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Functional Effects of Carbon Black Nanoparticles on Primary Airway Epithelial Cells *in vitro*

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UNIKLINIK RWTH AACHEN

Klinik und Poliklinik für Hals-, Nasen- und Ohrenkrankheiten, plastische und ästhetische Operationen
Direktor: Prof. Dr. Dr. h.c. R. Hagen

HNO

UFP Conference 2022

Sources

Climate effects

Air quality

Characterization

International Symposium **5-6 July2022** Brussels, Belgium

Epidemiology

Health effects and mechanisms

Abatement strategies and policies

Decision finding

UFP research initiative in Germany

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BAY UFP
BAYERISCHER PROJEKTVERBUND ULTRAFEINE PARTIKEL

„…close existing knowledge gaps, to support risk analysis of ultrafine particles (UFP)“

(free translation)

- Measurement techniques
- Chemical characterization
- Epidemiology
- Molecular mechanisms
- Toxicology

HNO

UFP research initiative in Germany

Uniklinikum Würzburg

BAY UFP

BAYERISCHER PROJEKTVERBUND ULTRAFEINE PARTIKEL

Project partners

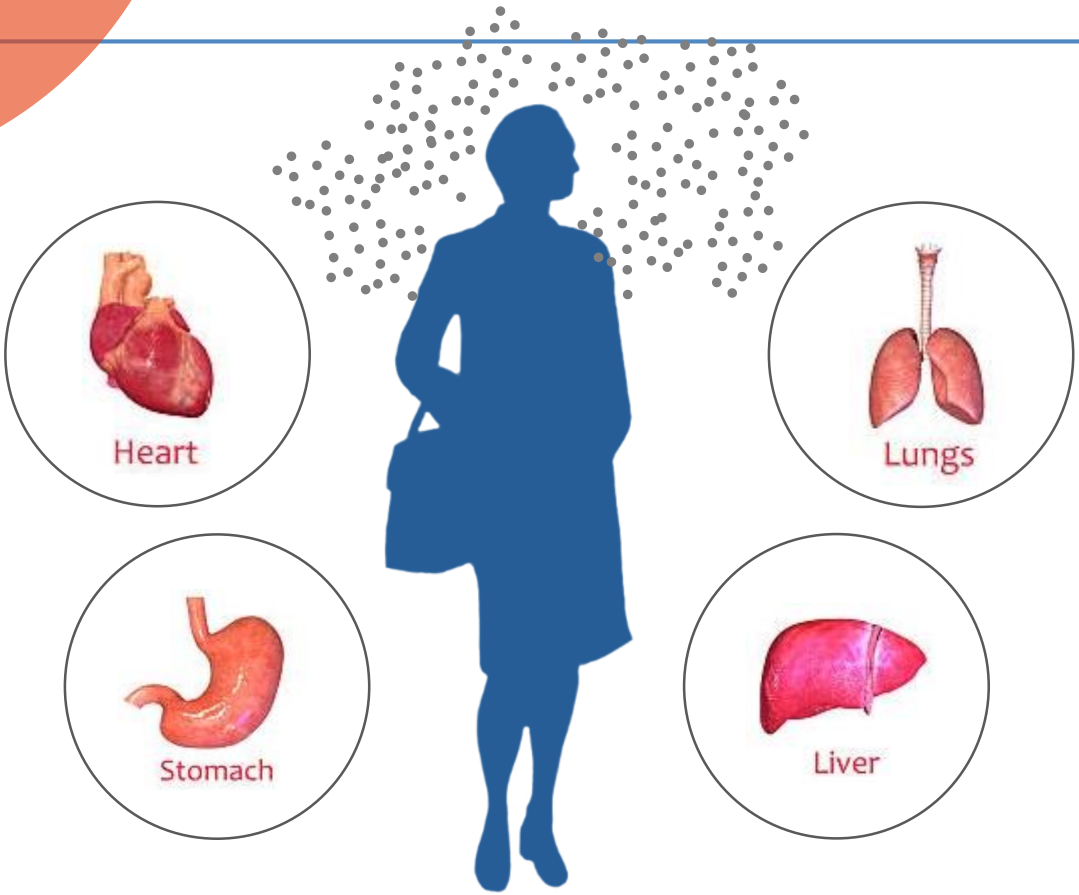
- **Friedrich-Alexander-Universität Erlangen-Nürnberg**
  - Institut und Poliklinik für Arbeits-, Sozial- und Umweltmedizin
    Prof. Dr. Hans Drexler
    Prof. Dr. Simone Schmitz-Spanke
- **Ludwig-Maximilians-Universität München**
  - Lehrstuhl für Epidemiologie am Institut für Medizinische Informationsverarbeitung, Biometrie und Epidemiologie
    Prof. Dr. Annette Peters
  - Institut und Poliklinik für Arbeits-, Sozial- und Umweltmedizin
    PD Dr. Stefan Karrasch
- **Universität Bayreuth**
  - Atmosphärische Chemie
    Prof. Dr. Anke Nölscher
- **Universitätsklinikum Würzburg**
  - Klinik und Poliklinik für Hals-, Nasen- und Ohrenkrankheiten, plastische und ästhetische Operationen
    Prof. Dr. Stephan Hackenberg
    (seit 2021 am Universitätsklinikum Aachen)

Collaborating partners

- **Helmholtz Zentrum München**
  Deutsches Forschungszentrum für Gesundheit und Umwelt
  - Cooperation Group „CMA" Prof. Ralf Zimmermann
  - Institut für Epidemiologie
- **Friedrich-Alexander-Universität Erlangen-Nürnberg**
  - Lehrstuhl für Kommunikationswissenschaft
- **Ludwig-Maximilians-Universität München**
  - Meteorologisches Institut
- **Universität Augsburg**
  - Wissenschaftszentrum Umwelt
- **Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit (LGL)**
- **Bayerisches Landesamt für Umwelt (LfU)**
- **NAKO e.V.**

HNO

Health effects and mechanisms

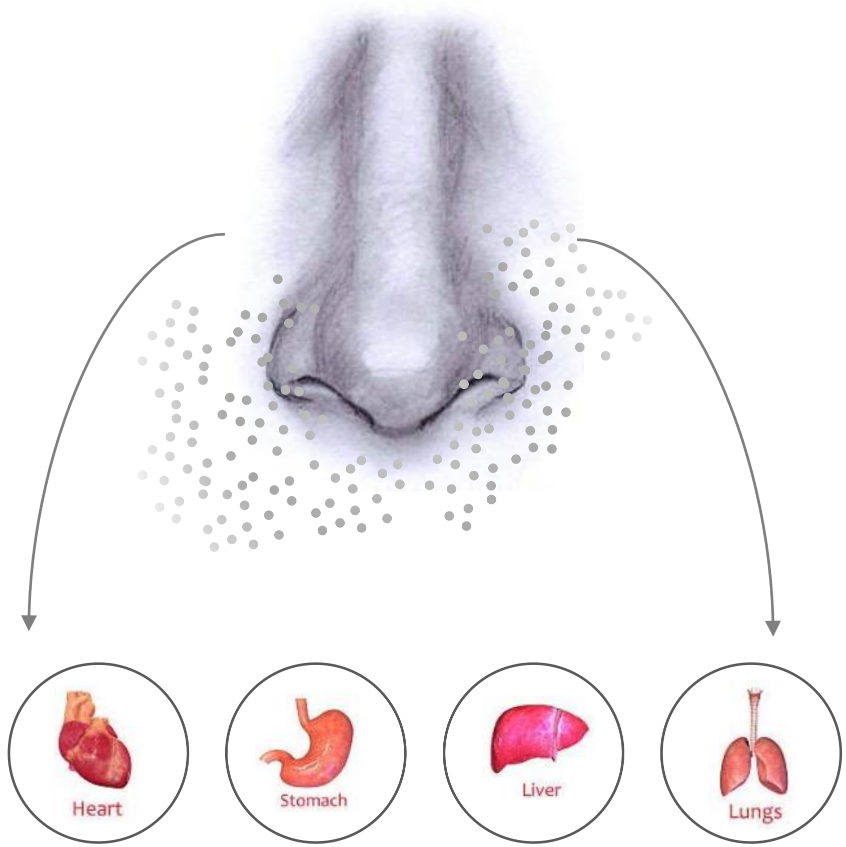


Determine risks: toolkit

For mechanisms, (sensible) simplification is necessary

Person: www.clipground.com; organs: media.istockphoto.com

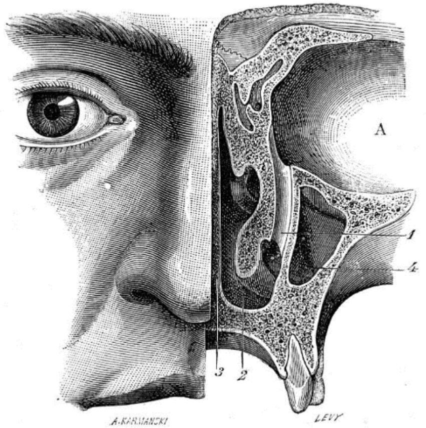
The nose is the entry point for aerosols

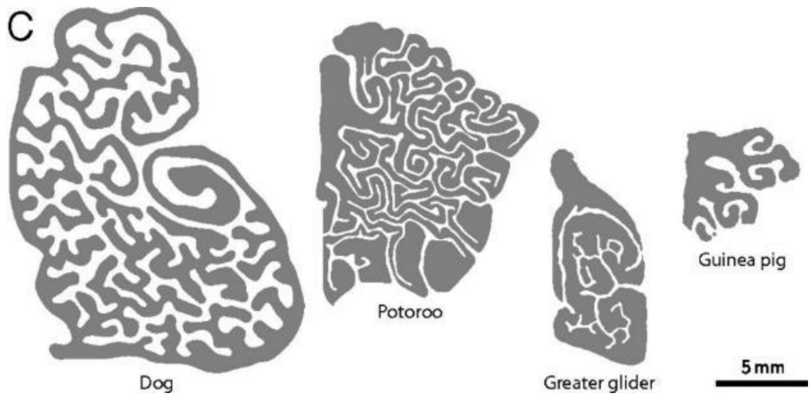


What happens to particles in the nose?

Nose drawing: apkpure.com nose drawing tutorials

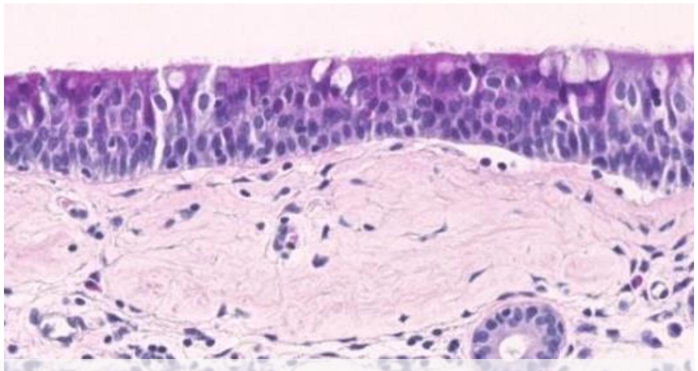
Transport through the nose



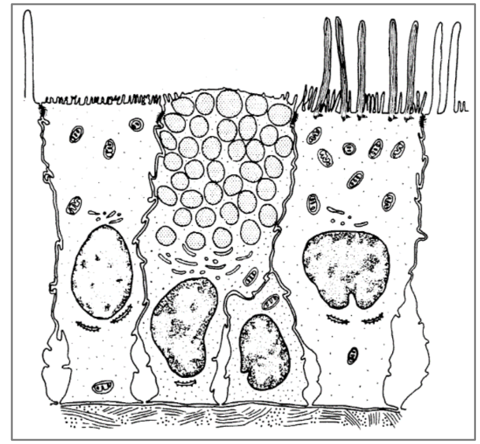


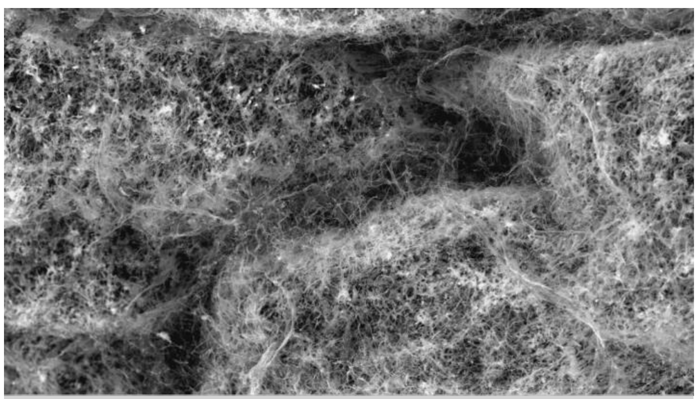
Which model options are available (and which make sense)?

