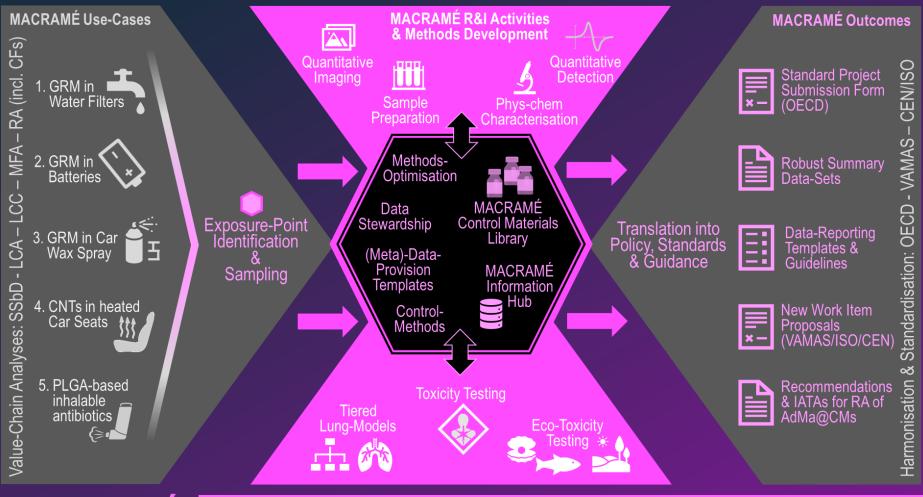
The MACRAMÉ project has received funding from the European Union's Horizon Europe Research and Innovation programme under grant agreement No. 101092686.

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 valuechain,
- 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



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).

Illustration of the MACRAMÉ R&I



... building on real, market-relevant Products

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market-relevant industrial MACRAMÉ Use-Cases:

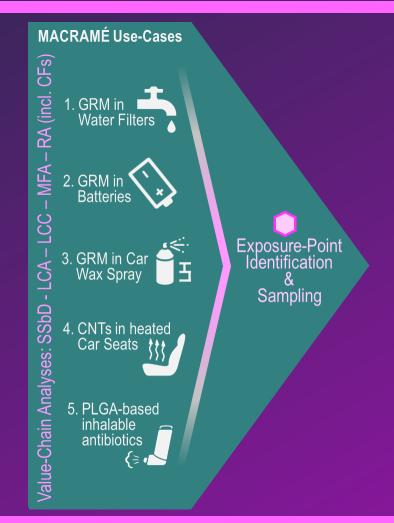
- Graphene oxide (GO) flakes in drinking-water filters;
- Few-layer graphene (FLG) in battery management systems (BMS);
- Graphene-related materials (GRM) in car polish consumer sprays;
- Carbon nanotubes (i.e. CNTs) in car-seats; and
- Poly lactic-co-glycolic acid (PLGA) for inhalable antibiotics.

... selected to conduct:

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



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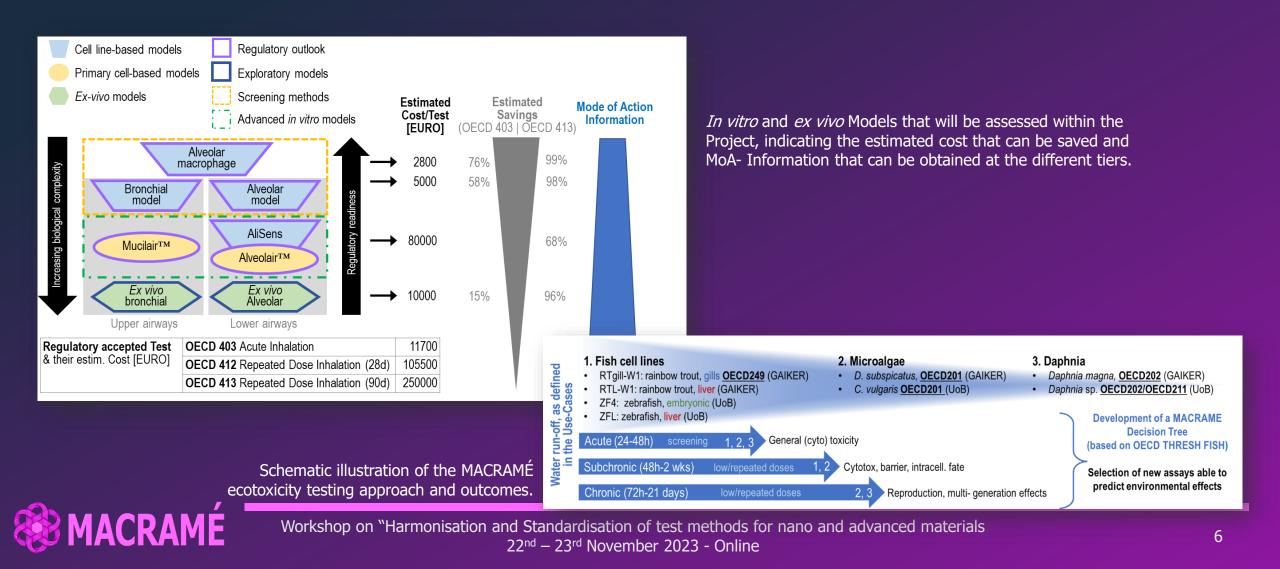


