Environmental, Health and Safety Considerations

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Outline

- Brief historical perspective of Nanotoxicology
- Evaluating the EHS Hazards and Risks of Nanomaterials - Nano Risk Framework
- Pulmonary bioassay studies in rats with – Fine/Ultrafine (Nano) TiO_2 particle types;

- Developing *in vitro* assays for lung toxicity studies with fine and nanoscale particulates
- suggested Strategy for developing reliable and predictive high throughput screenings (HTS) assays for nanomaterial toxicity [addressing Tox21 issues]

Common Perceptions on Pulmonary Toxicity of Nanoparticles

- Nanoparticles are more toxic (inflammogenic, tumorigenic) than fine-sized particles of identical composition.
- Concept generally based on 3 particle-types:
  - Titanium Dioxide particles
  - Carbon Black particles
  - Diesel Particles

Take home points regarding the toxicity of ultrafine TiO_2 particles

- On a per mass basis, inhaled ultrafine particles are more inflammogenic, fibrogenic and tumorigenic than chemically identical fine particles in the lungs of rats.
- On a surface area basis, the potency of ultrafine particles to produce adverse pulmonary effects is similar to larger particles of similar composition.
- Unintended incidental exposures (occupational/consumers) vs. Intentional exposures (Nanomedicine/diagnostic apps.)

Talking Points US Congress– Feb, 2008

- No Novel Toxicity with NP
- Current Risk Assessment methodologies appear to be adequate
- Current hazard assessments can define the toxicity
- Not a lot of data on NP
- Importance of Particle Characterization
- Particle Size vs. Surface Reactivity
- Proposed Risk Assessment Methodologies
- Risk = Hazard + Exposure
- Base Set concepts + Benchmarks

Environmental Health and Safety Considerations

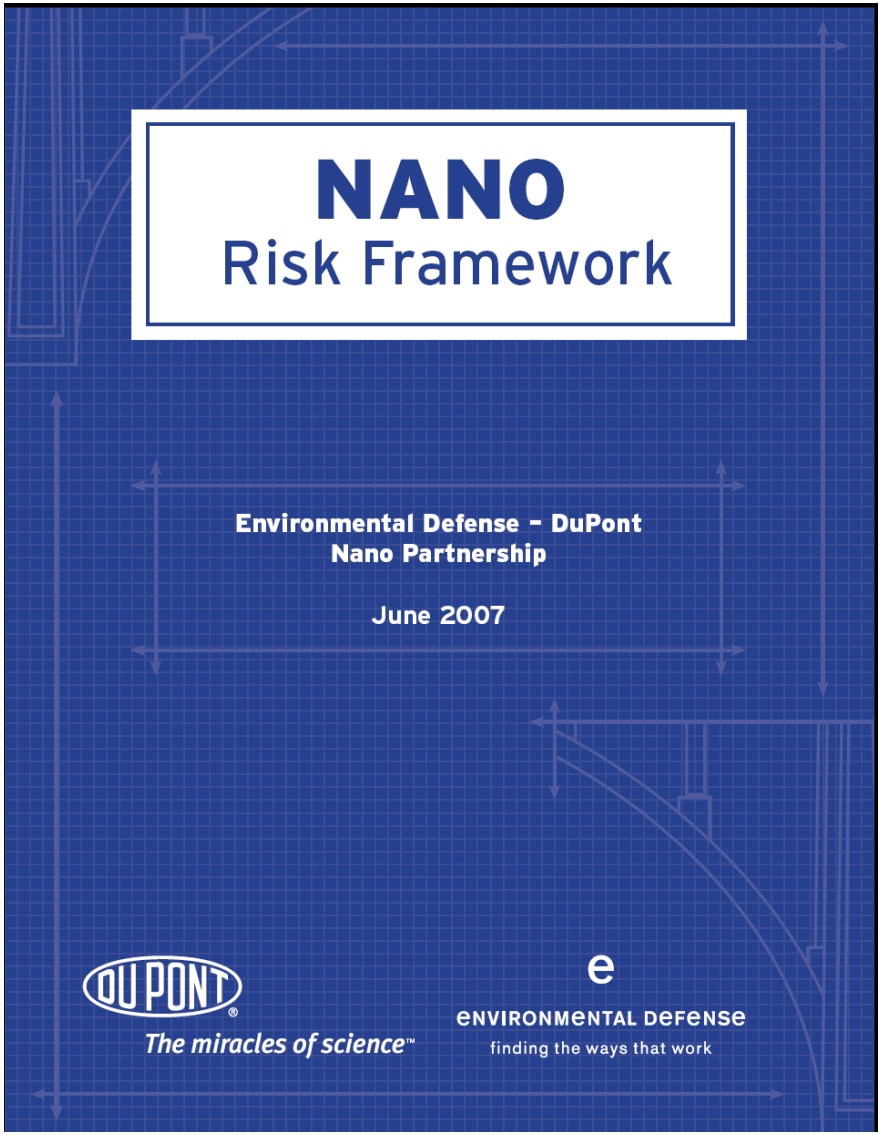
Practical Considerations for Developing Commercial Products

Nano Risk Framework

Objective: To develop and deliver a systematic and disciplined process for identifying, managing, and reducing potential environmental safety and health risks of engineered nanomaterials across all stages of a product's lifecycle.

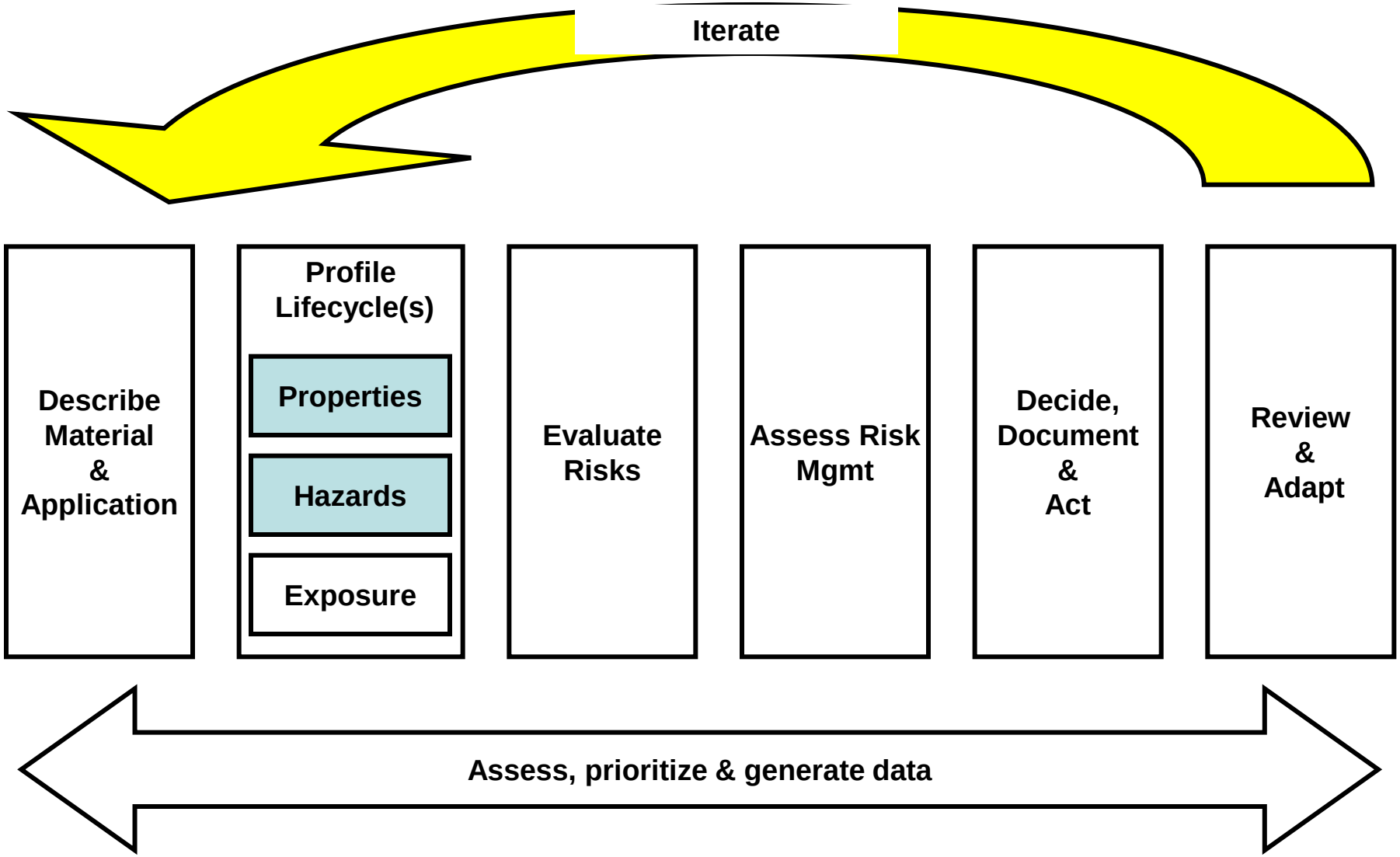
Scope: Offers guidance on the **key questions** an organization should consider in developing applications of nanomaterials, and on the **information needed** to make sound risk evaluations and risk-management decisions.

Audience: Primary audiences are organizations such as companies and public and private research institutions that are **actively working** with nanomaterials and developing associated products and other applications. Framework can also be **useful to other stakeholders**, such as government officials, academia, financial institutions, and nongovernmental public-interest organizations.



www.NanoRiskFramework.com

Nano Risk Framework
Draft Outline



Development of a base set of toxicity tests using ultrafine TiO_2 particles as a component of nanoparticle risk management

(Warheit et al. Tox Letters 171: 99-110, 2007)

Justification for the base set hazard tests rests on the following criteria

1) Potential routes of exposures related to human health effects (i.e., pulmonary, dermal, oral and/or ocular);
2) Screening for potential carcinogenic effects (i.e., utilizing well established mutational and chromosomal aberration screening assays);
3) Screening for potential toxic effects in representative aquatic organisms (i.e., exposures of nanomaterials to rainbow trout, Daphnia, and algae)

Base Set – Guidelines and Methods

- Pulmonary Bioassay - + full histopathology + NM Charact – BALF + BrdU + histopath
- Skin Irritation Test – OECD 404
- Skin Sensitization – LLNA- OECD 429
- Acute Oral Toxicity Test – OECD 425
- Eye Irritation Test – OECD 405
- Genotoxicity Tests (2) - Ames – OECD 471 Chromosomal Ab Study – OECD 473
- Aquatic Battery (3) - Rainbow Trout – OECD 203; Daphnid –OECD 202; Green Algae – OECD 201

Ultrafine TiO_2 Studies

Minimum Base Set – Tox Results

- Pulmonary Bioassay - low toxicity
- Acute Oral Toxicity Test – low toxicity
- Skin Irritation Test – not a skin irritant
- Skin Sensitization – LLNA – not a sensitizer
- Genotoxicity Tests - Ames – negative Chromosomal Ab Study - negative
- Aquatic Battery - Rainbow Trout – low hazard
- Daphnia - low hazard concern
- Algae- medium concern
- Eye Irritation – minor ocular conjunctival redness

Studies to Assess Pulmonary Hazards to Nanoparticulates

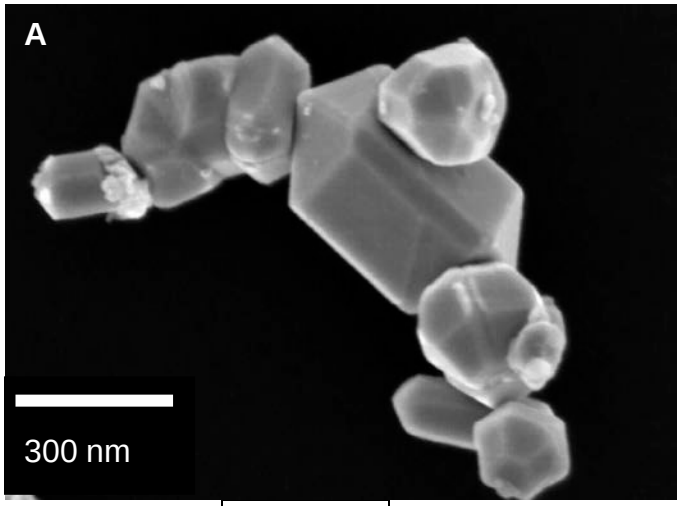
- **Key Features of Nanotoxicology Studies**