Cellular level: what must we mimick?

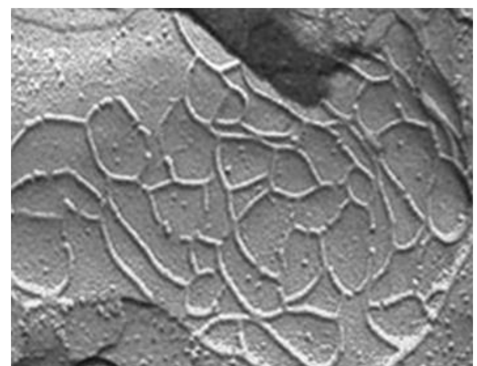


H&E staining, human nasal mucosa.
Image: Renate Bausch/Hieke ,Oberwinkler





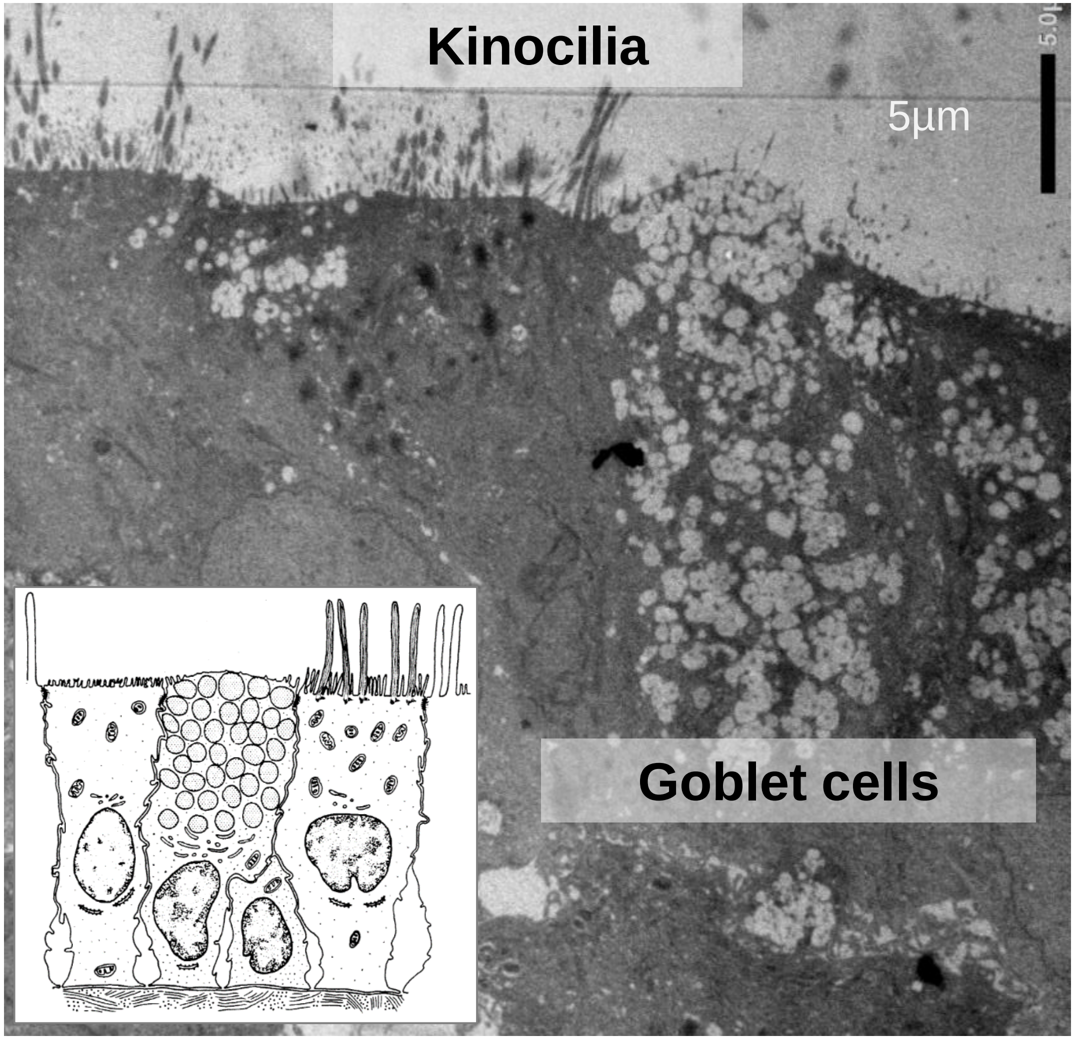
Mucin polymers. Image by Katharina Ribbeck lab.

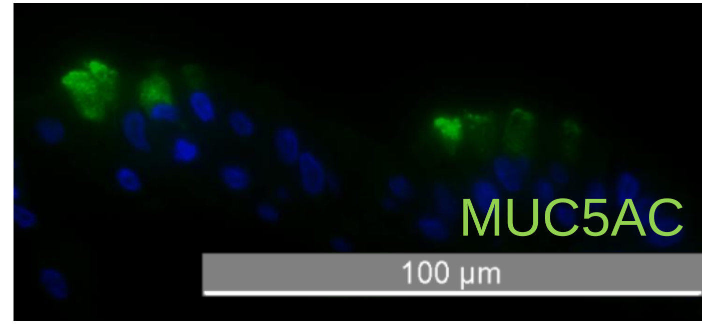


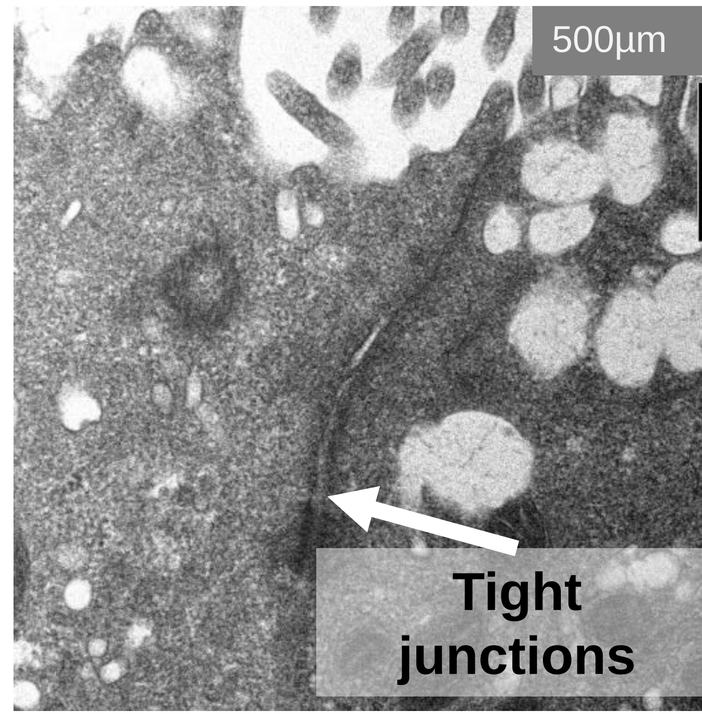
Freeze-fracture image of tight junctions in native nasal tissue by Kojima et al.

Model selection should be based on the objectives of the project

Human mucosal model of the upper airways







Cell types sketch: Mygind, Niels, and Ronald Dahl. "Anatomy, physiology and function of the nasal cavities in health and disease." Advanced drug delivery reviews 29.1-2 (1998): 3-12.

Our upper airway mucosa model

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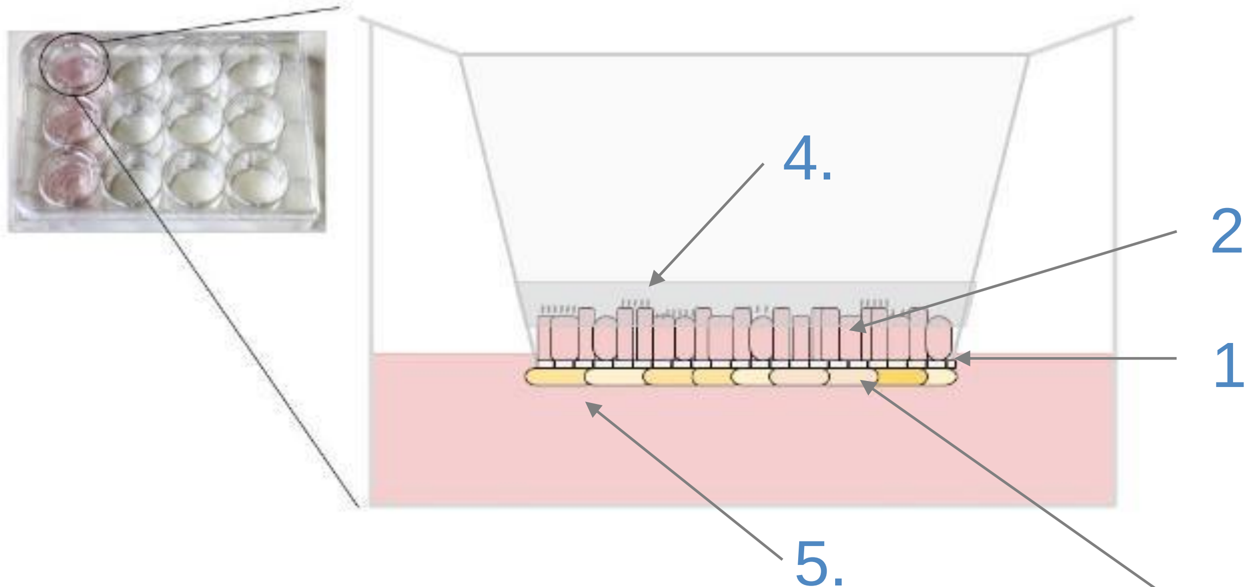
Human biopsies used to establish model from primary cells

Lower image from K. Ickrath's Doctoral Thesis



1. PET filter with 0.4 µm pores (collagen-coated)
2. Primary epithelial cells
3. Primary fibroblasts
4. Air interface with mucus → ALI
5. Liquid interface

Ultrafine particle exposures

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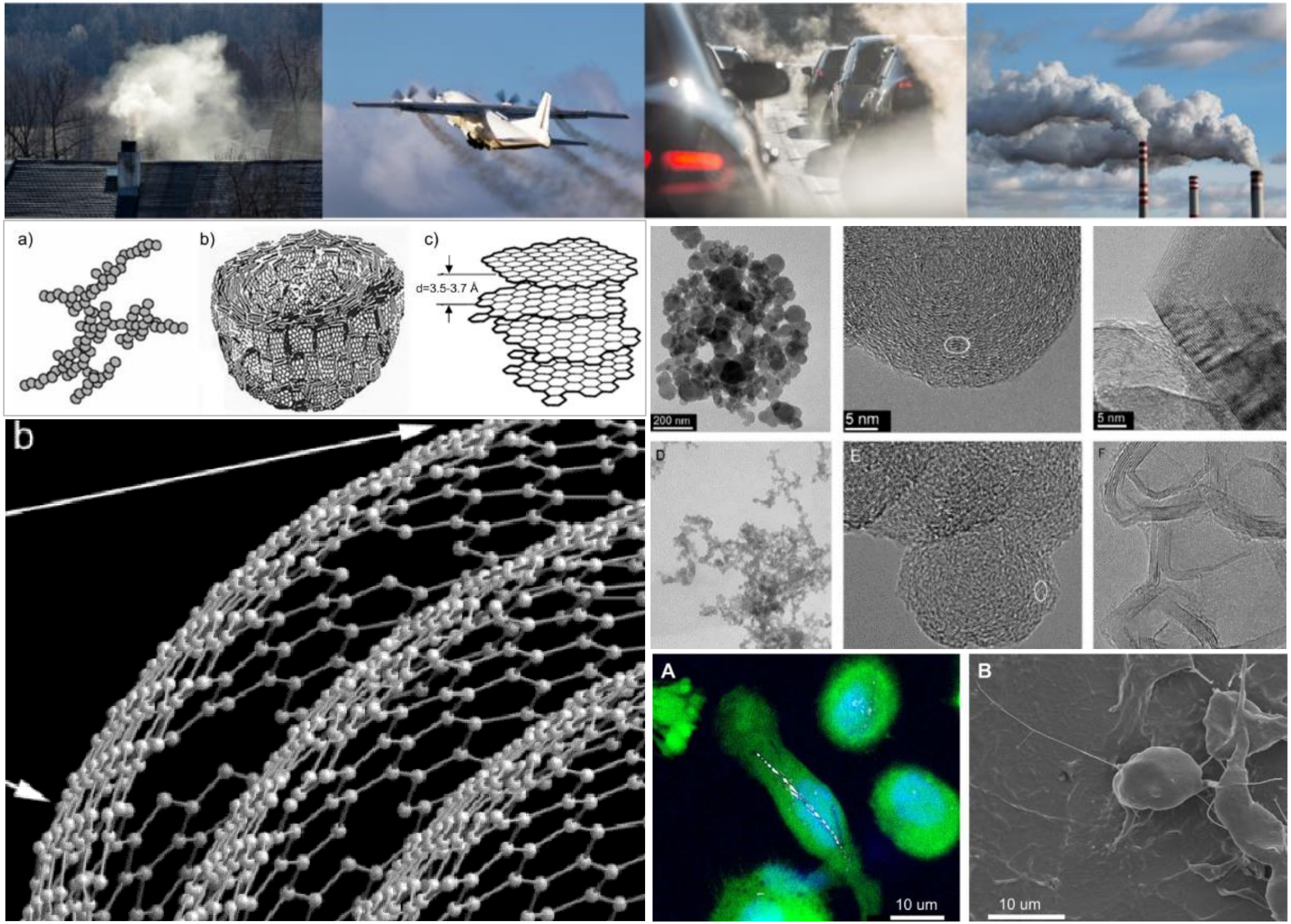
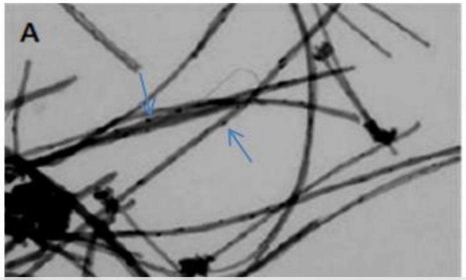