The MACRAMÉ's Demonstrator – Analyses, Assessments & Validations in industrial Value-Chains –

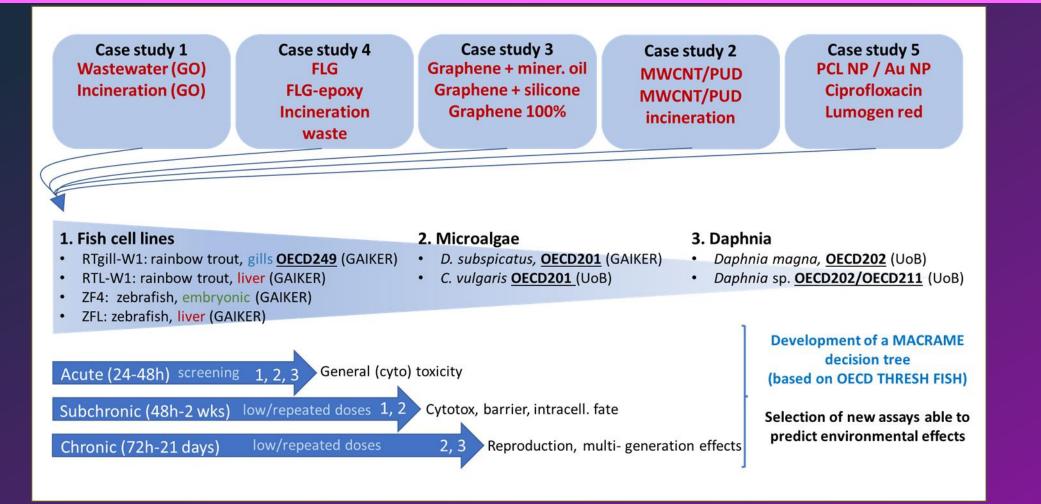
Samples & Constituents] (adva)	\bigcirc	MACRAMÉ Pristine AdMa AdMa (Un-)Bagging Product Reactor/ Machine (Un-)Bagging & (Professional) End-of-Life												
UC1: CO In Water, Filters Generation Sprays Pristine GL, polysidine, (matrix) CO@Polysidine-Fibre, Water from the spinning-process (solid samples) and reliculated (solid samples) and reliculated (solid samples)) (Hiered-water) Incinentiation products: Fl/segepoxy (stredded) Incinentiation products: Fl/segepoxy (stredded) Legend: Matrix UC2: CO in Water, Filters Gender Ge	Exposure Points		Manufacture & Transport		Manufacture Cleaning Transport		Transport	Intended Use	Shredding	Incineration	Release/Leakage			
Water-Filters FLG@epoxy (non-reliculated (fquid samples))** FLG@epoxy (contreated) FLG@epox (contreated)	[Sam	iples & Constituents	[AdMa]		[AdMa] - [AdMa@CPM] - [CPM]				[AdMa@EoL(CPM)] - [CPM(EoL)]					
AddMa AddMa@CMS AddMa@CSS AddMa@CSS AddMa@CSS AddMa@CSS AddMa@CSS	Jse-Cases & @ Exposure		Pristine GO, polysulfone (matrix)		GO@Polysulfone-Fibre, Water from the spinning-process		(filtered water) *	-						
AddWa Commercial CNTs (NanoCyl SA), polyurethane (PU)matrix CNTs@PU (masterbatch & cured composite) CNTs@PU (worheated, abraded (Taber)) Inimeration products (0depletion): Fly Ash, Flue-gas AddMa = Advanced Mit Commercial CNTs (NanoCyl SA), polyurethane (PU)matrix CMTs@PU (masterbatch & cured composite) CNTs@PU (worheated, abraded (Taber)) Inimeration products (0depletion): Fly Ash, Flue-gas AddMa = Advanced Mit Commercial CNTs (NanoCyl SA), polyurethane (PU)matrix AddWa = Advanced Mit CNTs@PU (worheated, abraded (Taber)) UC5: PLGA-particles (different sizes) Ciprofloxacine Controls: Au@PGLA, FeQ, QePLGA, PLGA (labeled with Lumogen red®) All UCs: - Aerosol generation for <i>in vitro</i> exposure - Characterisation of the generated aerosol - Characterisation of the generated aerosol - Characterisation at the generation for <i>in vitro</i> exposure - Characterisation at the generater of GRM - Adma@CMA		UC2: FLG in BMS	Pristine FLG, e	poxy resin	FLG@epoxy (non		oles) and reticulated	FLG@epoxy (overheated)		1				
Udd: CMIs in polymetriane (PU) matrix Commercial CMIs (ManCV) SA), polymetriane (PU) matrix CMIs (QPU) (sherefated, abraded (Taber)) CMIs (QPU) (sherefated, abraded (Taber)) Increation products (Q-depletion): Phy Ash, Flue-gas CM CM<			Ĭ		•			GRM@aerosol	GRM@aerosol(mate	I (in container), EoL according to container terial and/or special instructions				