1) Rigorous physicochemical characterization of particle-types
2) Dose response characteristics
3) Time course experimental protocol
4) Utilization of benchmark particulate controls (positive and/or negative)

Pulmonary Toxicity Study in Rats with Three Forms of ultrafine-TiO_2 Particles: Differential Responses related to Surface Properties

Warheit *et al*., Toxicology 230: 90-104, 2007

Characterization of Ultrafine TiO_2 Particle-types - 1



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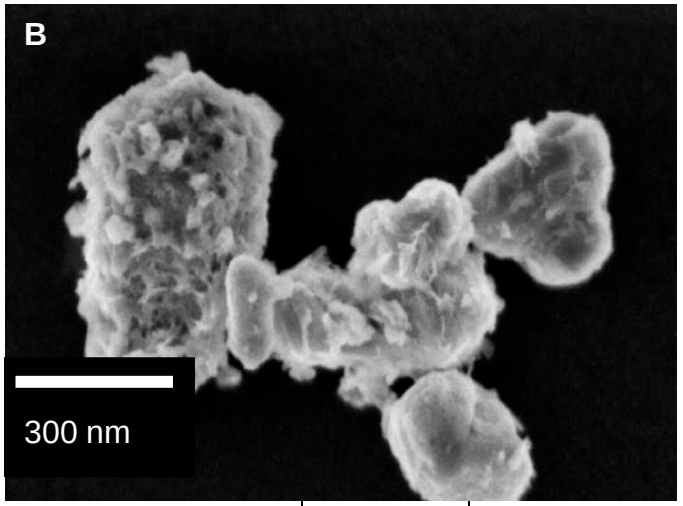
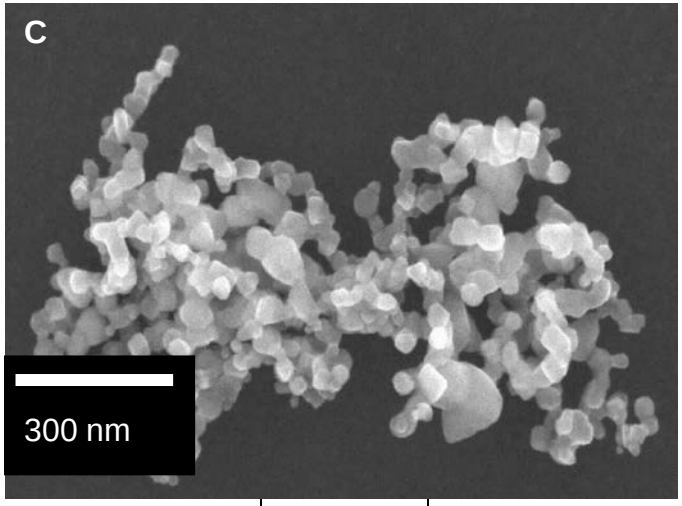


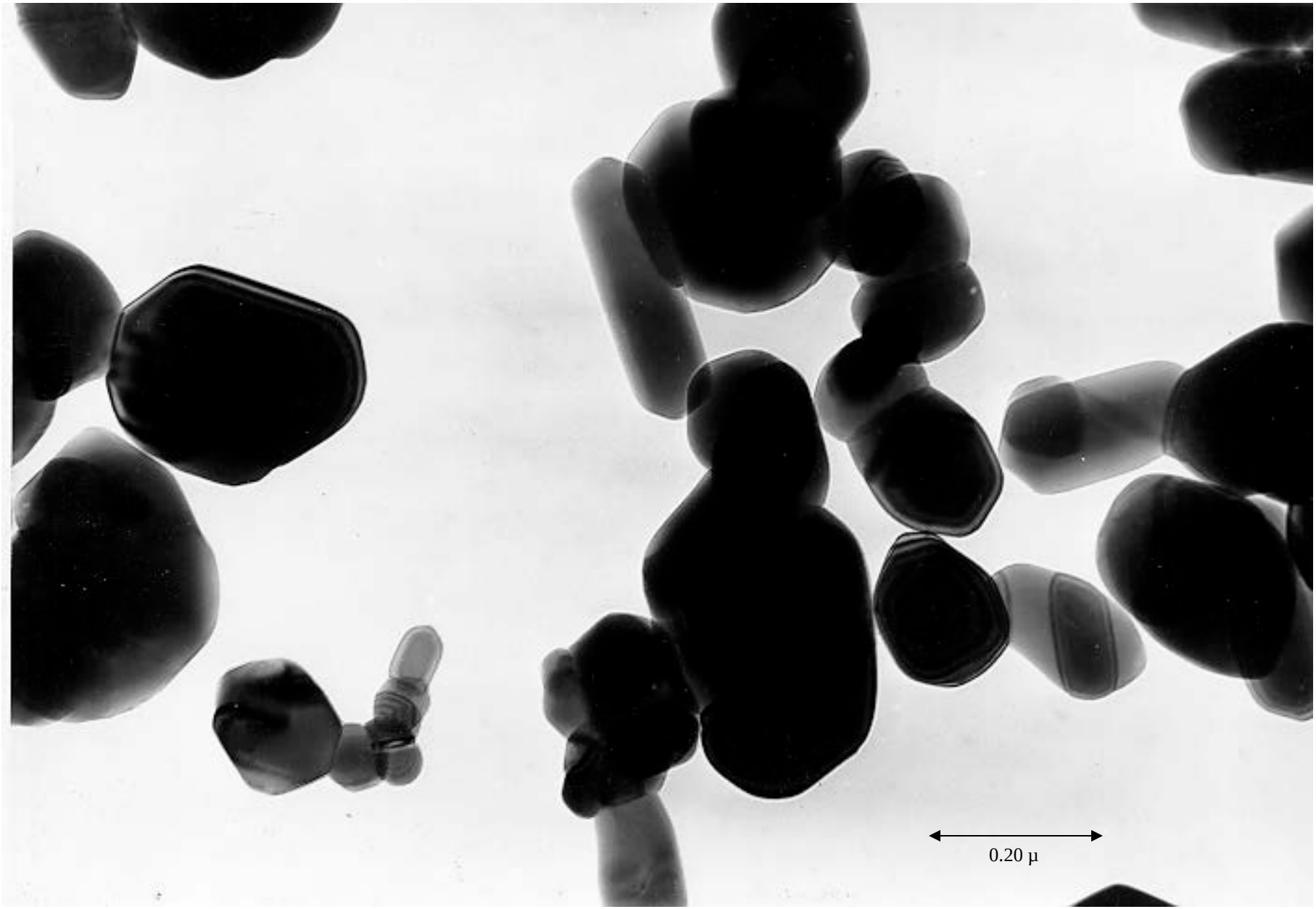
uf-2



uf-3

Ti-Pure®
titanium dioxide

TiPure® R-100



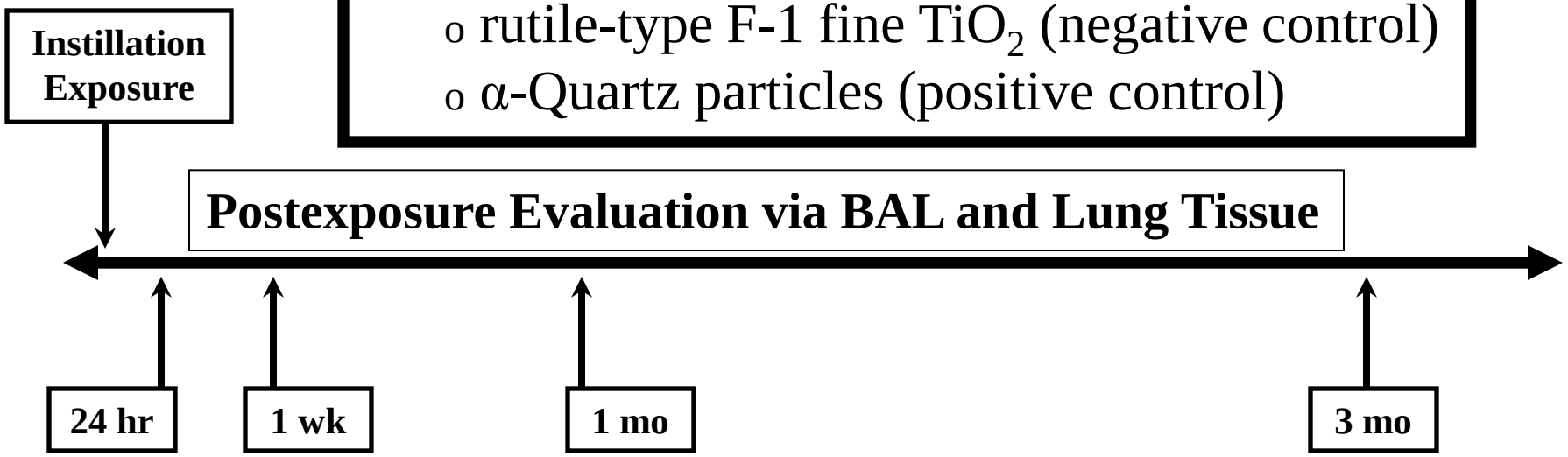
Characterization of Ultrafine TiO_2 Particle-types - 2

Sample	Crystalline phase	Median size and width distribution (nm)		Surface area (m^2/g)	pH		Chemical reactivity
		in water*	in PBS		deionized water	in PBS	delta b*
F-1	rutile	382.0 ± 36%	2667.2 ± 35%	5.8	7.49	6.75	0.4
uf-1	rutile	136.0 ± 35%	2144.3 ± 45%	18.2	5.64	6.78	10.1
uf-2	rutile	149.4 ± 50%	2890.7 ± 31%	35.7	7.14	6.78	1.2
uf-3	80/20 anatase/ rutile	129.4 ± 44%	2691.7 ± 31%	53.0	3.28	6.70	23.8

Protocol for ultrafine TiO_2 Pulmonary Bioassay Study

Exposure Groups

- PBS (vehicle control)
- Particle-types (1 and 5 mg/kg)
  - rutile-types uf-1 TiO_2
  - rutile-type uf-2 TiO_2
  - anatase/rutile-type uf-3 TiO_2
  - rutile-type F-1 fine TiO_2 (negative control)
  - α-Quartz particles (positive control)