BOTTOM RIGHT confocal and SEM: Cheng, Crystal, et al. "Toxicity and imaging of multi-walled carbon nanotubes in human macrophage cells." Biomaterials 30.25 (2009): 4152-4160. ALL OTHER: Jurkiewicz, Karolina, Mirosława Pawlyta, and Andrzej Burian. "Structure of carbon materials explored by local transmission electron microscopy and global powder diffraction probes." C 4.4 (2018): 68.

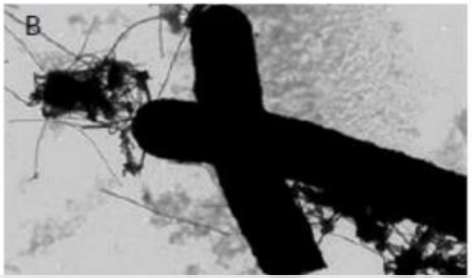
Submerged exposure

1. **Submerged exposure**
2. Nebulizer / Cloud system (Vitrocell)
3. Aerosol exposure (Vitrocell)

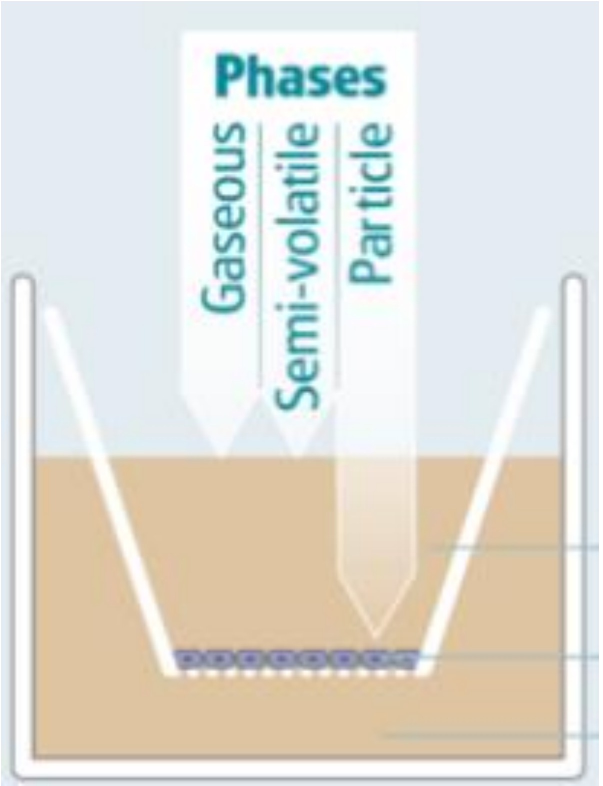
Example: Multi-walled carbon nanotubes (MWCNT NM-401)



Approx. 70nm wide, 4µm long



Agglomerates in sample



Kirsten, Rasmussen., et al. "Multi-Walled Carbon Nanotubes, NM-400, NM-401, NM-402, NM-403: Characterisation and Physico-Chemical Properties." (2014).

Cloud exposure

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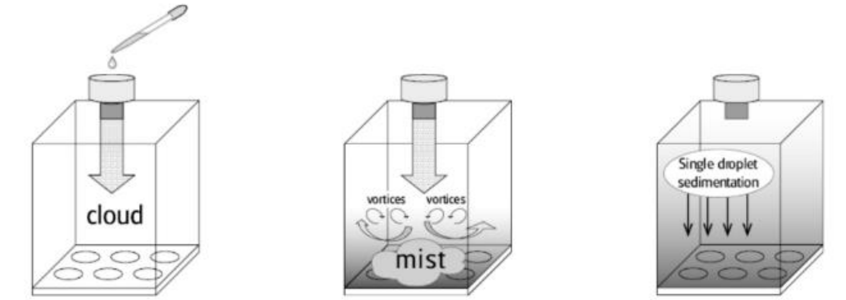
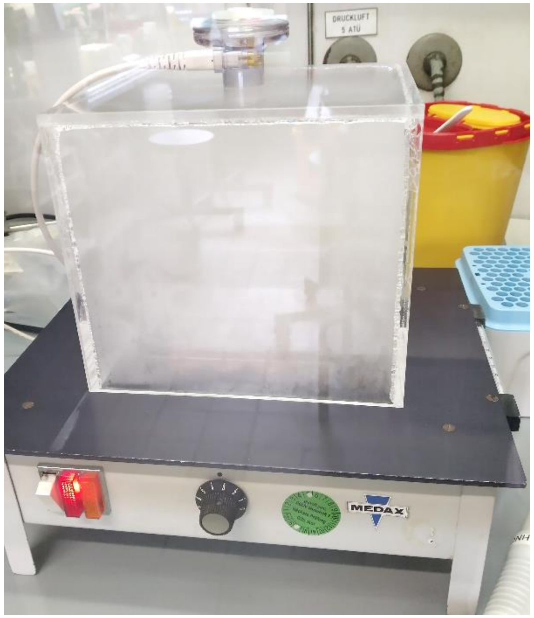
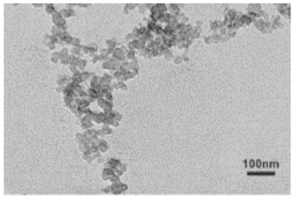
1. Submerged exposure
2. **Nebulizer / Cloud system (customized Vitrocell)**
3. Aerosol exposure (Vitrocell)



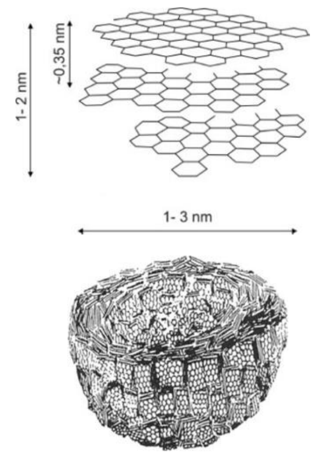
Phase 1: Emission Of Cloud Phase 2: Homogeneous Mixing Phase 3: Gravitational Settling



Example: Carbon black / Printex90



Basic structural units

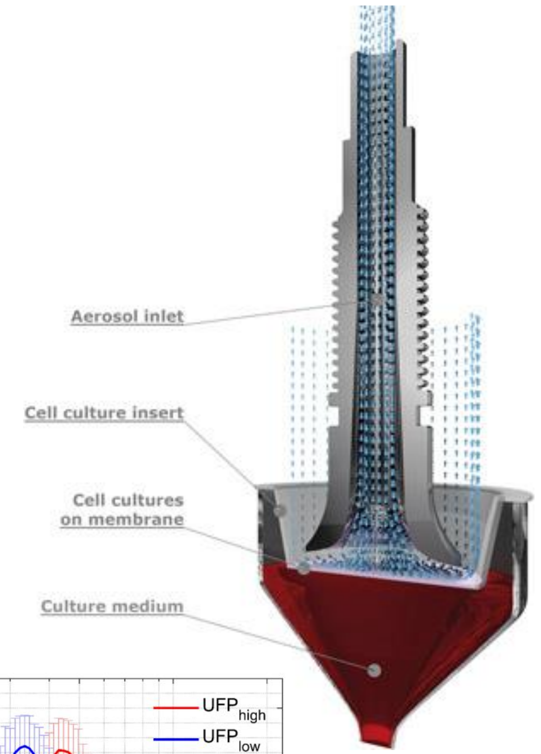


Printex90 TEM: Shen, Y et al.: Particle and fibre toxicology, 15. BSU and particle sketch: Pawlyta, Miroslawa, Jean-Noël Rouzaud, and Stanislaw Duber. "Raman microspectroscopy characterization of carbon blacks: Spectral analysis and structural information." Carbon 84 (2015): 479-490.

Aerosol exposure

Uniklinikum Würzburg UK

1. Submerged exposure
2. Nebulizer / Cloud system
3. **Aerosol exposure (Vitrocell) –** *collaboration Zimmermann/ di Bucchianico/ Delaval (Helmholtz German Research Center for Environmental Health)*