All UCs: All		UC4: CNTs in Polymer Foils	Commercial CNTs polyurethane((NanoCyl SA), PU) matrix	CNTs@P	U (masterbatch & cured	l composite)	(overheated,				CM = Complex Matrix		
All UCs: - characterisation: - exactorisation: - exactor		UC5: PLGA-based inhal. Antibiotics			controls: Au@	≥PGLA, Fe _x O _y @PLGA,	ine@PLGA , PLGA (labelled with l	Lumogen red®)			ciprofloxacine@PLGA	Matrix		
Characterisation - Imaging (by Attributes) *high resolution imaging in cells UC1, UC2, UC4, UC5; UC1, UC2, UC4, UC5; UC1, UC2, UC4; ·characterisation ipresence of GRM ·characterisation of release of LoPPH ·characterisation of release of	Characterisation - Detection – Imaging (by Attributes)		 characterisation; aerosol generation & characterisation; stability in environmental & biological matrices; high resolution imaging in cells 		 Aerosol generation Characterisation or 	Aerosol generation for <i>in vitro</i> exposure Characterisation of the generated aerosol			EoL leaking from container/matrix Aerosol generation for <i>in vitro</i> exposure			 CPM = Complex Product Matrix 		
Human Toxicity Testing (in-vivo – ex-vivo) [Samples & Constituents] In case of inhalable release: Scheduled Tiered Lung-Model Approach (see Section 1.2, III.vii) AdMa@EoL(CPM) Fuedwide_EMI_ Individe_CPM_GEMI_ CPM@BM] [AdMa@EoL(CPM]					UC5: U • Identification, quantification &		 Identification: presence of GRM YES: quantification, 	entification: esence of GRM YES: quantification, • characterisation after mechanical abrasion (Taber) • characterisation after mechanical abrasion of		characterisation	 Identification of release of EoL- products (in land- 	AdMa@CMs o AdMa@EM		
Human Toxicity Testing (in-vivo – ex-vivo) [Samples & Constituents] In case of inhalable release: Scheduled Tiered Lung-Model Approach (see Section 1.2, III.vii) AdMa@EoL(CPM) [AdMa@BM] [AdMa@CPM] [AdMa@CPM@BM]- [CPM@BM] [AdMa@EoL(CPM]			[AdMa] - [AdMa@EM] - [AdMa@BM]		[AdMa			M@BM]	3		oL(CPM)@EM]			
E cotox Testing Strategy	(i	in-vivo – ex-vivo)								 AdMa@EoL(CPM)@EM 				
	[Sam	ples & Constituents]	[AdMa@	BM]		[AdMa@CPM] [AdM	la@CPM@BM]- [AdM	1@BM] - [CPM@BM]		[AdMa	@EoL(CPM]			
	Ecotoxicity Testing [Samples & Constituen		••	••		E	Ecotox Testing Strateg (see Figure 5)	ау 🔴 🔴 🖉						
[AdMa@EM] [AdMa@EM] - [AdMa@CPM@EM] - [CPM@EM] [AdMa@EoL(CPM)] [AdMa@EoL(CPM]]			[AdMa@EM]		[AdMa@EM] – [AdMa@CPM@EM] - [CPM@F			M] [AdMa@EoL(CPM)] [AdMa@EoL(CPM]			@EoL(CPM]			