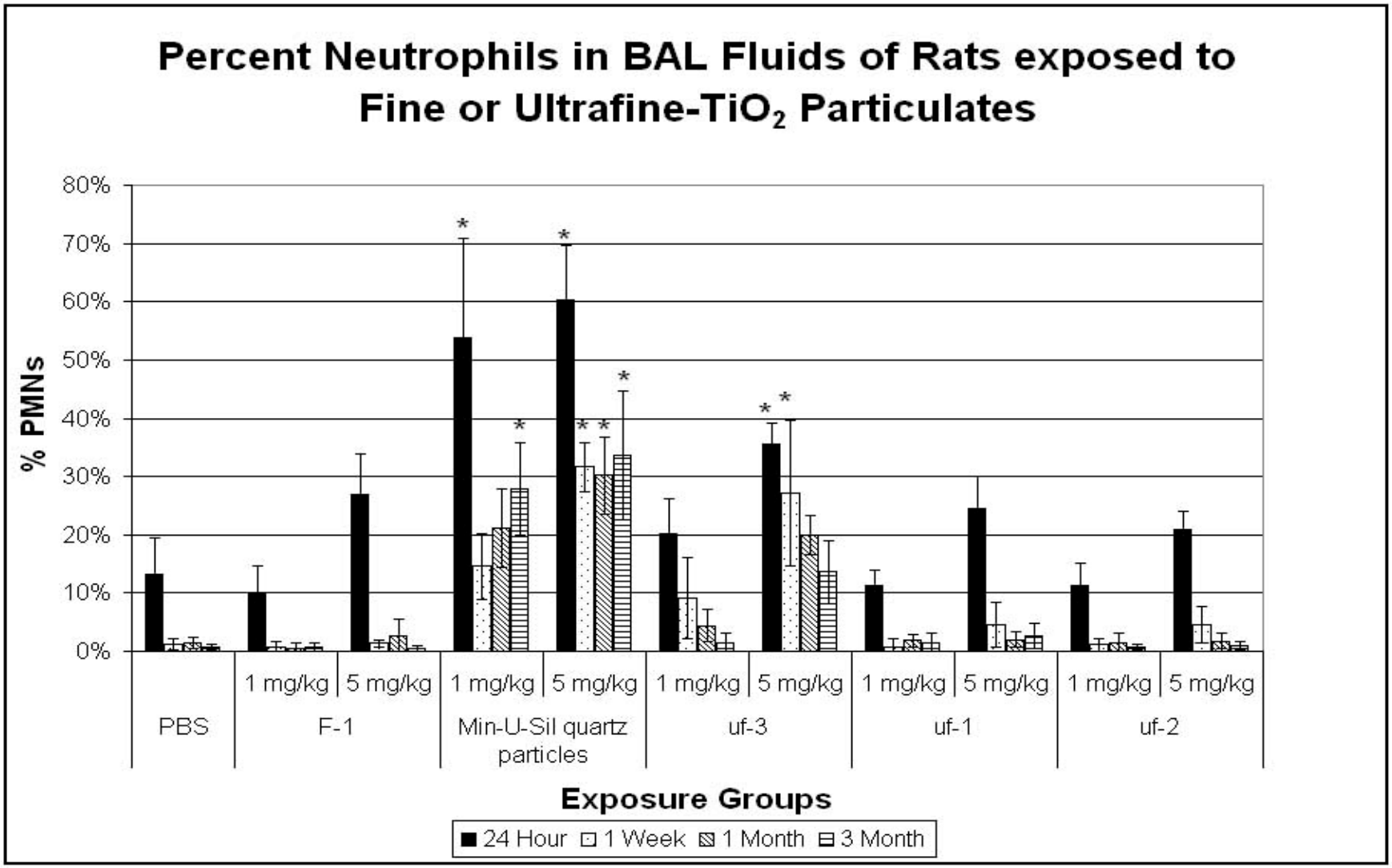
RESULTS

Biomarkers

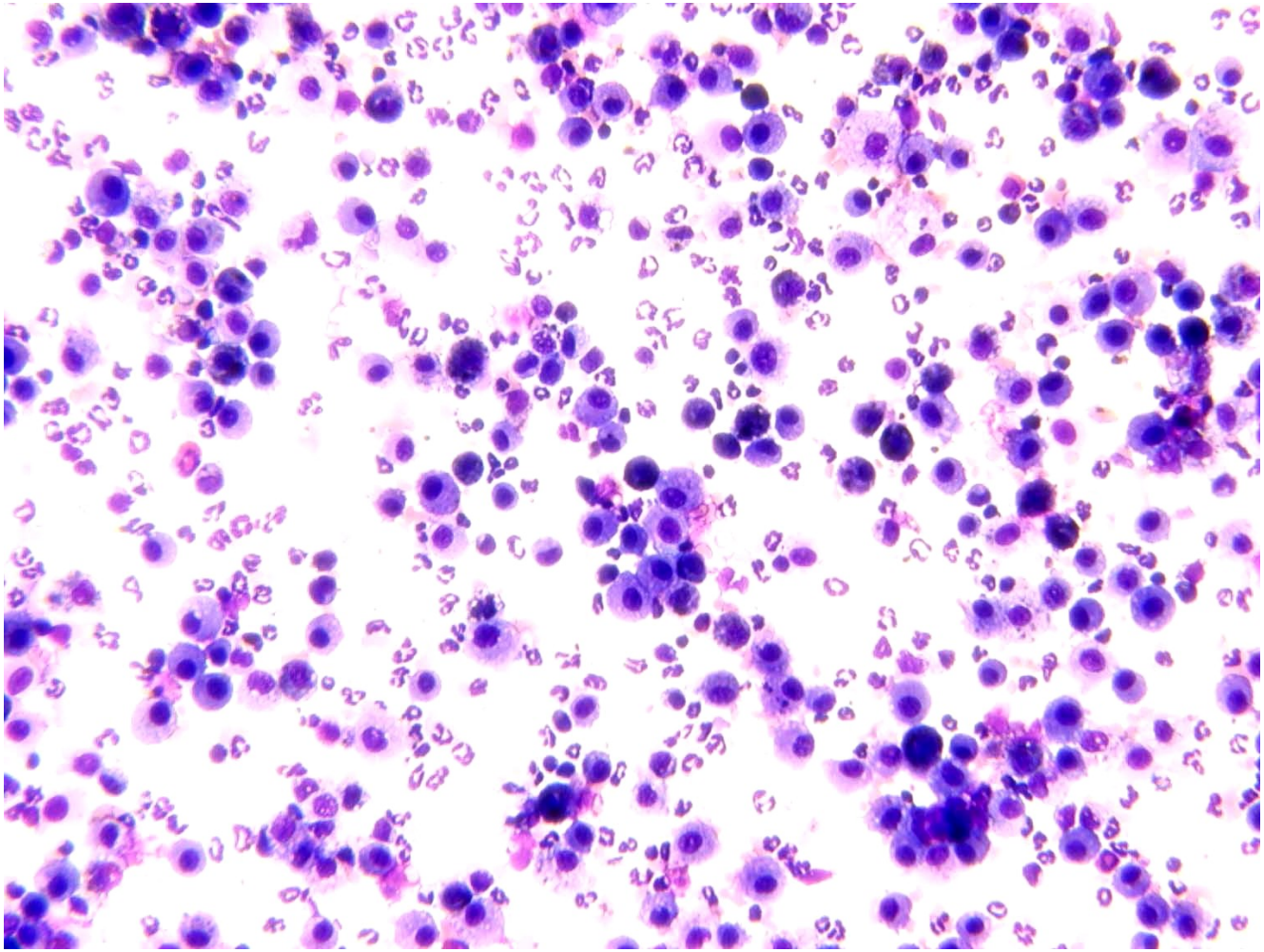
Pulmonary Inflammation

Pulmonary Cytotoxicity

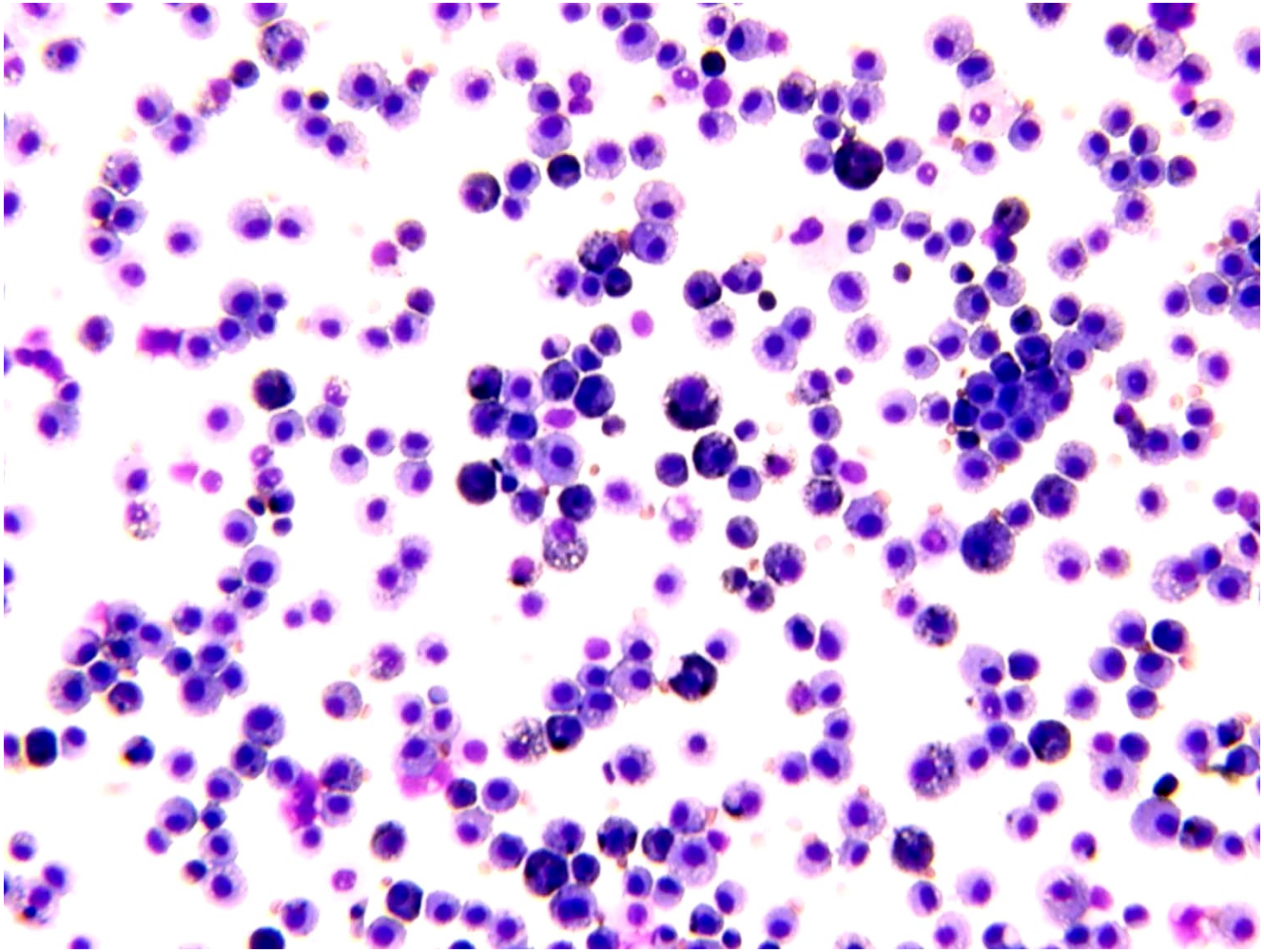
Pulmonary Inflammation



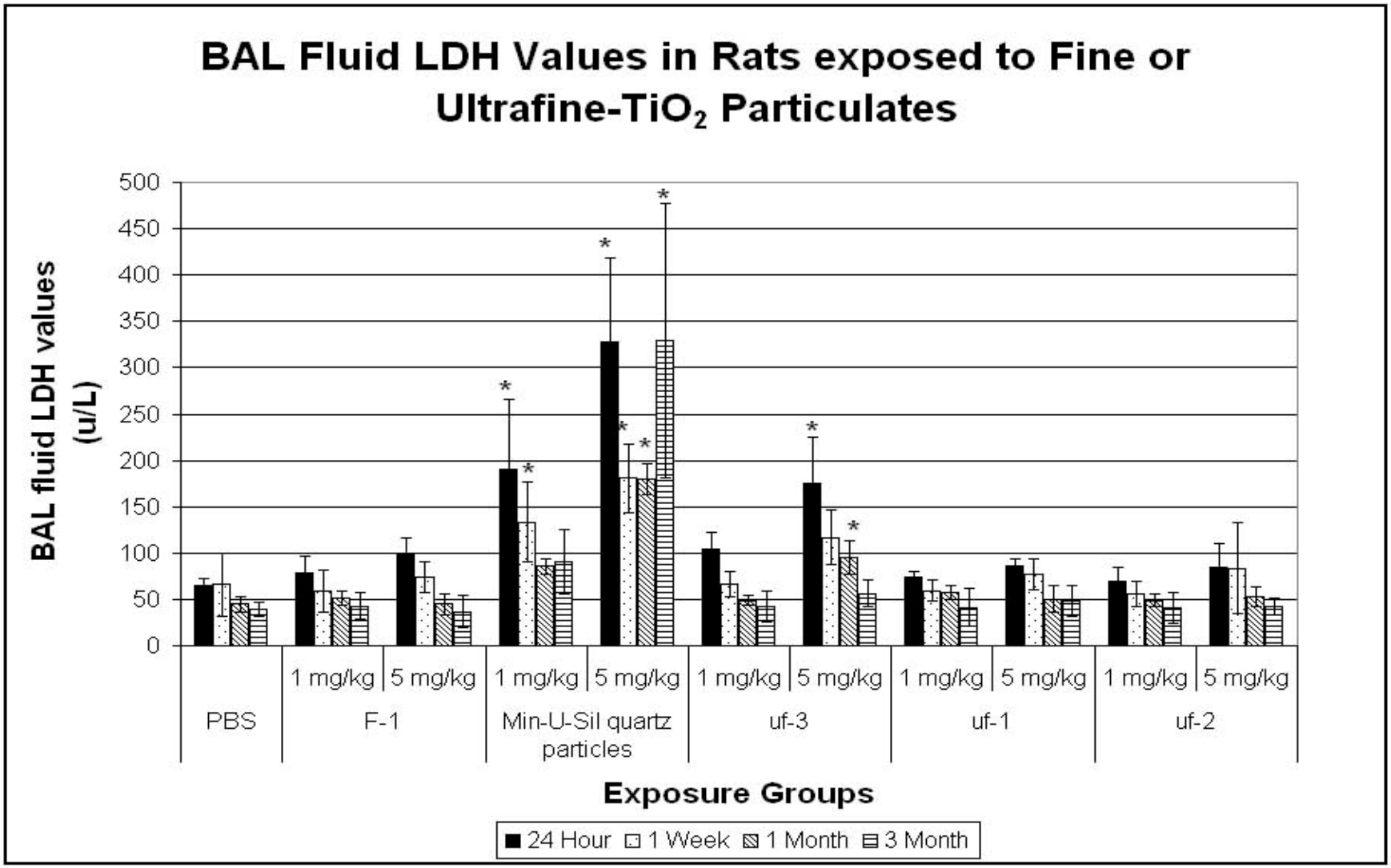
Cytocentrifuge Prep of BALF Cells - Rat Exposed to Nanoscale TiO_2 Dots –24 hr pe