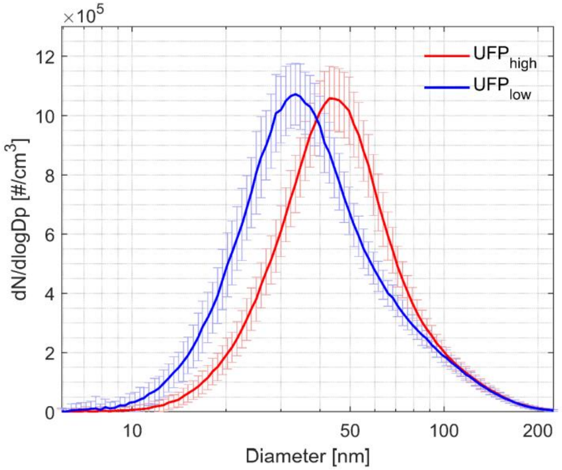


Generated Black carbon

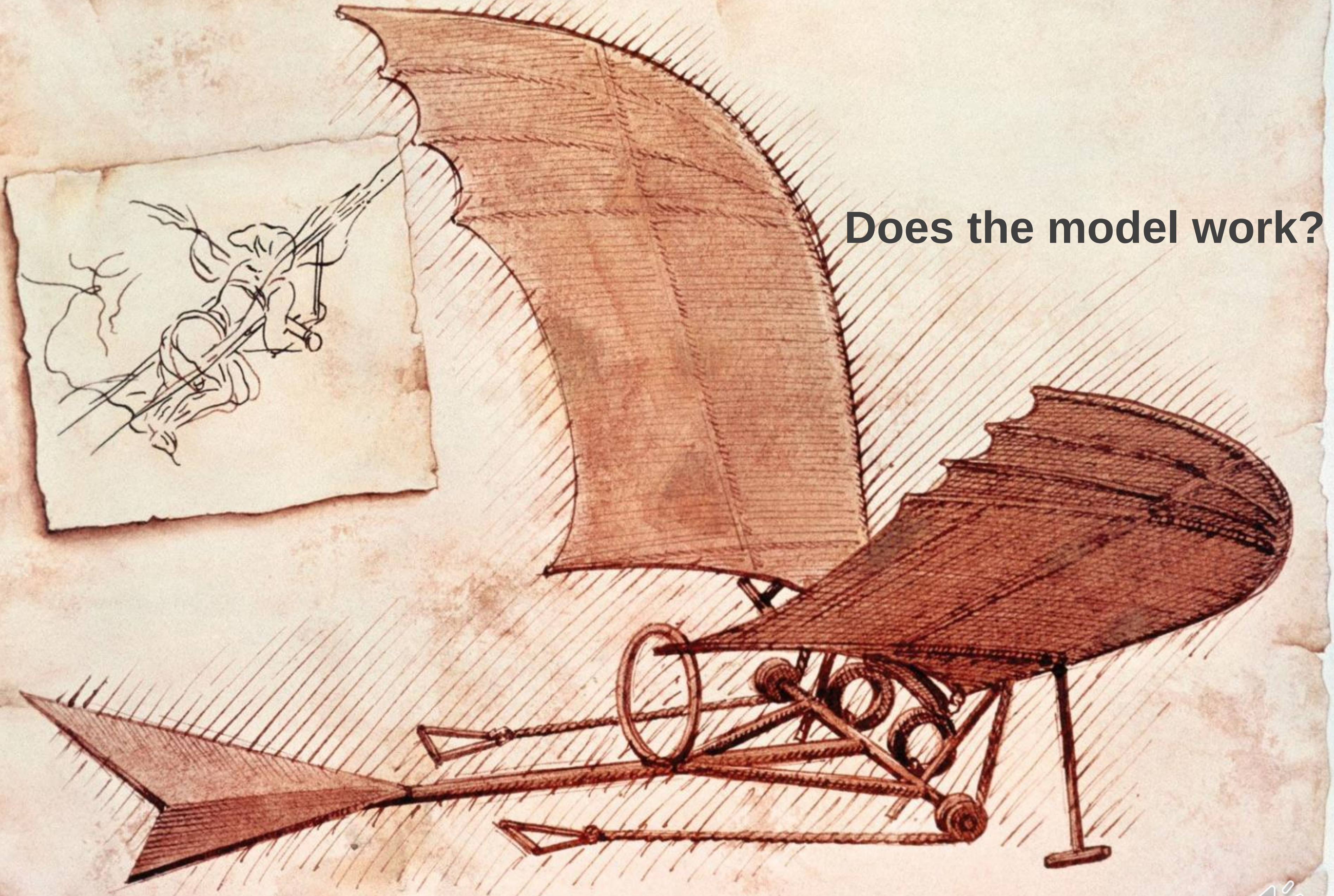
~40% elemental carbon, 60% organic carbon compounds

~45 nm diameter

Approximated dose: 1.5 ± 0.2 ng/cm^2

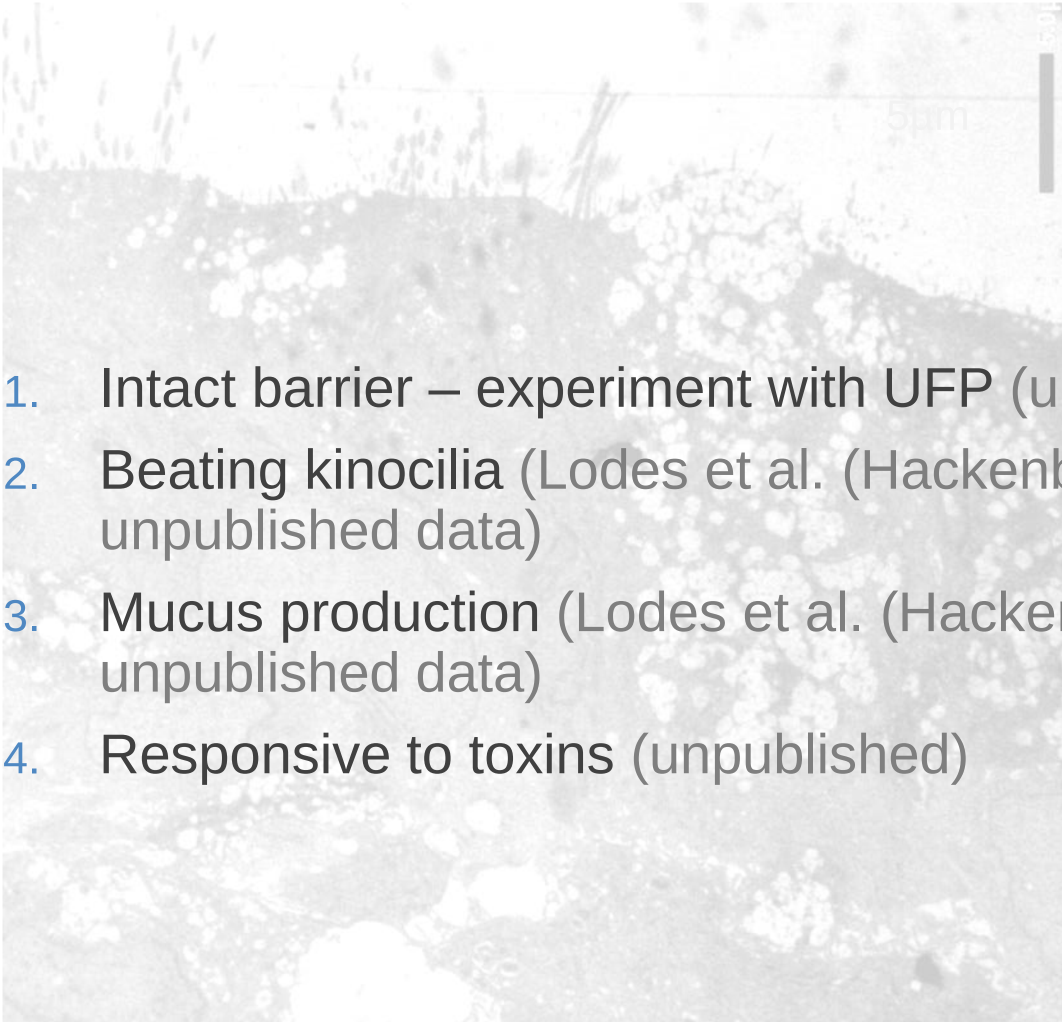


Does the model work?



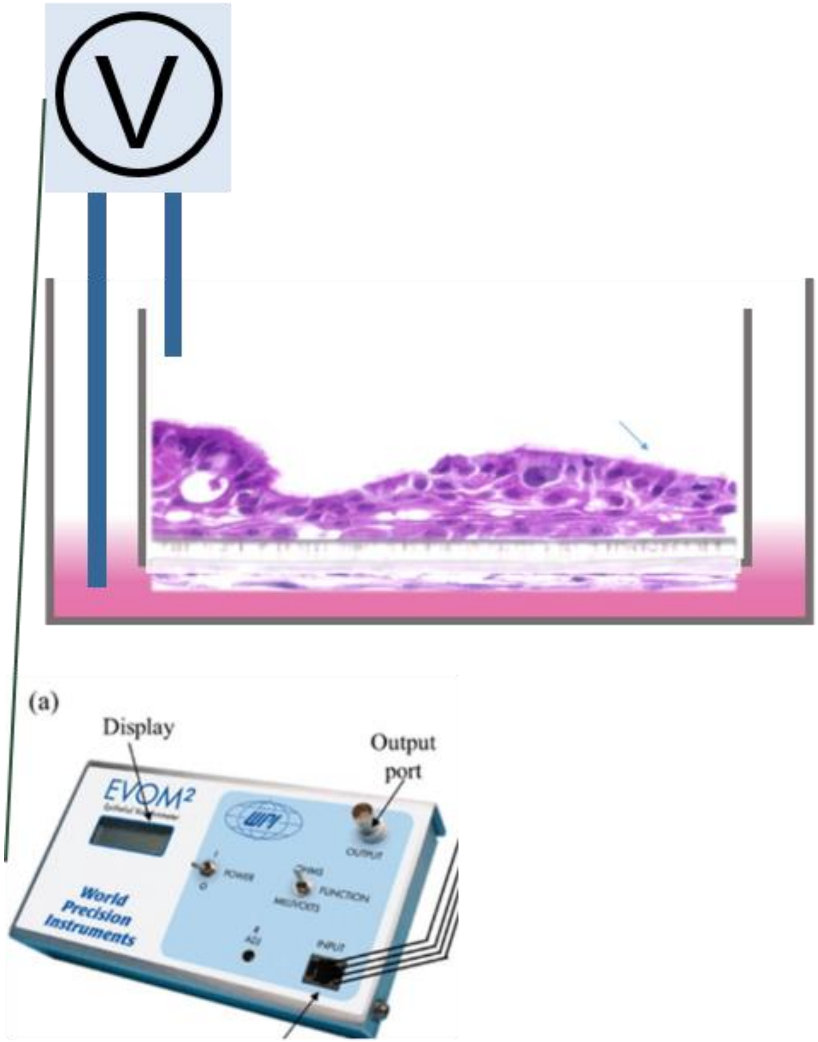
Copy of drawing by Leonardo da Vinci from
www.britannica.com/summary/Leonardo-da-Vincis-Achievements

Mucosal model functionality - data



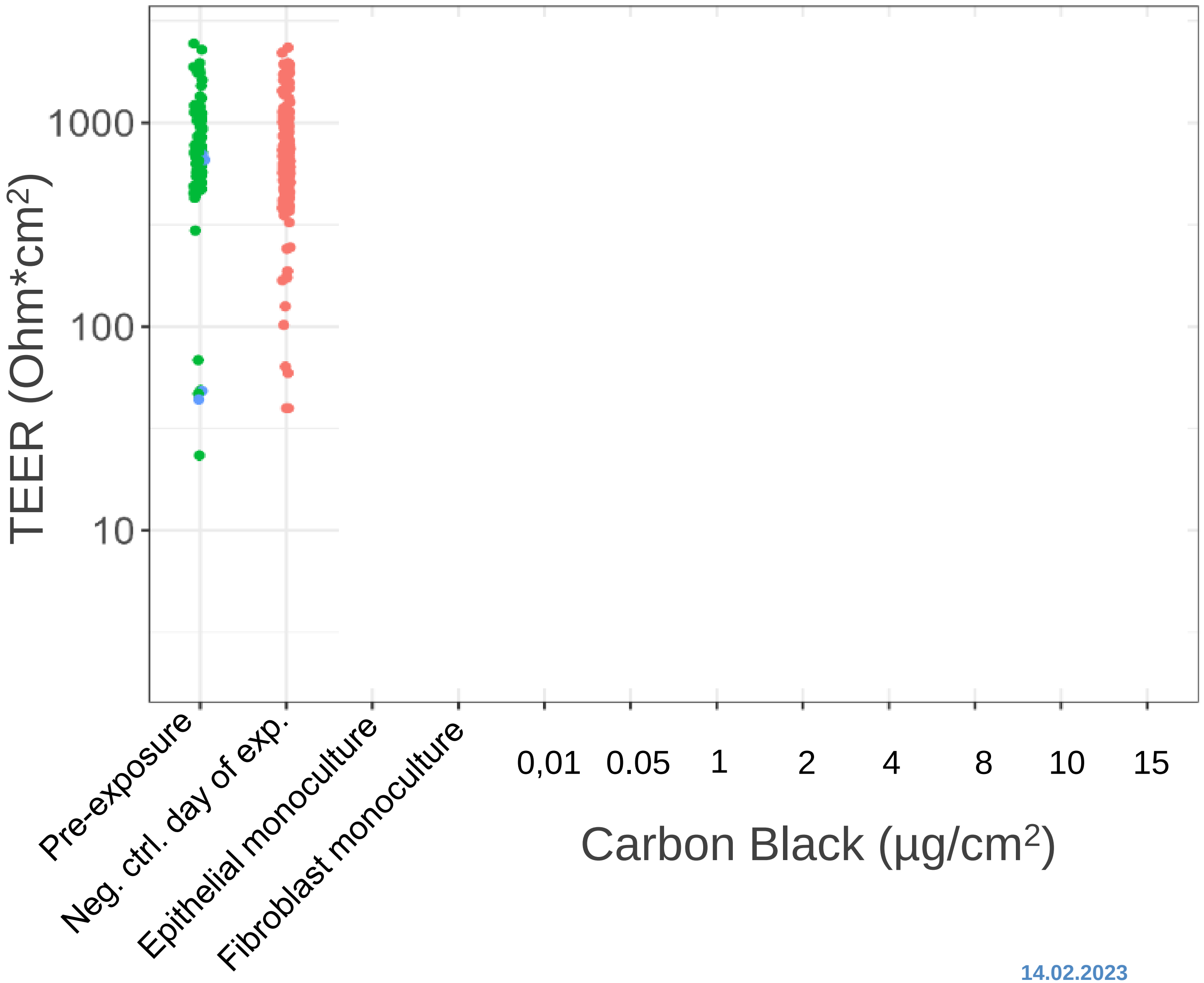
1. Intact barrier – experiment with UFP (unpublished)
2. Beating kinocilia (Lodes et al. (Hackenberg), 2020 + unpublished data)
3. Mucus production (Lodes et al. (Hackenberg), 2020 + unpublished data)
4. Responsive to toxins (unpublished)