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



The MACRAME's tiered eco-toxicological approach





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

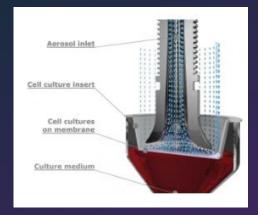
How to generate reproducible and realistic aerosol?
How to generate appropriate suspension for submerged test (e.g. the AMA assay)

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

Towards harmonized methods

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 				
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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.

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Further testing of the NebuLIST



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

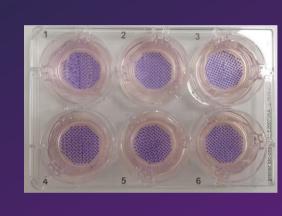
- higher deposition efficiency
- faster set-up and cleaning operations
- increased stability and reproducibility
- complete separation of the apical side from the basolater side
- reduced risk of biological contamination
- reduced risk of chemical carry over

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



TECAN D300e digital dispenser







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%		

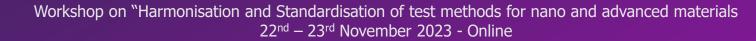
DMSO dispensing

Selected dispensehead cassette specifications for DMSO dispensing:

• 70-100% DMSO.

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• Nanoparticles < 1 micron in diameter in stable suspension at concentrations $\leq 0.5\%$.



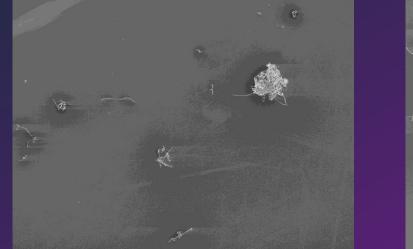
Some very preliminary results for the PowderX

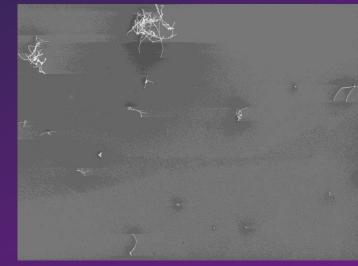
NM-401

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
- SiO₂ NM-201, NM-203) JRC
- CeÓ2 (NM-212) JRĆ
- BaSO4 (NM220) JRC
- Mn₂O₃ 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
- 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

Materials selected for the validation of AMA (Tier 1)