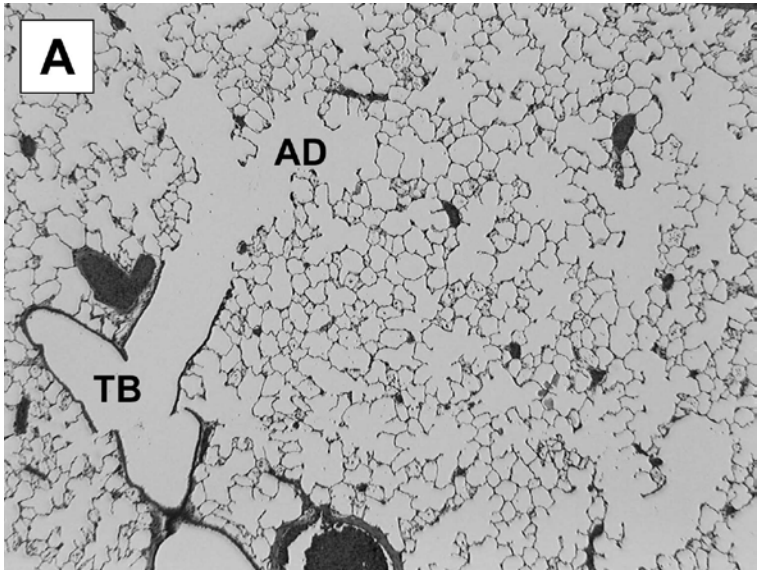
Cytocentrifuge Prep of BALF-derived Cells From a Rat Exposed to Nanoscale TiO_2 Dots–1 wk pe

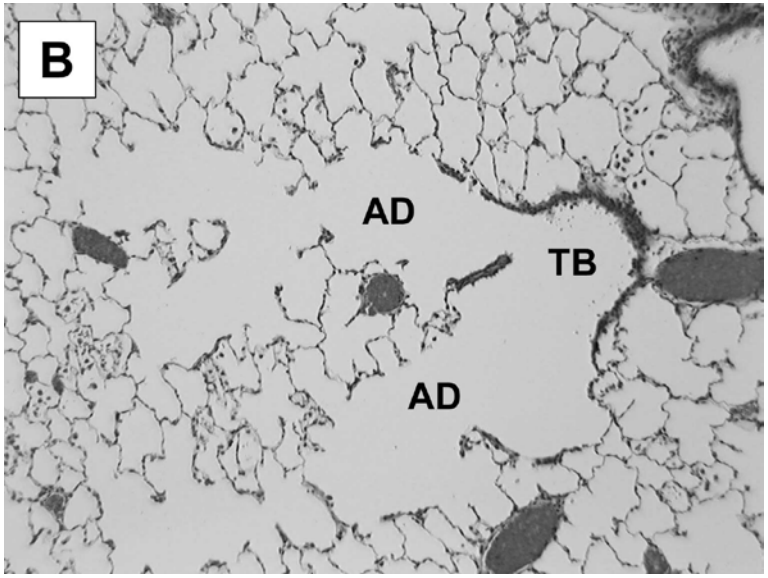


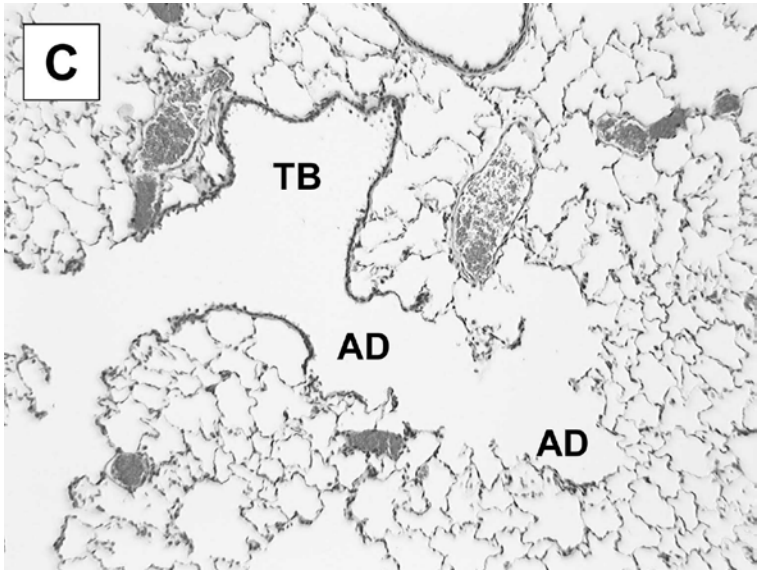
BAL Fluid LDH Values (cytotoxicity)



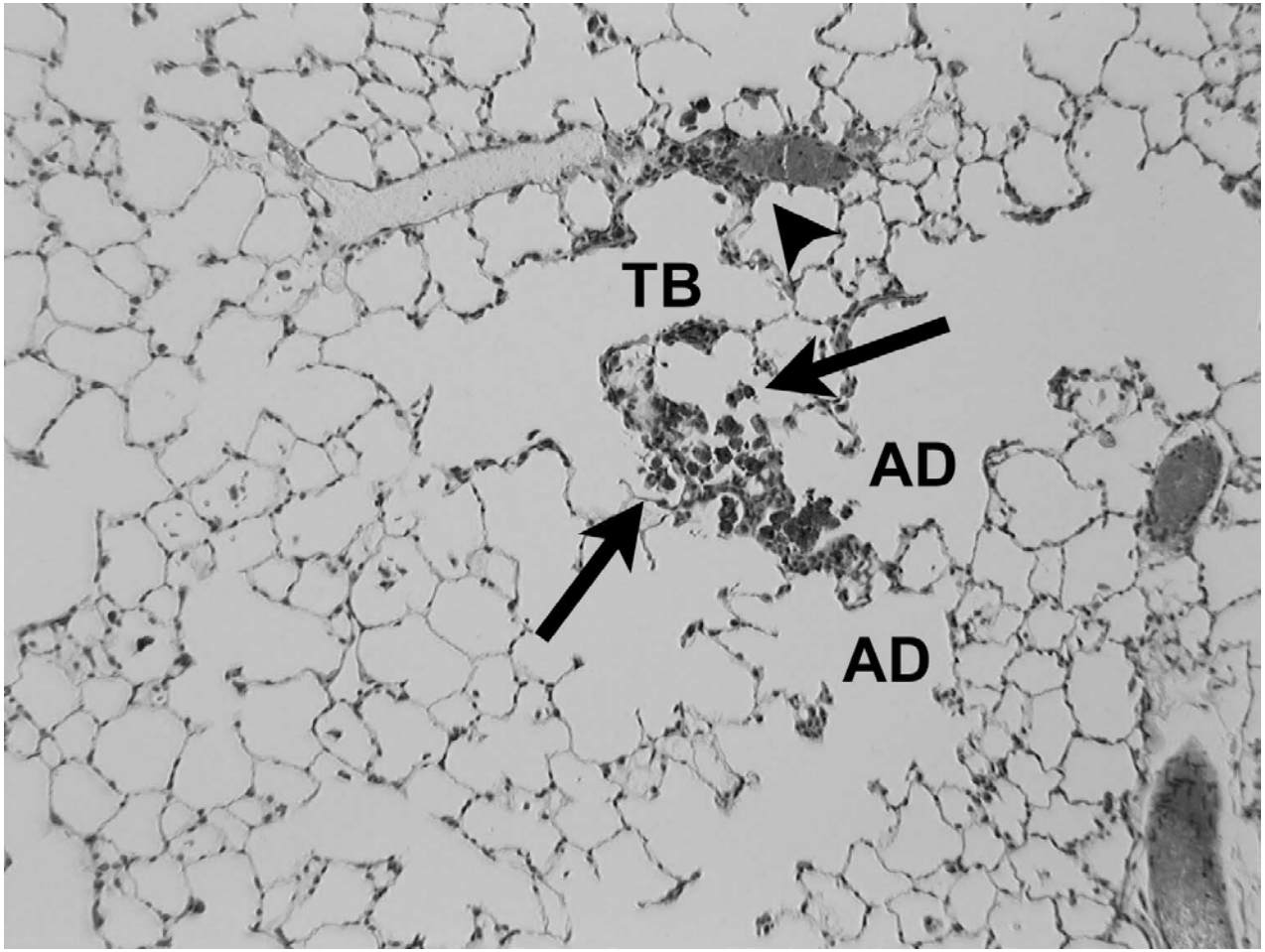
Lung Sections of Rats exposed to uf-1 (A); uf-2 (B); or F-1 (C)- 3 months pe