1. Intact barrier – TEER data



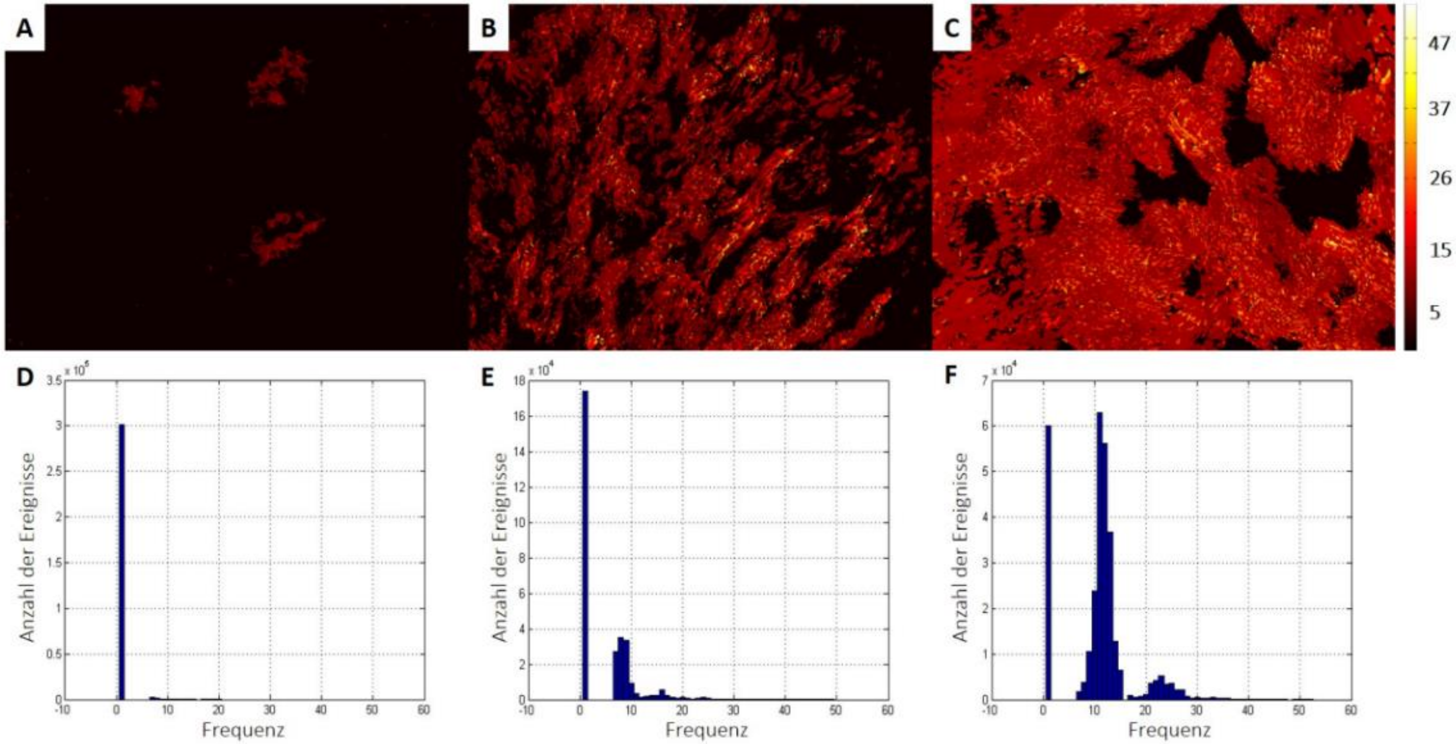
1. Intact barrier – potential effect of UFP

Transepithelial Electrical Resistance (TEER)



2. Beating kinocilia

Cell line HBEC3-KT | Primary bronchial cells | Primary nasal cells



Ciliary beat frequency and heat-maps of beating kinocilia: Dissertation by Nina Lodes, 2019 (co-supervised by S. Hackenberg)

2. Beating kinocilia

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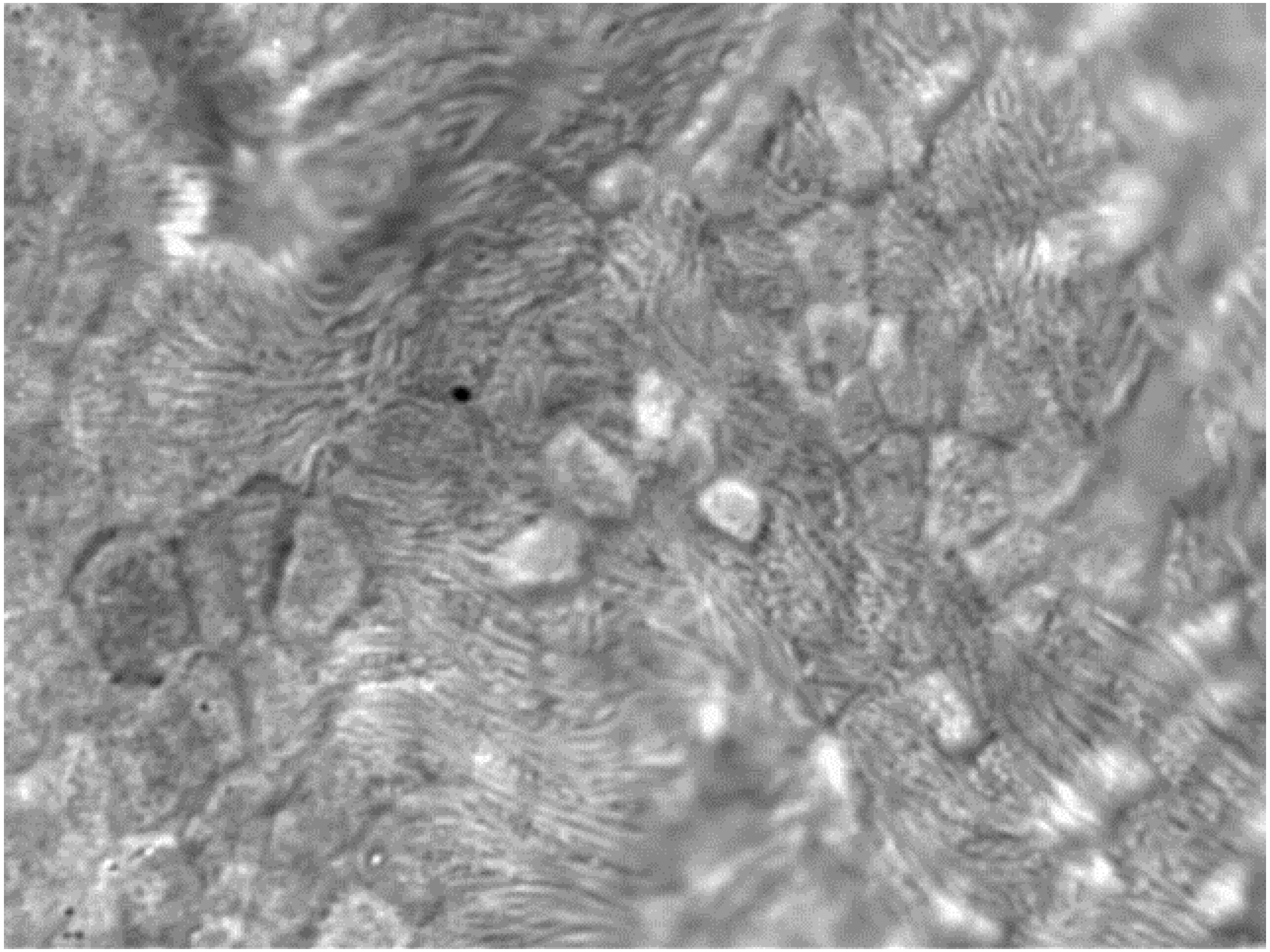
Video: Nina Lodes / David Kessie in Steinke Lab, Fraunhofer ISC



2. Beating kinocilia

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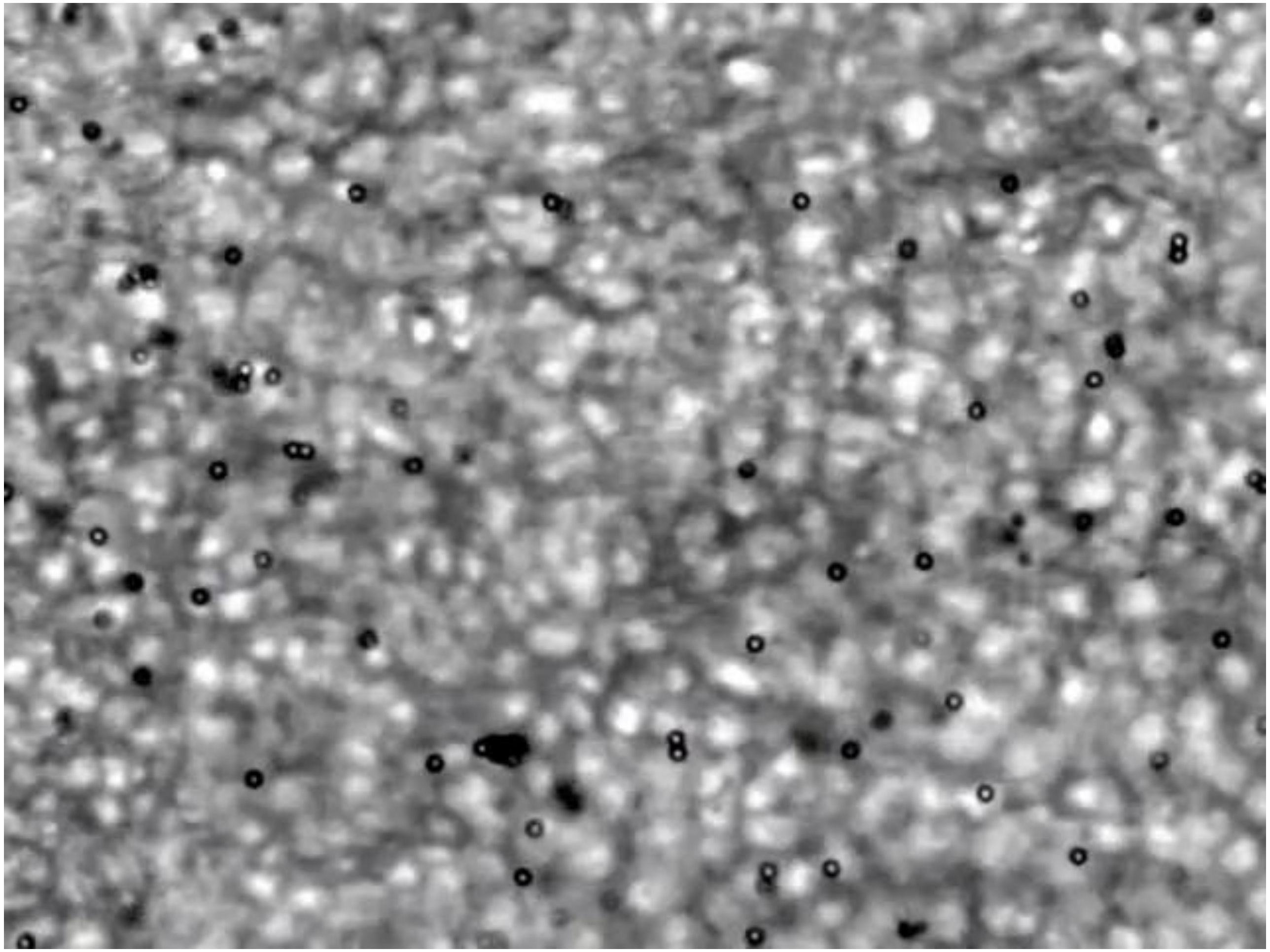
Video: Nina Lodes / David Kessie in Steinke Lab, Fraunhofer ISC



3. Mucus production

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TABLE I.
Currently Cloned Human Mucin Genes: Main Tissue Expression and Chromosomal Localization