A subset will be used for higher Tiers testing

Dispersed UC5 materials, partly modified for bioimaging studies



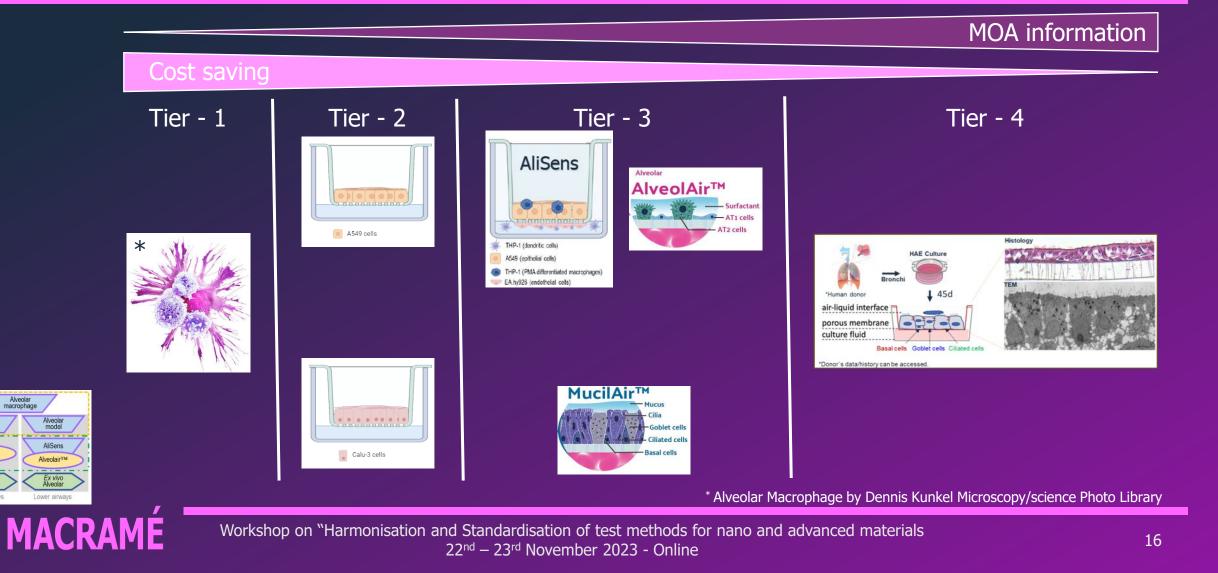
The MACRAME's tiered lung approach

Bronchial model

Mucilair™

Ex vivo bronchial

Upper airways

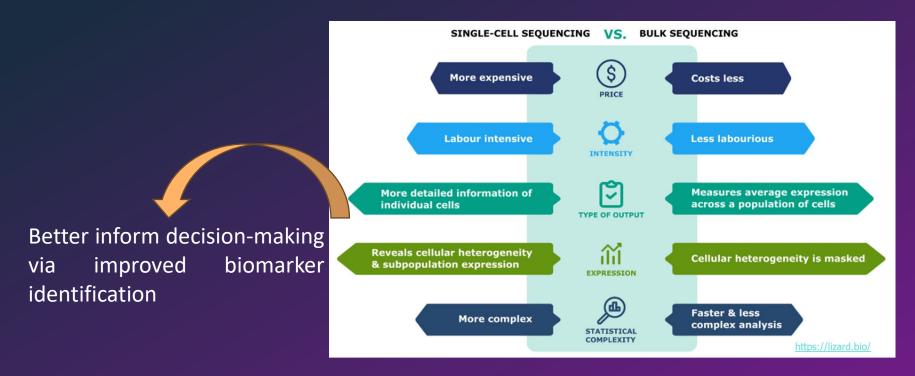


Qualification of the biological systems using reference materials

- 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.

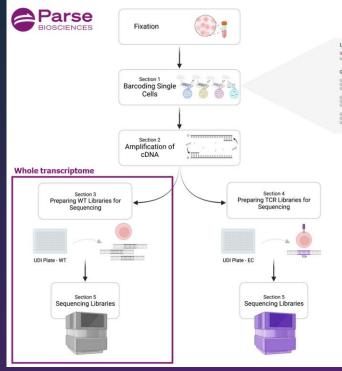
Advanced characterization of the biological systems and molecular techniques – scRNA-seq applied to NAMs

Single cell transcriptomics vs bulk transcriptomics





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

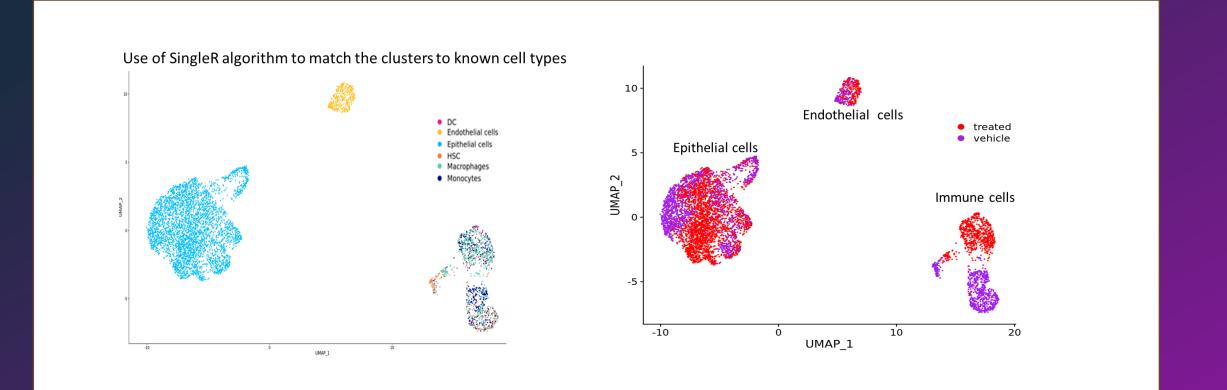


library	Prep		
	library prepara any illumina si		adapters ready for
Genes	Barcodes 1 2 3 4		
iene A - iene B - iene C -			Cell 1
iene A - iene B - iene D -			Cell 2
iene E - iene F - iene G -	-	1	Cell 3

		Sc-RNA-Seq Bulk RNA-Sec							
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
Mercaptobenzot hiazole	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



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Thank you ③



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