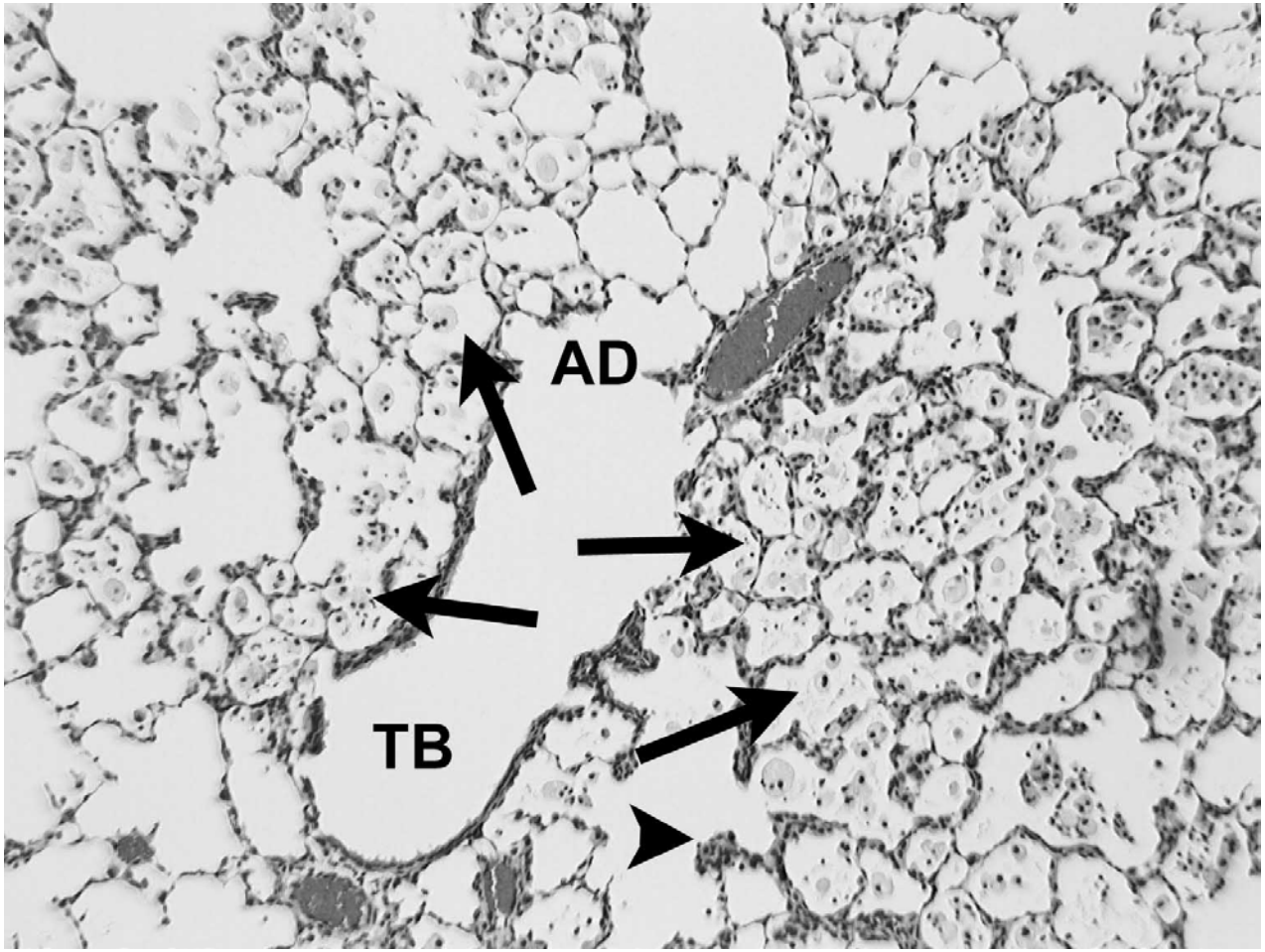




Lung Section of Rat exposed to uf-3 @ 3 months postexposure



Lung Section of Rat exposed to Quartz particles @ 3 months postexposure



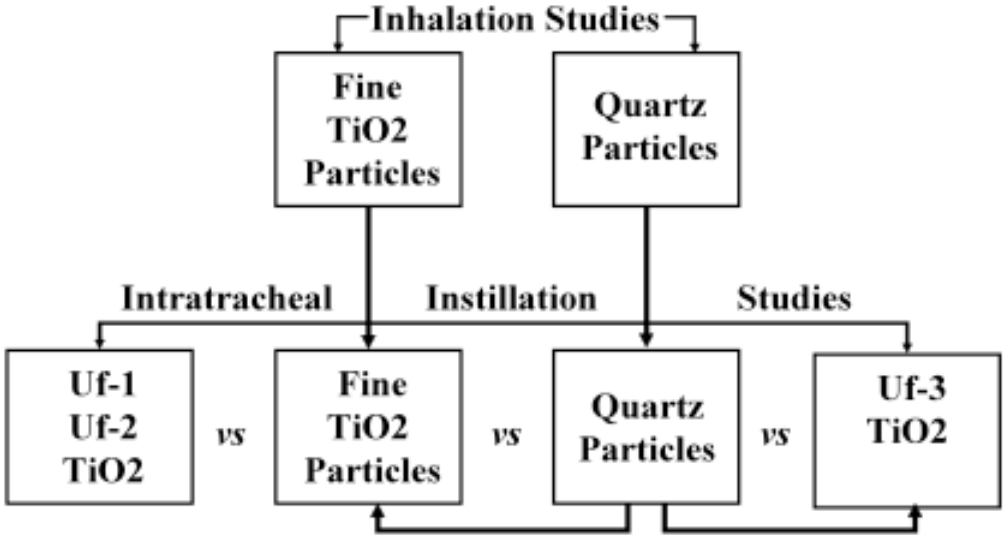
Summary - Important Particle Characteristics

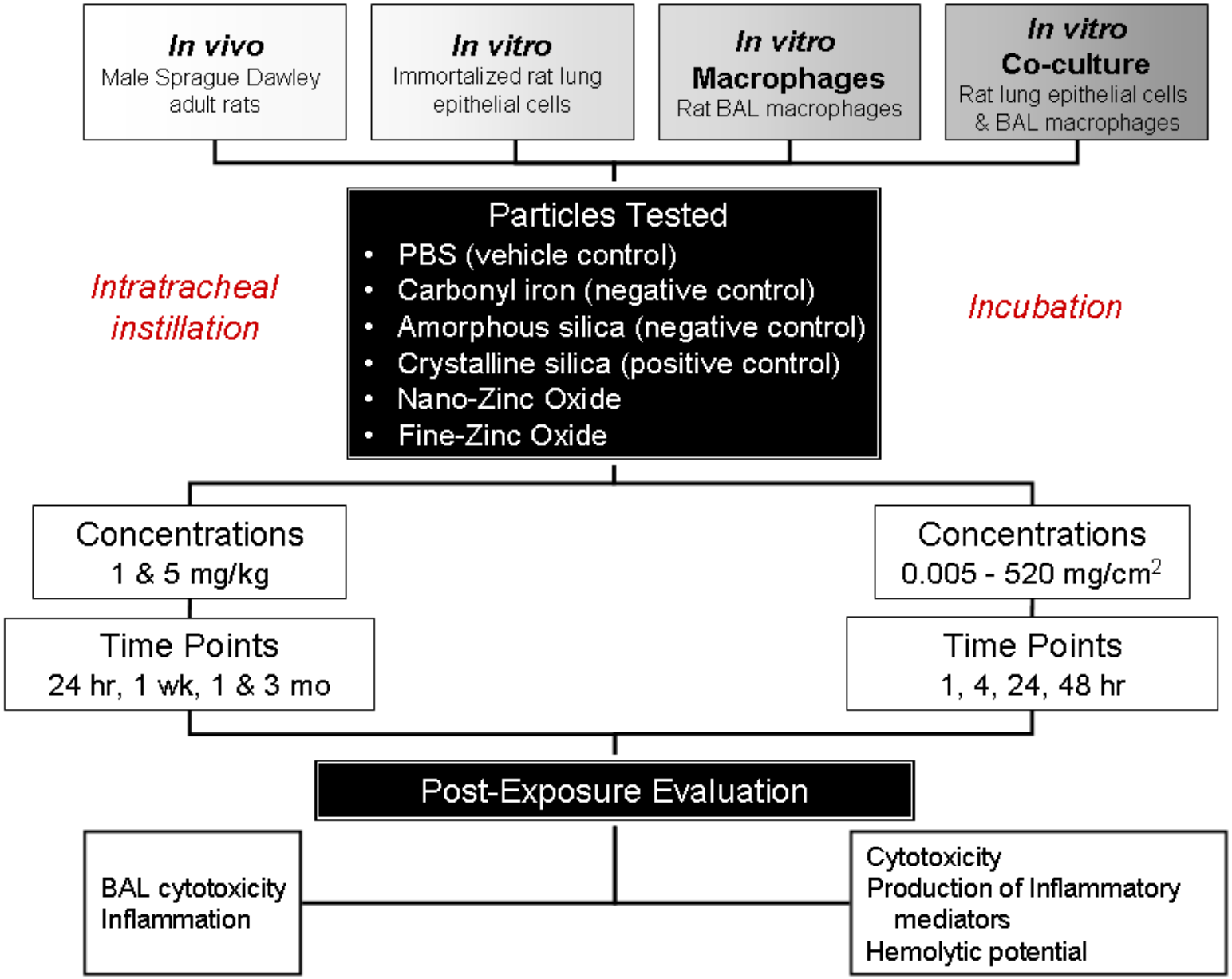
- Primary particle size
- Particle shape (SEM)
- Surface area
- Surface charge
- Composition- e.g., crystalline vs. amorphous
- Surface Coatings
- Aggregation status
- **Particle surface reactivity**

Conclusions

- The ultrafine TiO_2 particle-types used in these base set hazard tests generally have a low hazard potential.
- Risk = Hazard x Exposure
- Played a significant role in the commercialization of DuPont DLS-210 ultrafine/nano TiO_2.

Pulmonary Bioassay Bridging Studies

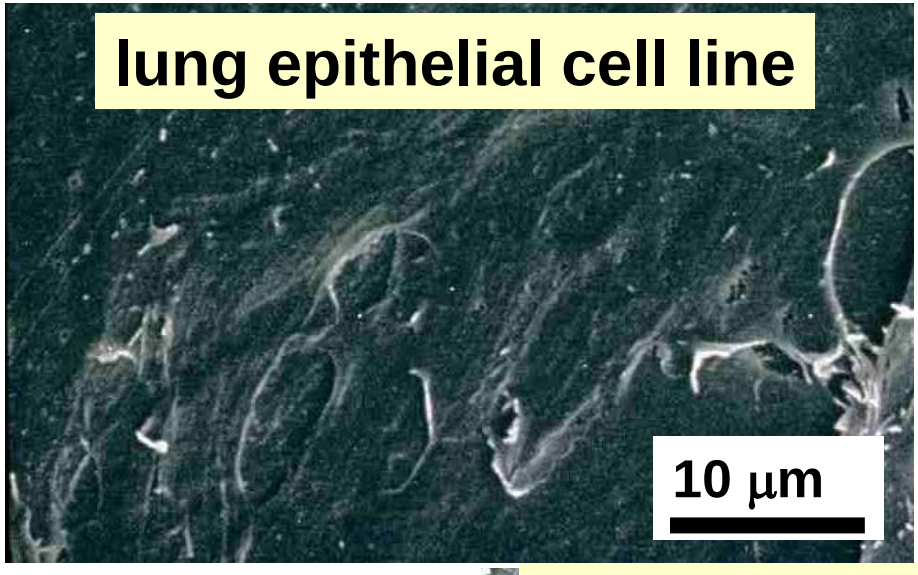


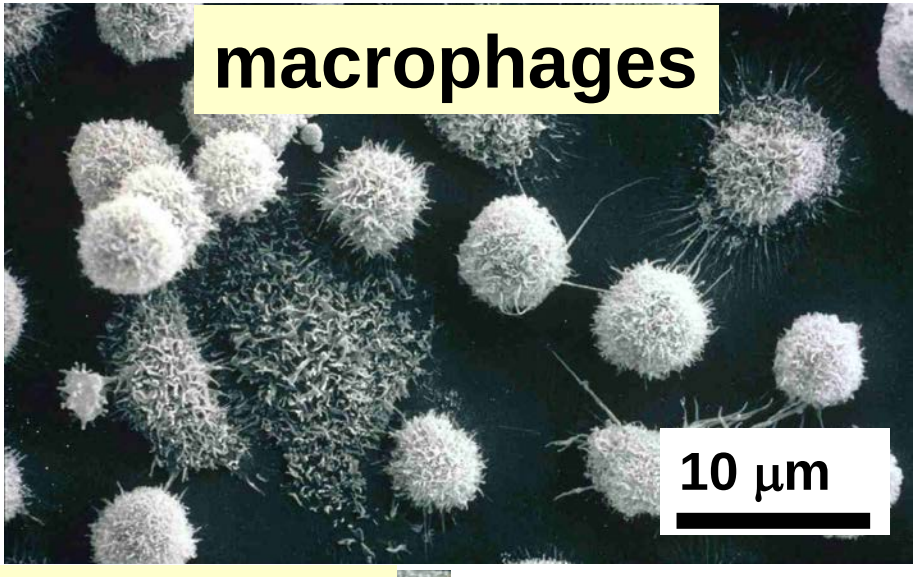

In vivo
Male Sprague Dawley adult rats
In vitro
Immortalized rat lung epithelial cells
In vitro
Macrophages
Rat BAL macrophages
In vitro
Co-culture
Rat lung epithelial cells & BAL macrophages
Particles Tested
PBS (vehicle control)
Carbonyl iron (negative control)
Amorphous silica (negative control)
Crystalline silica (positive control)
Nano-Zinc Oxide
Fine-Zinc Oxide
Intratracheal instillation
Incubation
Concentrations
1 & 5 mg/kg
Concentrations
0.005 - 520 mg/cm2
Time Points
24 hr, 1 wk, 1 & 3 mo
Time Points
1, 4, 24, 48 hr
Post-Exposure Evaluation
BAL cytotoxicity
Inflammation
Cytotoxicity
Production of Inflammatory mediators
Hemolytic potential