Gene	Mucus Type	Mucin	Main Tissue Expression
MUC1	Transmembrane	Pan-epithelial	Breast, pancreas
MUC2	Secreted	Intestinal	Jejunum, ileum, colon
MUC3A	Transmembrane	Intestinal	Colon, small intestine, gall bladder
MUC3B	Transmembrane	Intestinal	Colon, small intestine, gall bladder
MUC4	Transmembrane	Airway	Airways, colon
MUC5AC	Secreted	Airway	Airways, stomach
MUC5B	Secreted	Airway	Airways, submandibular glands
MUC6	Secreted	Gastric	Stomach, ileum, gall bladder
MUC7	Secreted	Salivary	Sublingual and submandibular glands
MUC8	Secreted	Airway	Airways
MUC9	Both	Reproductive	Fallopian tubes
MUC11	Transmembrane	Colonic	Colon, airways, reproductive tract
MUC12	Transmembrane	Colonic	Colon, pancreas, prostate, uterus
MUC13	Transmembrane	Colonic	Colon, trachea, kidney, small intestine
MUC15	Transmembrane	Colonic	Colon, airway, small intestine, prostate
MUC16	Transmembrane	Reproductive	Ovarian epithelial cells
MUC17	Transmembrane	Intestinal	Duodenum, colon, stomach
MUC18	Transmembrane	Airway	Lung, breast
MUC19	Secreted	Salivary	Salivary glands, trachea
MUC20	Transmembrane	Renal	Placenta, colon, lung, prostate, liver

Laryngoscope 117: May 2007 Ali and Pearson: U

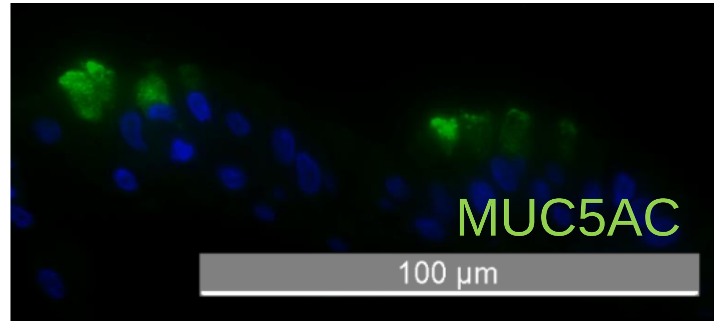
Table I adjusted from from Ali, Mahmoud S., and Jeffrey P. Pearson. "Upper airway mucin gene expression: a review." The Laryngoscope 117.5 (2007): 932-938.

3. Mucus production

Uniklinikum Würzburg

Proteome by F. Neiers and J-M. Heydel, Center for Taste and Feeding Behaviour, Dijon, France in collaboration with **S. Hackenberg** and M. Steinke

Genes detected in mucus secreted from *in vitro* mucosal model
MUC1
MUC4
MUC5AC
MUC5B



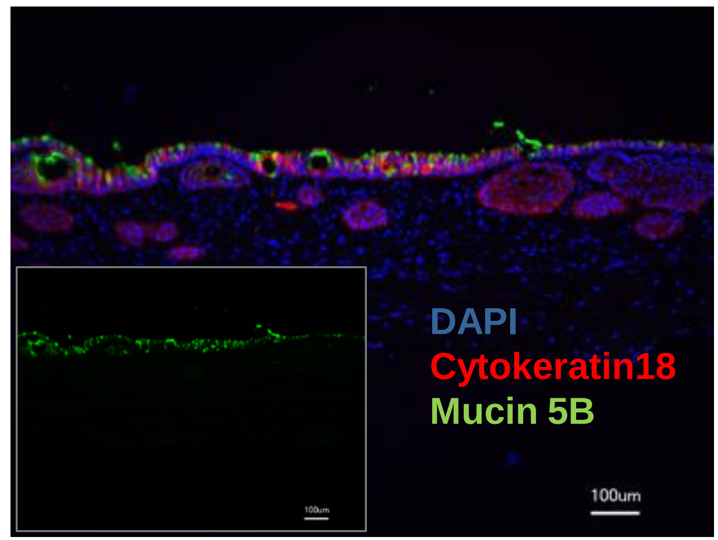


TABLE I.
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MUC4	Transmembrane	Airway	Airways, colon
MUC5AC	Secreted	Airway	Airways, stomach
MUC5B	Secreted	Airway	Airways, submandibular glands
MUC6	Secreted	Gastric	Stomach, ileum, gall bladder
MUC7	Secreted	Salivary	Sublingual and submandibular glands
MUC8	Secreted	Airway	Airways
MUC9	Both	Reproductive	Fallopian tubes
MUC11	Transmembrane	Colonic	Colon, airways, reproductive tract
MUC12	Transmembrane	Colonic	Colon, pancreas, prostate, uterus
MUC13	Transmembrane	Colonic	Colon, trachea, kidney, small intestine
MUC15	Transmembrane	Colonic	Colon, airway, small intestine, prostate
MUC16	Transmembrane	Reproductive	Ovarian epithelial cells
MUC17	Transmembrane	Intestinal	Duodenum, colon, stomach
MUC18	Transmembrane	Airway	Lung, breast
MUC19	Secreted	Salivary	Salivary glands, trachea
MUC20	Transmembrane	Renal	Placenta, colon, lung, prostate, liver

Laryngoscope 117: May 2007 Ali and Pearson: U

Now mucus functionality can be investigated

Table I adjusted from from Ali, Mahmoud S., and Jeffrey P. Pearson. "Upper airway mucin gene expression: a review." The Laryngoscope 117.5 (2007): 932-938.

4. Responsive to toner particles

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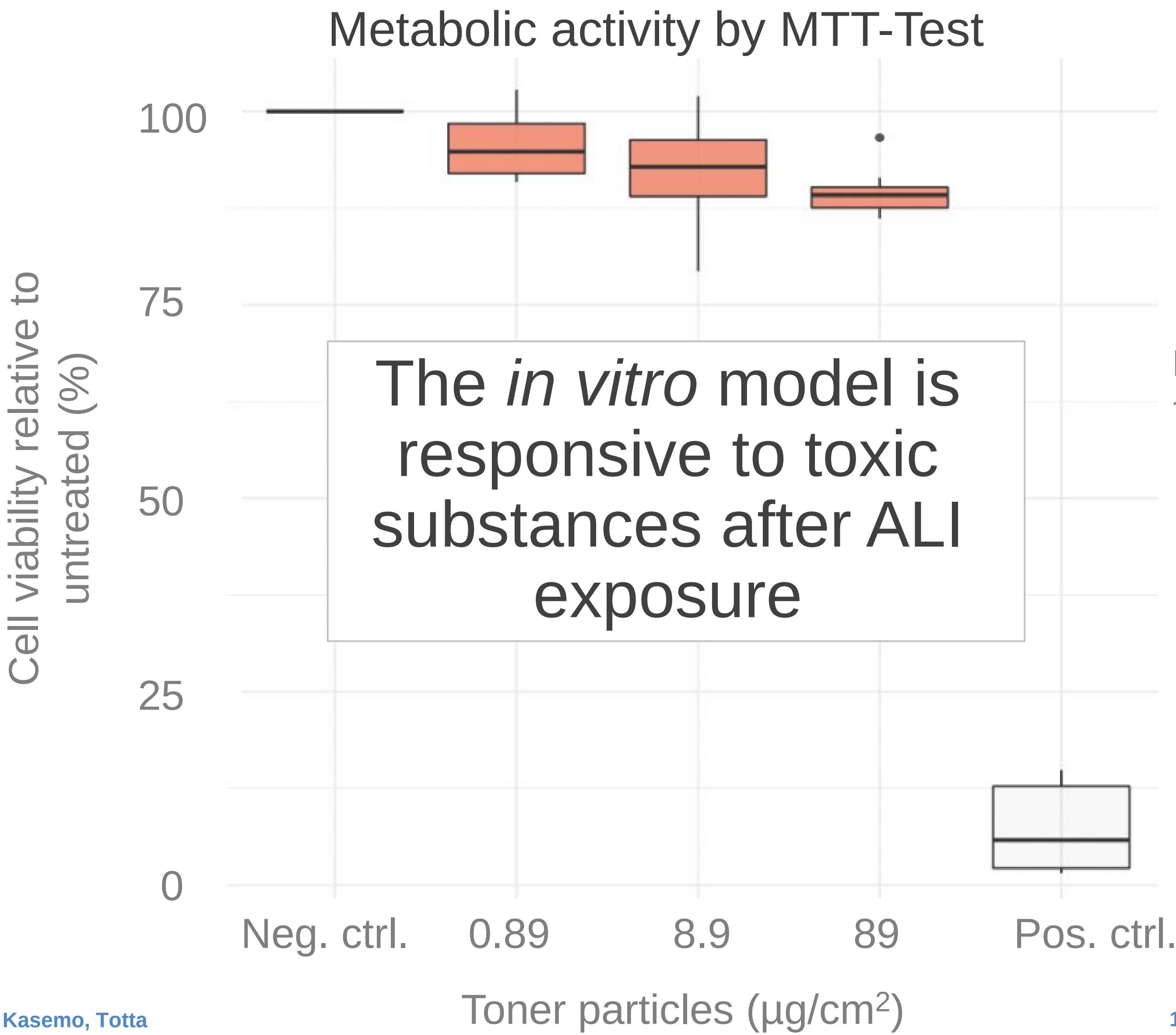

The *in vitro* model is responsive to toxic substances after ALI exposure

Data by Nursen Tekin and Dr. Till Meyer, HNO Würzburg

Particle size by TEM: ~5-10µm

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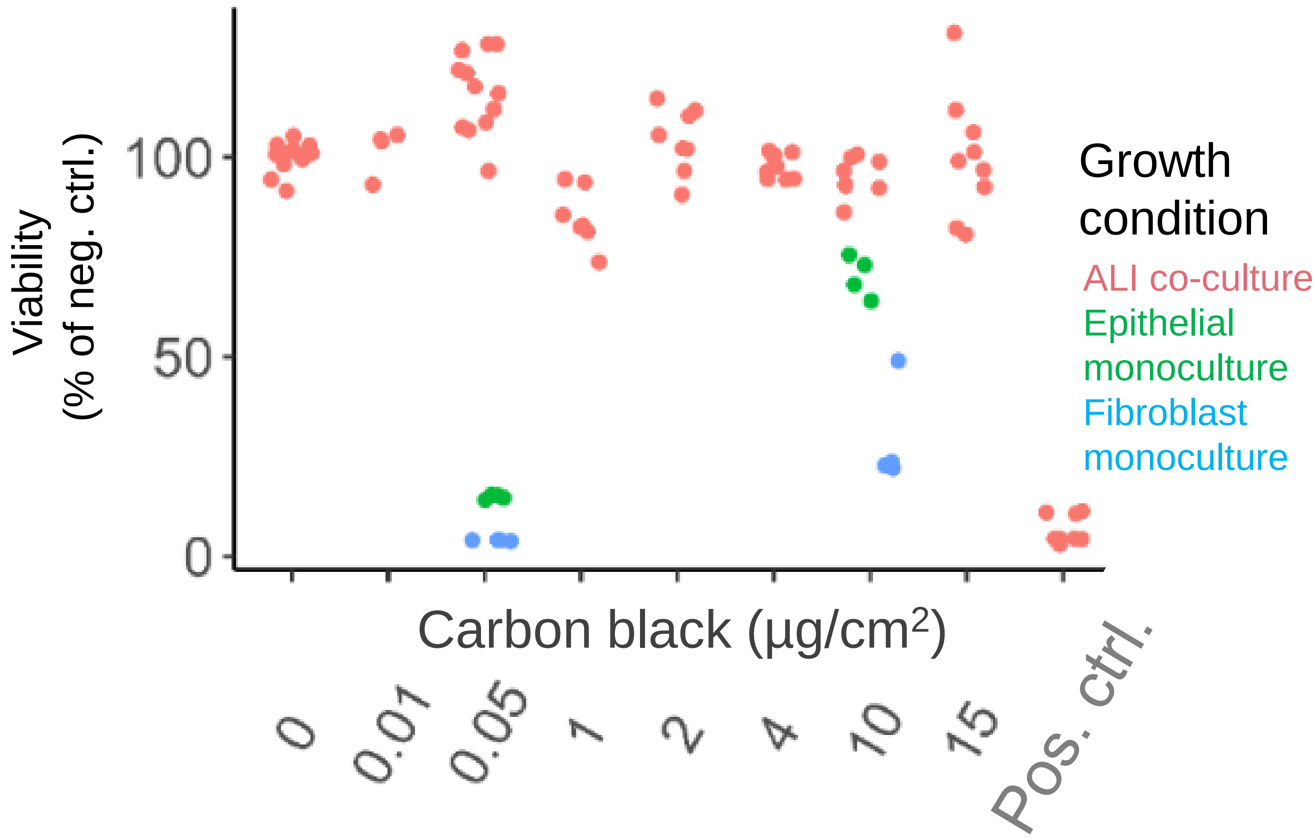
Model UFP induces no **cyto**toxicity after 24h (MTT)