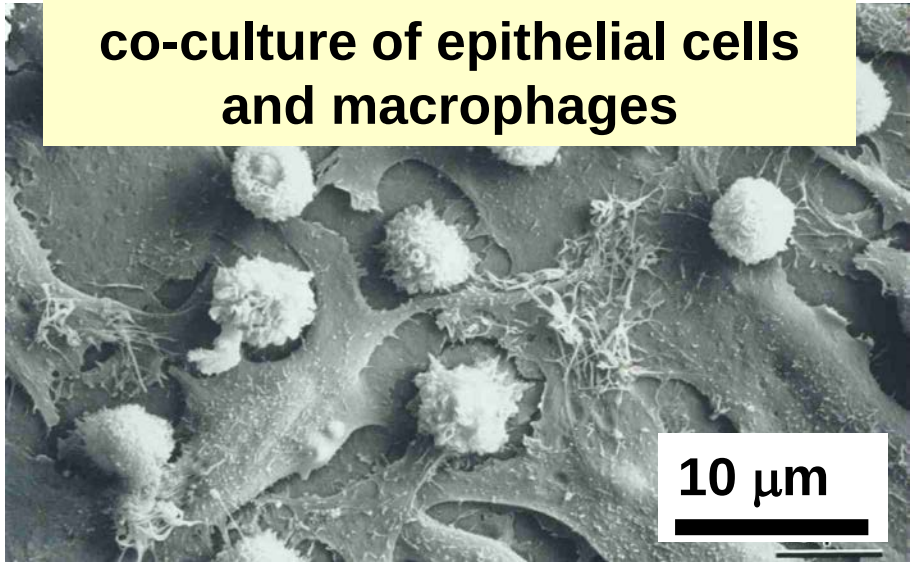
Variables assessed in this *in vitro* vs. *in vivo* study

- 5 different fine and nanoscale particle-types
- Time course studies – 1, 4, 24, or 48 hrs
- Cell-types utilized *in vitro* – epithelial, macrophage, and epithelial/MΦ co-culture
- Endpoints – inflammation and cytotoxicity
- *In vivo* biomarkers – BALF LDH and PMNs
- *In vitro* biomarkers – LDH and MTT (cytotox)
- *In vitro* biomarkers – MIP-2, TNF-α, IL-6

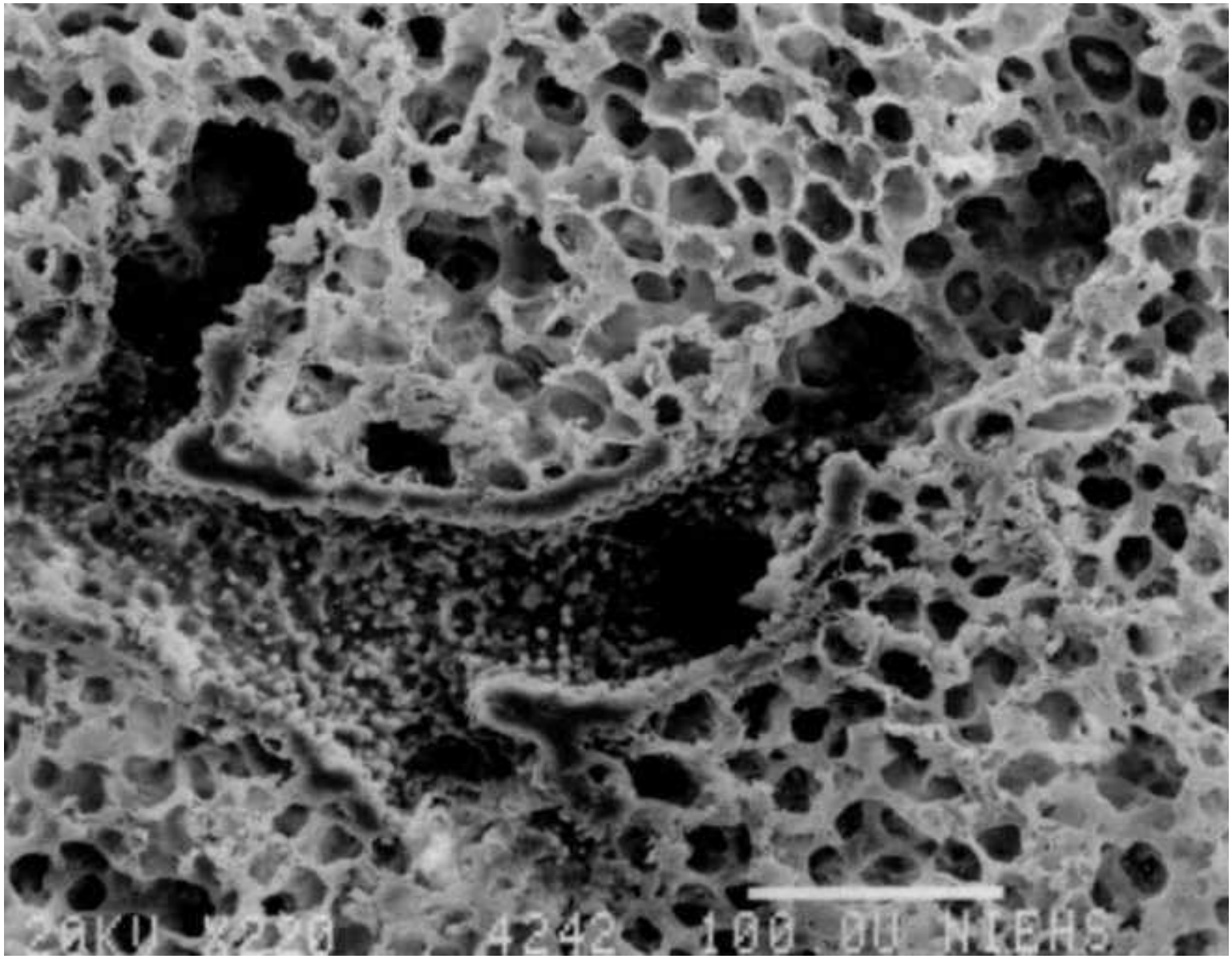
Case Study 1: *in vitro* vs. *in vivo*



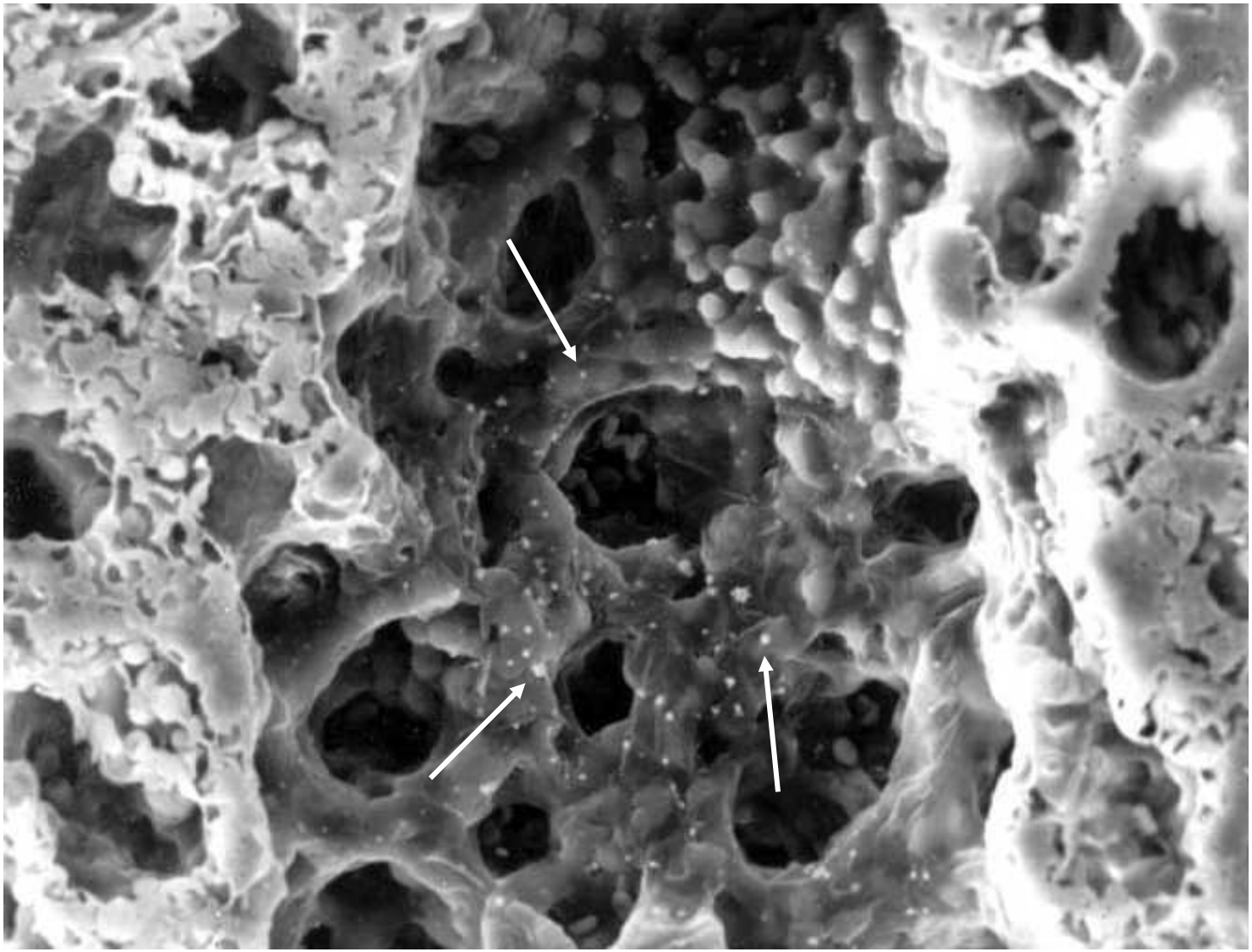




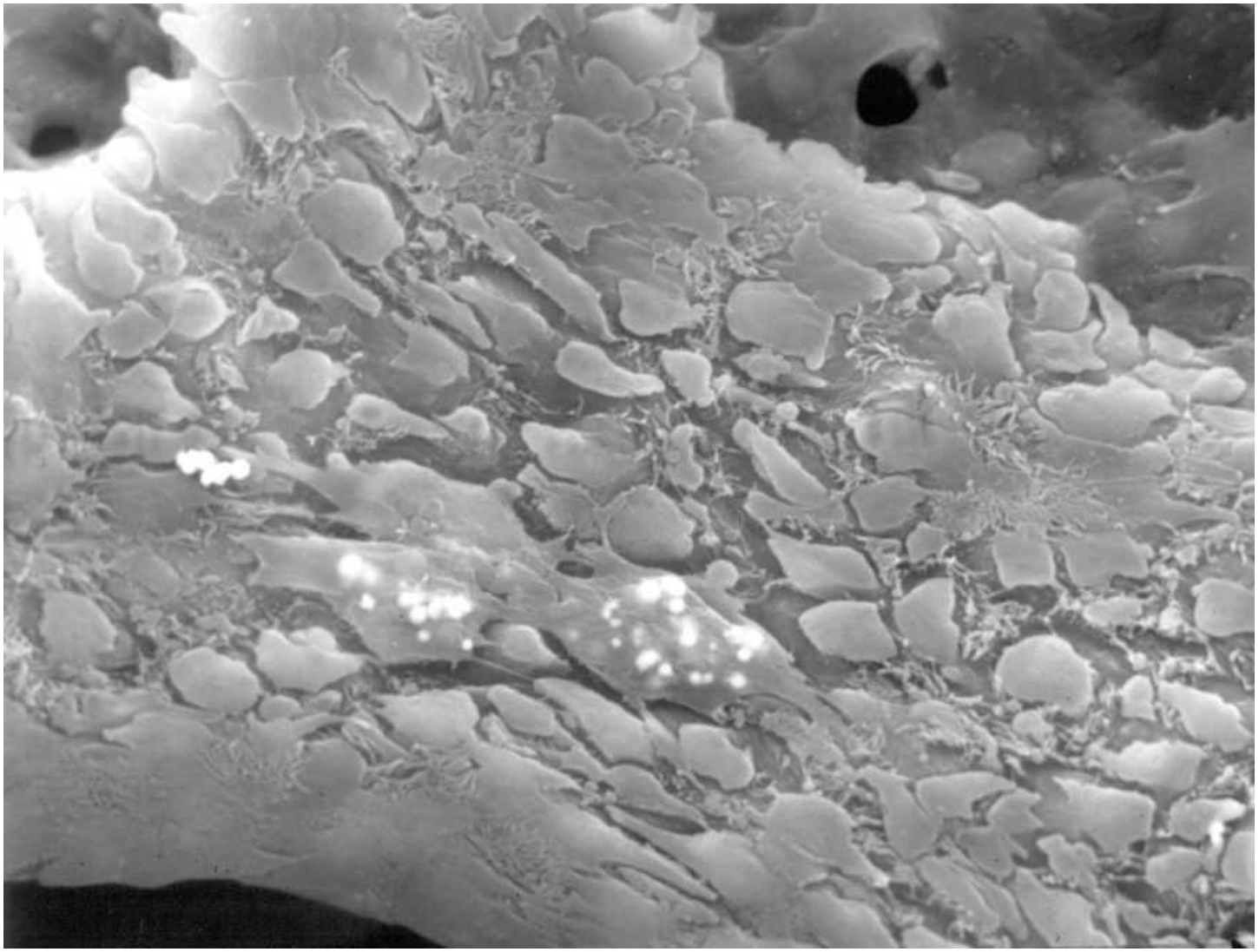
Rat Lung Tissue Dissected to Demonstrate the Junction of the Terminal Airway and Proximal Alveolar Region



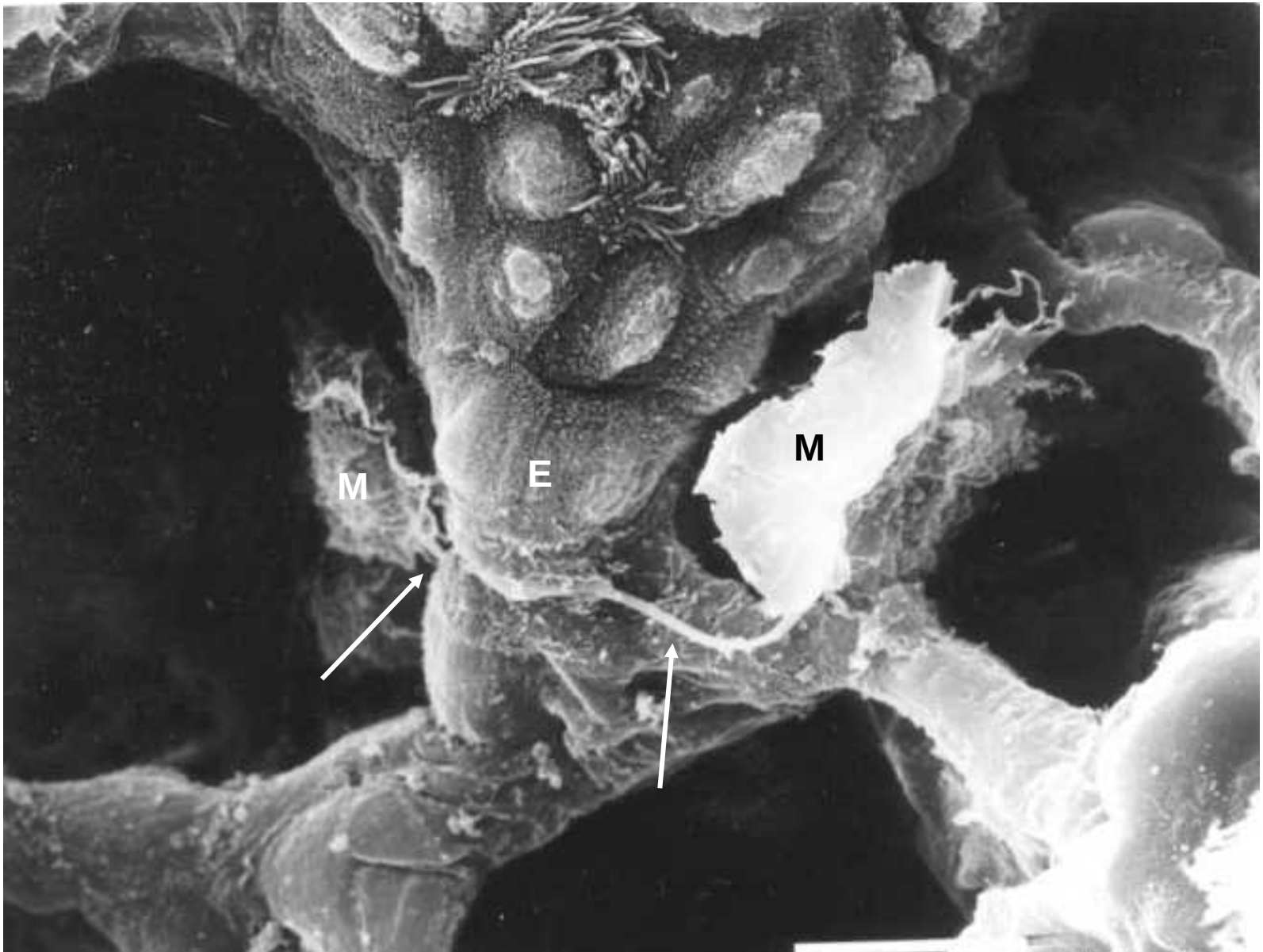
Iron Particle (↑) Deposition in the Lungs of Exposed Rats



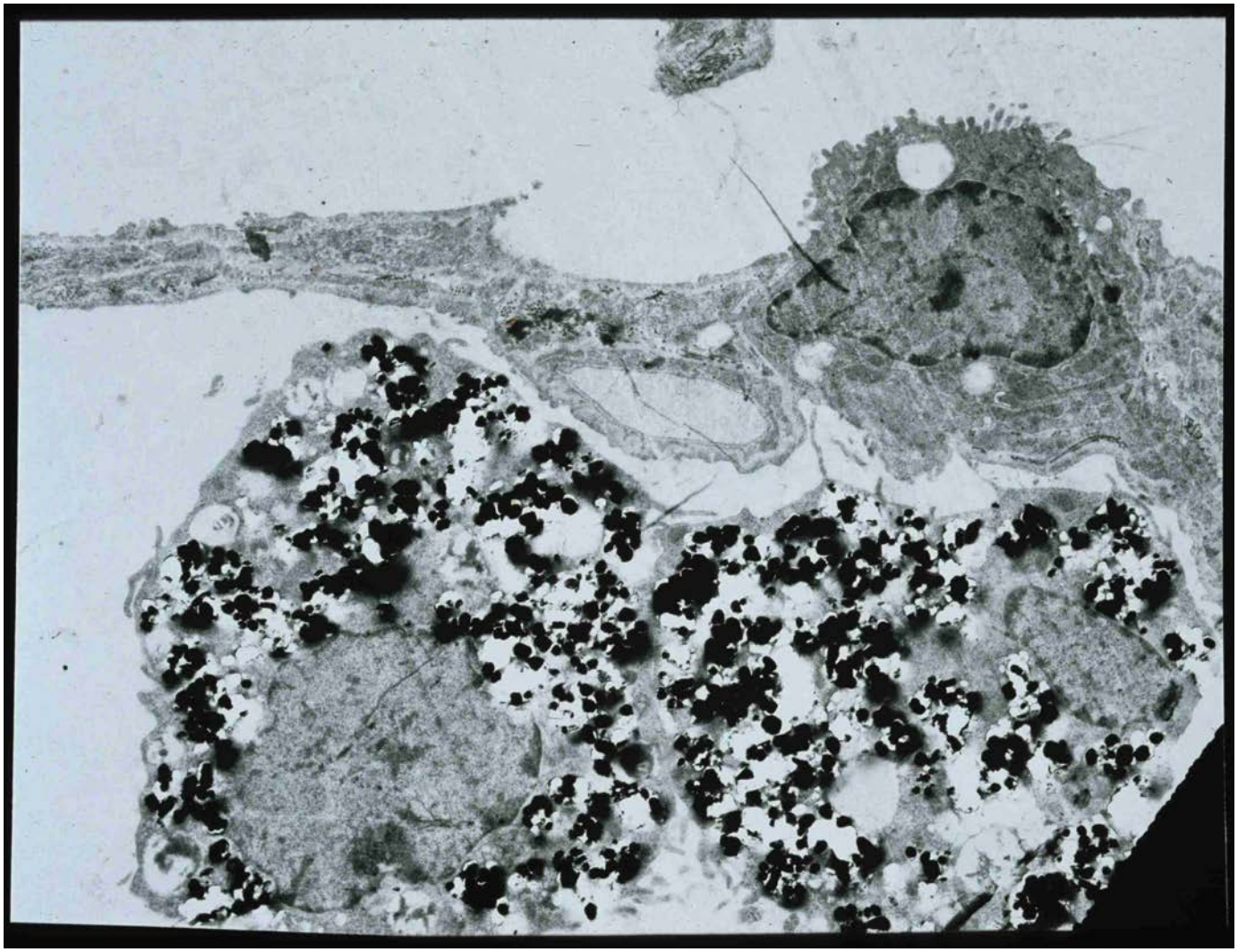
Alveolar Macrophage Migration to Iron Particle Deposition and Phagocytosis

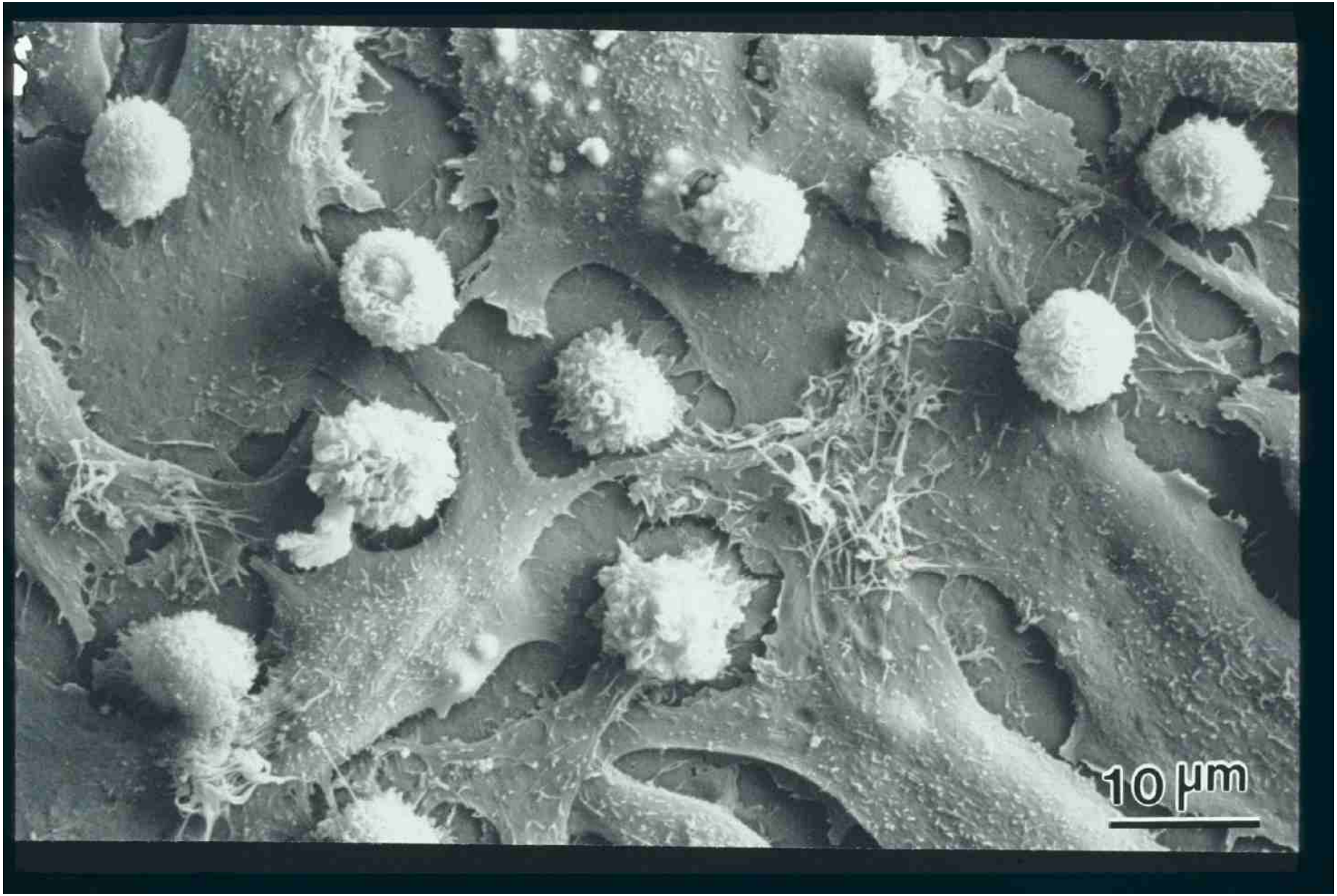


Alveolar MΦs (M) Sharing a Chrysotile Asbestos Fiber (↑) with an Alveolar Epithelial Cell (E)



Macrophage phagocytosis of TiO_2 particles




10 µm

Comparisons of *in vivo* and *in vitro toxicity* results using the same particle-types – Sayes *et al*. 2007

- In two separate studies assessing the capacity of *in vitro* studies to predict the pulmonary toxicity of particles *in vivo* – **the tests systems utilized were not accurate screens for inflammatory potential or cytotoxic effects *in vivo*.**

- *In vitro* studies may be more suitable for mechanistic toxicity studies wherein hypotheses are being tested.