… but monocultures are sensitive

HNO

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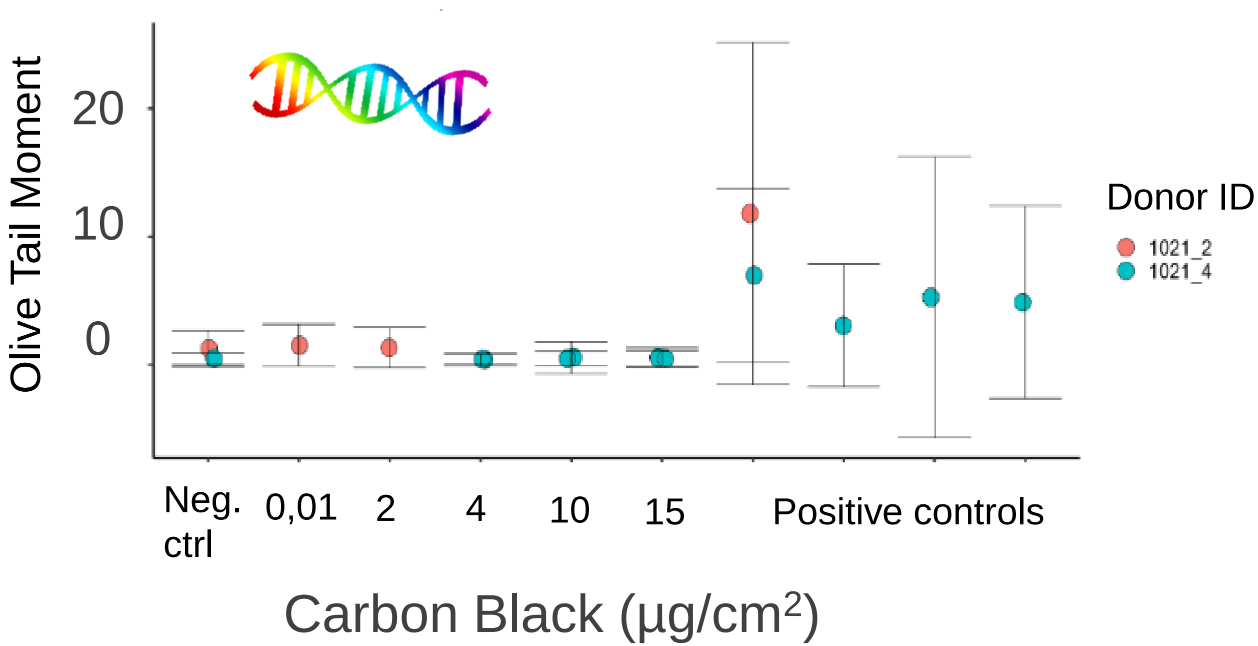
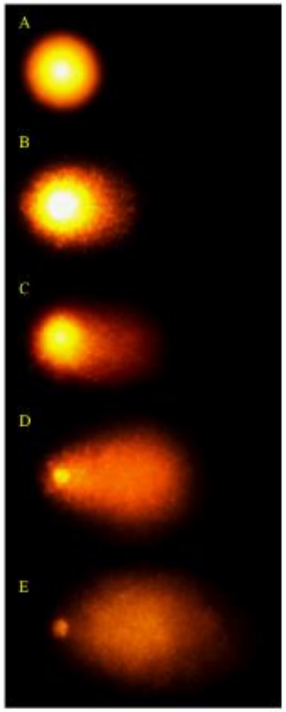
Model UFP induces no **geno**toxicity after 24h (Comet)





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Mucosal model suitablity

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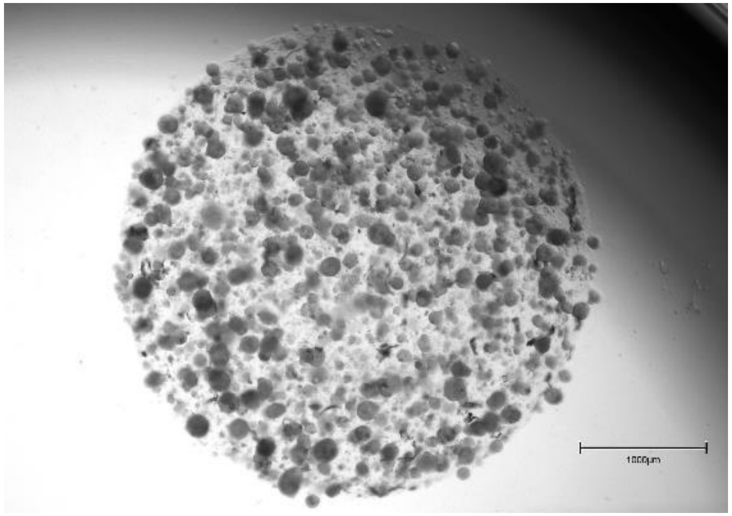
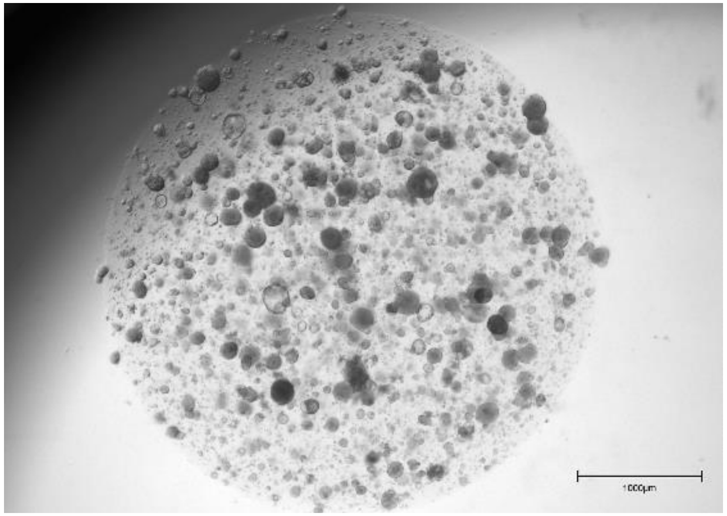
Parameter	Model suitable?	Methods
Barrier integrity	Yes	TEER, FD4, ultrastructure, IFA
Mucociliary clearance	Yes	Ciliary beating and frequency analysis
Mucus present	Yes	Histology, antibody detection, proteins, qPCR, rheology…
Toxicity	Yes	LDH, MTT, Comet assay…
Inflammation	Molecular analysis or co-culture	ELISA, Dotblot, qPCR, Western blot, FACS…
Cell composition	Yes	Histology, antibody detection, ultrastructure, RNA-seq
Morphology	Yes	Histology, ultrastructure, ciliary beating heat-maps
Regeneration	Most likely	TEER, histology, mucus assays…

Teaser: organoids



P0, day 6, donor 1

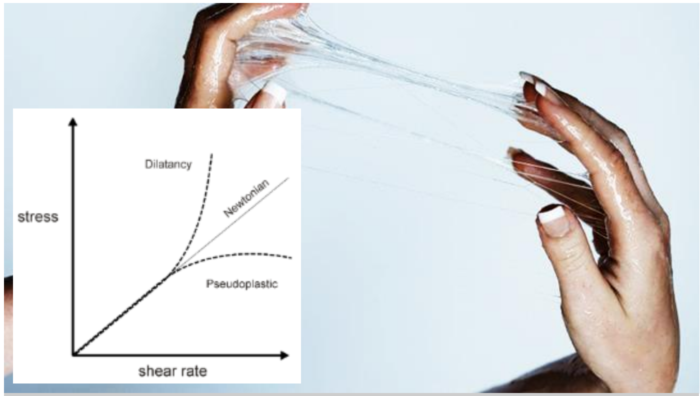


P2, day 9, donor 2

Images and cell cultures: Lili Szabo (Kai Kretzschmar Lab, Würzburg)

Stem cells

Exciting development



Mucus proterties and particle interactions?

Population/donor variation

- Barrier damage without toxicity – new approaches to risk assessment?
- Mucus-particle interactions
- Mucus rheology
- What is normal? Population variance

Slime illustration: www.seriouslynatural.org/. Viscoelasticity illustration: Picout, David R., and Simon B. Ross-Murphy. "Rheology of biopolymer solutions and gels." TheScientificWorldJOURNAL 3 (2003): 105-121. Population: www.dreamstime.com

Thank you for your attention

Collaborations

Fraunhofer ISC, Würzburg

- Maria Steinke
- Sofia Dembski

University of Burgundy Franche-Comté, Center for Taste and Feeding Behaviour, Dijon, France

- Fabien Neier
- Jean-Marie Heydel

MSNZ for Cancer Research Würzburg

- Kai Kretzschmar

Within BayUFP

Friedrich-Alexander-Universität Erlangen-Nürnberg

- Simone Schmitz-Spanke

Helmholtz Zentrum München – German Research Center for Environmental Health

- Ralf Zimmermann
- Sebastiano di Bucchianico
- Mathilde Delaval

- **Stephan Hackenberg**
- **Agmal Scherzad**
- **Till Meyer**
- **Totta Ehret Kasemo**

MWCNT 401

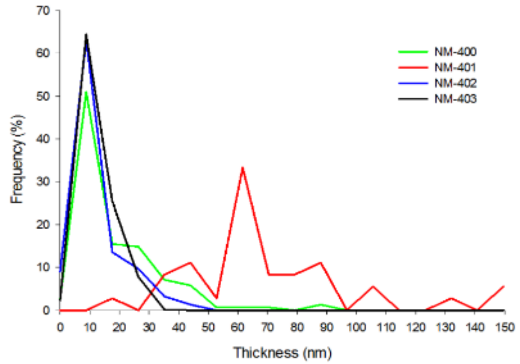


Figure 27. Qualitative TEM image analysis of MWCNT. Graph illustrates the primary thickness as a function of the frequency. Highly bent MWCNT: NM-400, NM-402 and NM-403; Straight-wall MWCNT: NM-401.

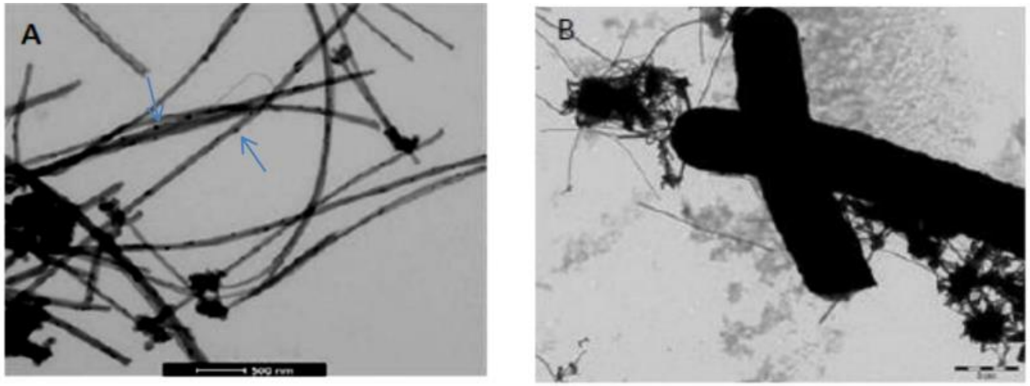


Figure 24. NM-401 A) Representative TEM micrograph of NM-401. Dark spots, of which two are indicated with blue arrows) in the MWCNT sidewall are catalyst impurities (scale bar 500 nm). B) Example of "megatubes" and µm-size dense aggregates and agglomerates in the NM (Scale bar 2 µm).

Table 27. Thickness, geodesic length, percentage of particles with a thickness lower than 100 nm and aspect ratio of NM-400, NM-401, NM-402, NM-403 (CODA-CERVA), and manually measured dimensions of the MWCNT nanomaterials (IMC-BAS).

Material	Thickness, CODA-CERVA ± SD (nm)*	Thickness of nanotube, IMC-BAS ± SD (nm)*	Thickness of tubewall, IMC-BAS ± SD (nm)*	Geodesic length, CODA-CERVA ± SD (nm)*	< 100 nm CODA-CERVA	Aspect ratio* CODA-CERVA	n& CC	n& IB
NM-400	11 ± 3	16.2 ± 3.5	5.1 ± 1.0	846 ± 446	100 %	79 ± 50	20	36
NM-401	67 ± 24	61.4 ± 24.4	-	4048 ± 2371	90 %	66 ± 46	43	358
NM-402	11 ± 3	14.3 ± 2.7	5.4 ± 1.2	1372 ± 836	100 %	125 ± 66	20	135
NM-403	12 ± 7	-	-	443 ± 222	100 %	42 ± 29	50	-

* Mean ± standard deviation (SD), n&: number of observations, CC: CODA-CERVA, IB: IMC-BAS

Table 15. Elements (impurities) detected in the MWCNT NMs according to analytical method.