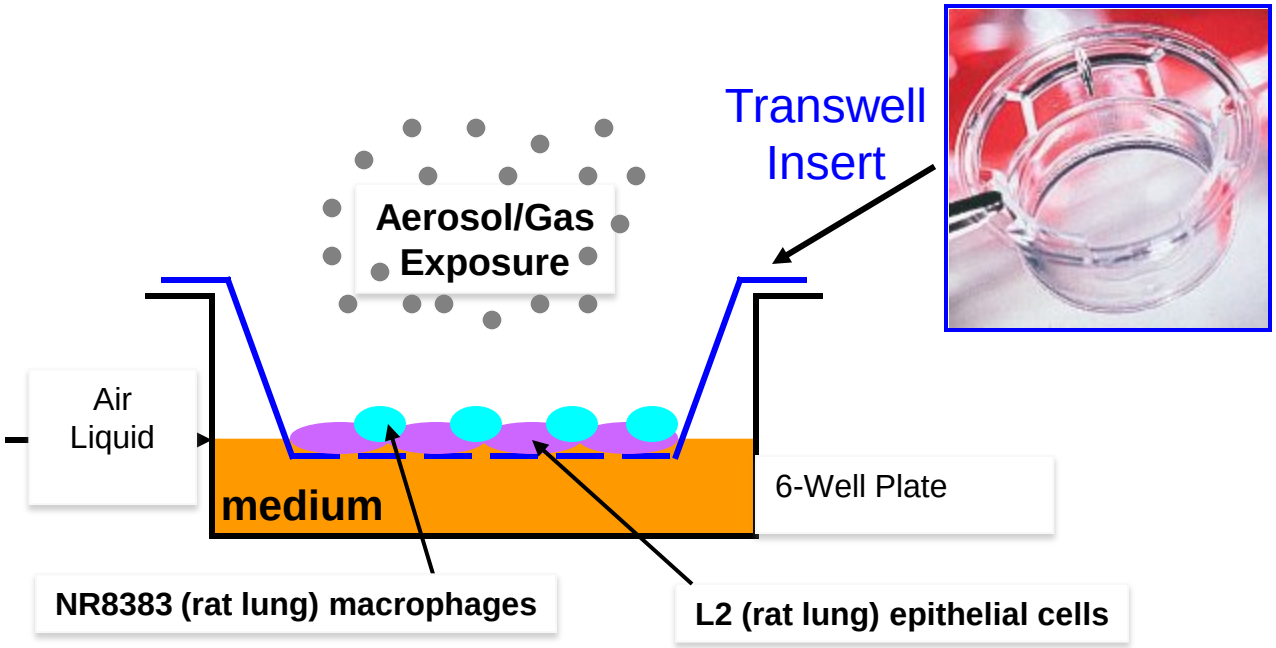
- Utilization of *in vitro* studies to serve as predictive toxicity screens need further development and validation.

How can we improve on the predictive value for developing *in vitro* pulmonary screening assays to assess toxicity of particulates?

S Ng and DB Warheit

Revising the *In vitro* strategy

- Need for *In vitro* cell-based assays for pulmonary toxicity tests / screening
- Conventional submerged system
- vs. Air-Liquid Interface (ALI)



Aims: (1) Optimize Cell Growth/Assay Conditions and Parameters

(2) Develop Toxicity End-points/Assays Applicable to ALI Co-Cultures

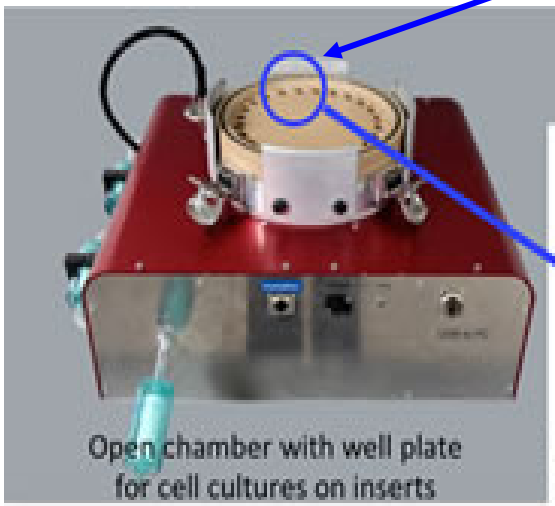
(3) Purchase & Set-up the In Vitro Aerosol Exposure Chamber

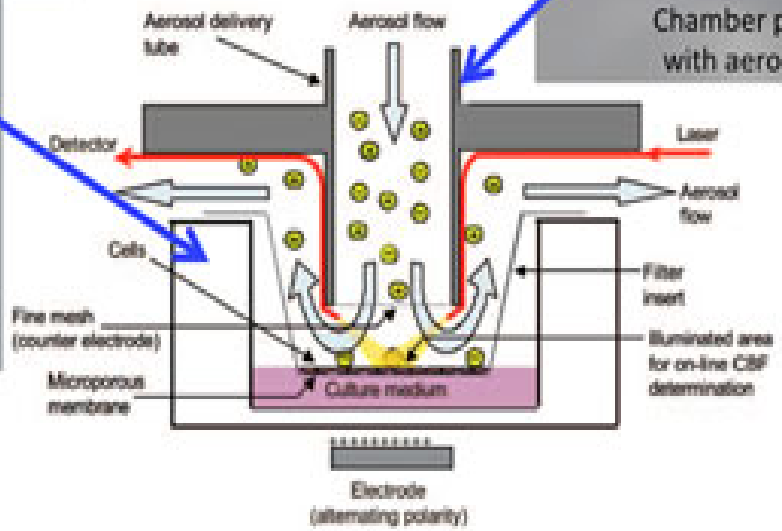
Nano-Aerosol Chamber for In Vitro Toxicology

Features:
Internal heater & humidifier
RH & T sensors
Integrated pumps & flow control
Aerosol charger

Savi et al., *Environ. Sci. Technol.*, 42, 5667–5674, 2008

Air-Liquid Interface (ALI) Co-Cultures





Aims / Method:

24-hr exposures to ZnO fine particles, applied in suspension, cells maintained on ALI

1. Optimization:

(a) Cell seeding density (0.25 or 0.5 x 10^5 total cells/cm^2)

(b) L2 (epithelial) to NR8383 (macrophage) ratio

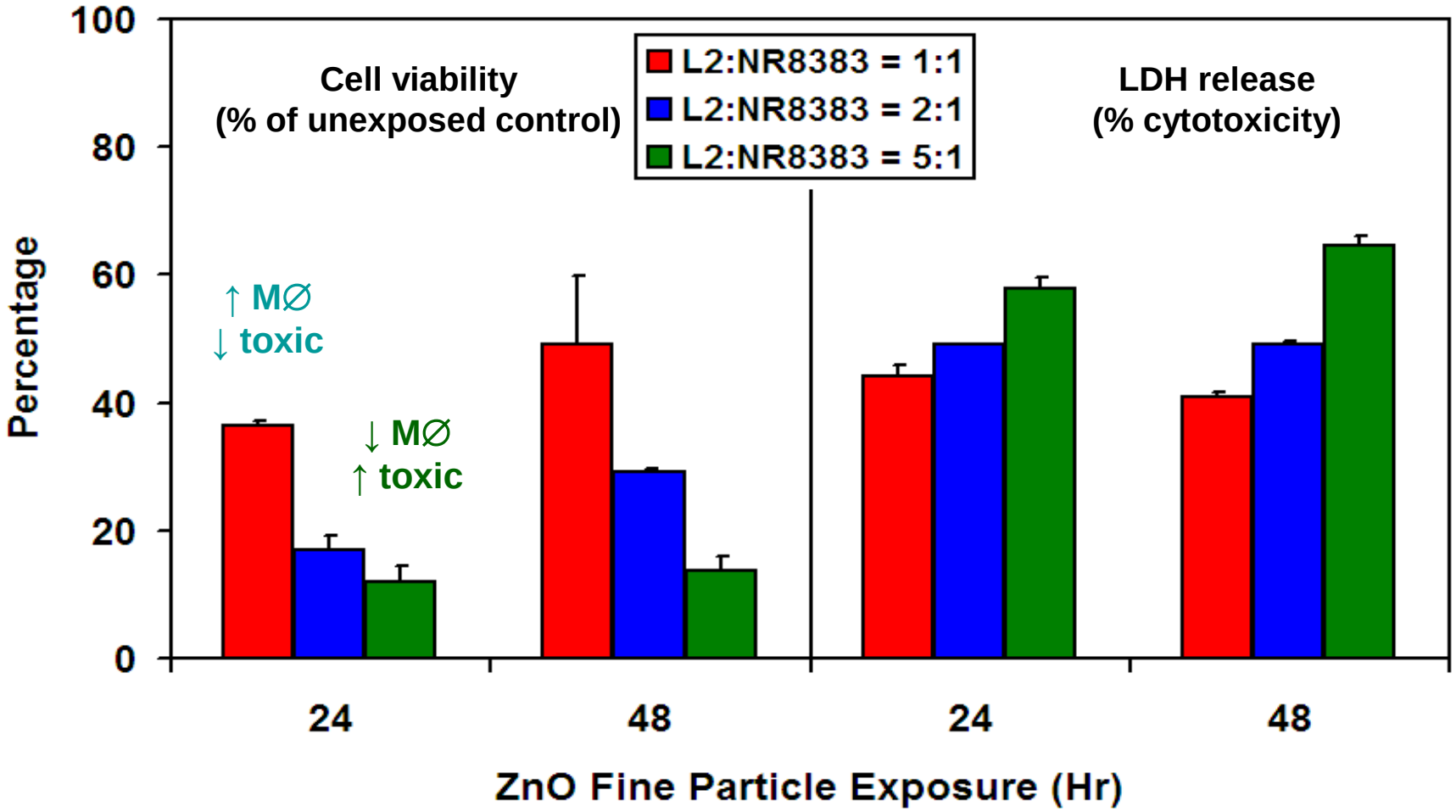
1:1, 2:1 (↓ MØ), 5:1 (↓ ↓ MØ)

(c) Immortalized NR8383 macrophages vs. MØ from rat lungs

- biologically relevant substitute

- **2. Develop Toxicity End-points / Assays:**
- **- XTT viability assay – metabolism (mitochrondrial dehydrogenase)**
- **- LDH release assay – cell lysis (lactate dehydrogenase)**
- **- Cytokines: IL-6, TNF-a, MIP-2**

Result : Percent cell viability and cytotoxicity of 20 μg/cm² fine-sized ZnO particle exposure varied with L2(epithelial):NR8383(M∅) cell ratios



Mean (n=2) ± SD; Seeding density = 0.25 x10^5 total cells/cm^2

Developing Reliable High Throughput Screening (HTS) Assays- How do we get there? [a suggested pathway]

- Absence of validated screening tools
- Many of the current published study designs incorporate high-dose overload acute exposures to single cells concomitant with a single postexposure time period and do not consider that underlying mechanisms are dose-dependent or time-course related.

How do we get there? (2) – suggested template

- Reliable *in vivo* data – as a foundation
- Currently – a paucity of useful *in vivo* hazard data to establish a foundation for development of better screening tools

- Need development of relevant *in vivo* hazard data based upon simulation of relevant routes of exposure and realistic exposure conditions as a starting point

How do we get there (3)

- Suggest building a relevant *in vivo* database of 5 – 10 representative nanomaterial-types and then benchmarking *in vitro* and *in silico* methodologies vs. the *in vivo* results for a particular simulated exposure route.

How do we get there (4)

- *In vitro* studies need to focus on more complex experimental design issues:
- More robust nanomaterial characterization;
- Relevant dose and dosimetry;
- Dose response and time course characteristics;
- Appropriate target cells;
- Development of standardized reference materials or benchmark (positive) controls.