Material	EDS	ICP-OES	XRD	ICP-MS
NM-400	Al, Si, Fe, Co, Cu, Zn	Al, Fe, Na, S (above 0.01%)	Al	Al, Fe, Na, Co
NM-401	Si, Cu, Zn	S	N.A.	Fe, Na, Co
NM-402	Al, Si, Fe, Cu	Al, Co, Mg, Mn, Ca	Fe	Al, Fe, Na
NM-403	N.A.	-	N.A.	Al, Mg, Na, Mn, Co

N.A. not applied

7.1. Size and structure of fractal aggregates by SAXS

All SAXS diffractograms and the corresponding representations in $I(q)q^4$ for MWCNT powders are displayed in Figure 18, Figure 19 and Figure 20. Among the carbon nanotube powders, NM-401 stands out from the others and does not display any real plateau. Therefore, its specific surface area is difficult to assess, but remains very low compared to other NMs, indicating the presence of true aggregates and/or large nanotubes.

Table 22. Specific surface area measured by SAXS for the MWCNT NMs.

NM	Lim Iq^4 (10^{-3} cm^{-1} A^{-4})	Σ (m^{-1})	Specific surface area (m^2/g)	Error on plateau (m^2/g)	+ 5% error on density (m^2/g)	Equivalent diameter for cylinders (nm)
NM-400	5.9	2.65E+08	189.3	± 8.1	± 27.1	15
NM-401	0.9	4.27E+08	>30.5	± 1.5	± 4.6	< 94
NM-402	4.0	1.82E+08	130.3	± 4.2	± 17.2	22
NM-403	5.9	2.65E+08	189.0	± 10.8	± 29.7	15

* *For NM-401 no real plateau is observed so the surface area calculation is only a minimum estimation.

Table 24. Results of the IMC-BAS BET measurements on the MWCNT NMs.

	BET surface m^2/g	Total pore volume mL/g	Micro surface area m^2/g	Micropore volume mL/g
NM-400	254.00	0.9613	0.0	0.0
NM-401	17.850	0.0776	0.0	0.0
NM-402	226.39	0.8892	15.747	00814

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Endpoints by usage in non-animal models

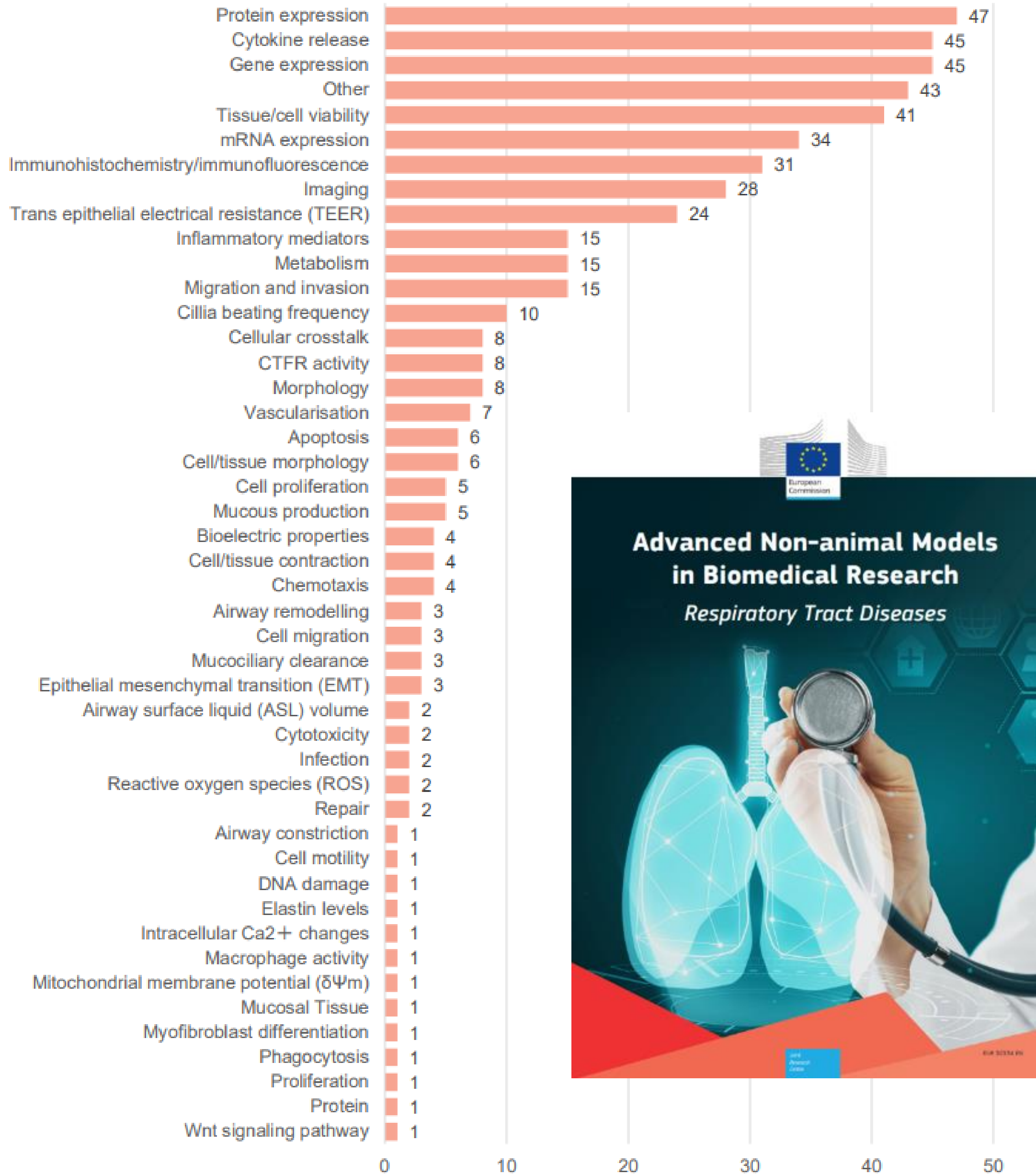

Figure 8: Number of unique models identified and extracted per biological endpoint.

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Table 4. MWCNT NMs: physico-chemical characterisation performed and institutions involved.

Physico-chemical Properties and Material Characterization (from OECD list)	NM characterised				Method	Institution(s)	Chapter
	400	401	402	403			
Agglomeration/ aggregation	x	x	x	x	TEM	CODA-CERVA, IMC-BAS	10
Water solubility *)	Endpoint not investigated						
Crystalline phase	x x	x	x x		XRD	IMC-BAS, NRCWE	9
	x	x	x		Raman	NRCWE	5
Dustiness	x	x	x	x	Vortex shaker method	INRS	9
Crystallite size	Endpoint not relevant						
Representative TEM picture(s)	x x	x x	x x	x	TEM	CODA-CERVA IMC-BAS	10
Particle size distribution	x x	x x	x x	x	TEM	CODA-CERVA, IMC-BAS	10
Specific surface area	x	x	x		BET	IMC-BAS	8
(SSA)	x	x	x	x	SAXS/USAXS	CEA	7
Zeta potential (surface charge)	Endpoint not investigated						
Surface chemistry (where appropriate).	Endpoint not investigated						
Photo-catalytic activity	Endpoint not relevant						
Porosity	x	x	x		BET	IMC-BAS	8
Octanol-water partition coefficient, where relevant	Endpoint not relevant						
Loss in ignition	Endpoint not investigated						
Redox potential (by O_2)	x	x	x		SDR	NRCWE	6
OH radical formation, acellular	x	x	x		SDR	NRCWE	6
Other relevant information (where available) Elemental analysis/impurities	x	x	x	x	Semi quantitative ICP-OES	CODA-CERVA	4.1
	x		x		Semi quantitative ICP-MS	LNE	4.1
	x	x	x	x	Quantitative ICP-MS	Duke University	4.1
	x	x	x		Semi quantitative EDS	IMC-BAS	4.1
Other relevant information (where available)	x	x	x	x	TGA	NRCWE	4.2
	x	x			DTA	IMC-BAS	4.2
Presence of catalysts	x		x		XRD	NRCWE	4.3
Solubility in Gambles solution	x	x	x	x		NRCWE	6
Caco2 medium	x	x	x	x		NRCWE	6
NANOGENOTOX batch dispersion medium	x	x	x	x		NRCWE	6

Table 6. Elemental concentrations by EDS measurements on NM-400, NM-401 and NM-402 performed at IMC-BAS.

Material	C (wt %)	Al* (ppm)	Si* (ppm)	Fe* (ppm)	Co* (ppm)	Cu* (ppm)	Zn* (ppm)	O calculated# (wt %)
NM-400	89.81	46100	400	7600	2500	2000	1900	4.15
NM-401	99.19		500			2300	2200	0.6
NM-402	92.97	21100	500	29800		400		1.93

* ppm by weight # calculated by difference

Table 7. Overview of impurities detected in the MWCNT NMs by semi-quantitative ICP-OES.

Material	Impurities > 0.01%	Impurities 0.005 – 0.01%	Impurities 0.001 – 0.005%
NM-400	Al, Fe, Na, S*	Co	Ca, K
NM-401	S*		Ag
NM-402	Al, Fe, S*	-	-
NM-403	Al, Co, Mg, Mn, Ca		

*Impurity content might be higher for sulphur

Table 10. Extraction procedure for NM-401.

NM-401			
5 mg	10 mg	5 mg	7 mg
9 mL HCl suprapur + 3 mL HNO_3 suprapur	7 mL HNO_3 suprapur + 1 mL H_2O_2 suprapur	12 mL HNO_3 suprapur + 1 mL H_2O_2 suprapur	3 mL HNO_3suprapur + 3 mL H_2O_2 suprapur
Intact particles	Intact particles	Intact particles	Mineralization Dissolution completed OK

Carbon black / Printex90

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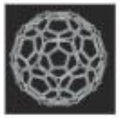

Journal of Carbon Research

MDPI

Review

Structure of Carbon Materials Explored by Local Transmission Electron Microscopy and Global Powder Diffraction Probes

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Abstract: Transmission electron microscopy and neutron or X-ray diffraction are powerful techniques available today for characterization of the structure of various carbon materials at nano and atomic levels. They provide complementary information but each one has advantages and limitations. Powder X-ray or neutron diffraction measurements provide structural information representative for the whole volume of a material under probe but features of singular nano-objects cannot be

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Projects – internal list to discuss

- Ultrafine particles in co-culture ALI model of upper airway mucosa
  - Carbon black/Printex90 with nebulizer, inhouse
  - Aerosols with Vitrocell system (collab. Helmholz München)
  - Particle characterization (collab. Sofia Dembski, Fraunhofer ISC)
- Mucus
  - Optimize model (e.g. induce goblet cells with IL-13, establish positive controls for expulsion (mechanical, LPS, ….. Co-supervised by Maria S.)
  - Mucus proteome (SH + MS collab. France)
  - Mucus rheology (upcoming - collab. Maria Steinke, Fraunhofer ISC)
- Organoid pre-cultures
  - More replicates from each donor → quality improvement (collab. Kai Kretzschmar, Würzburg
  - Biopsy VS brush based is an option (so far not explored)
- Microbiology
  - Fungi and bacteria in voice prostheses
  - So far a side project towards understanding challenges with the functional impact of biofilms on voice prostheses (map economic and health gains of improvements as another side project?)
- Population variance – „how different are people?“ → *New project to initiate? Possible grant topic, synergies with almost all other projects using ALI-models*
  - Analyze different aspects of nasal physiology to gain better understanding for what is normal and what is disease-related or related to harmful substance exposures
  - RNA abundance („gene expression“) i.e. RNA-seq
  - Mucus characterization
  - Comet+LDH responses to (toxic?) substances (establish lab standard, e.g. UFPs (Printex and ZnO), LPS, …. Viruses with Maria?)
  - Functional impact of (toxic?) substances → TEER, mucus production, ciliary beating (re-establish this protocol)
  - Statistical analysis with generalized linear models (GLMs) or similar → collab London (ask Alice Balard? Or ask David Zwicker)