FRAUNHOFER INSTITUTE FOR TOXICOLOGY AND EXPERIMENTAL MEDICINE ITEM

Research for human health

Alternative methods in inhalation toxicology

Prof. Dr. Armin Braun

Head of Pre-clinical Pharmacology and In Vitro Toxicology

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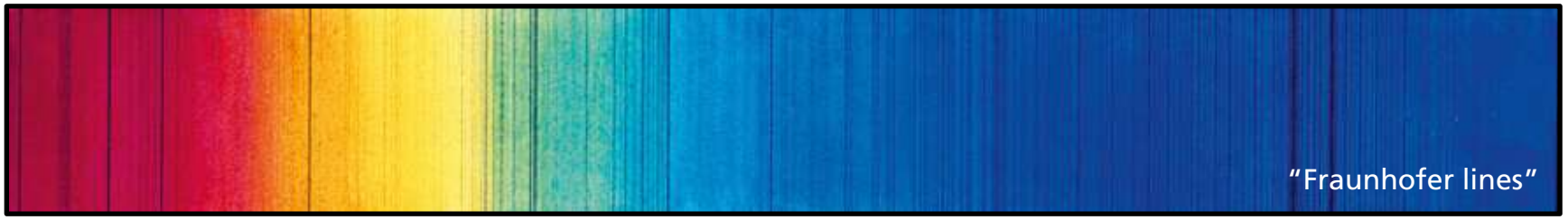
Fraunhofer-Gesellschaft, the largest organization for applied research in Europe

- 66 institutes
- 24,000 staff
- € 2 billion annual research budget totaling
  - two thirds contract research for industry and public
  - one third by the German governments base funding
- International cooperation



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Institute facts and figures 2013/2014

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Our focus, our aim

TABLE OF CONTENT

Alternative methods in inhalation toxicology

P.R.I.T. ExpoCube:
an innovative in-vitro exposure system

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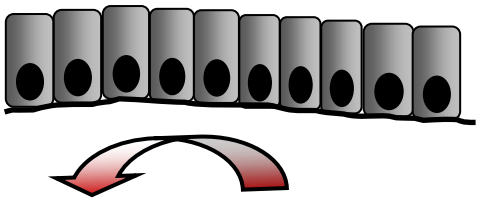
Air-lifted interface (ALI) cultures

Human lung

Biological barrier

Inhalable compounds "air"



"blood" / liquid

Inhalable compounds "air"

Culture medium "liquid"

ALI culture

Air-liquid/air-lifted interface cell culture technique

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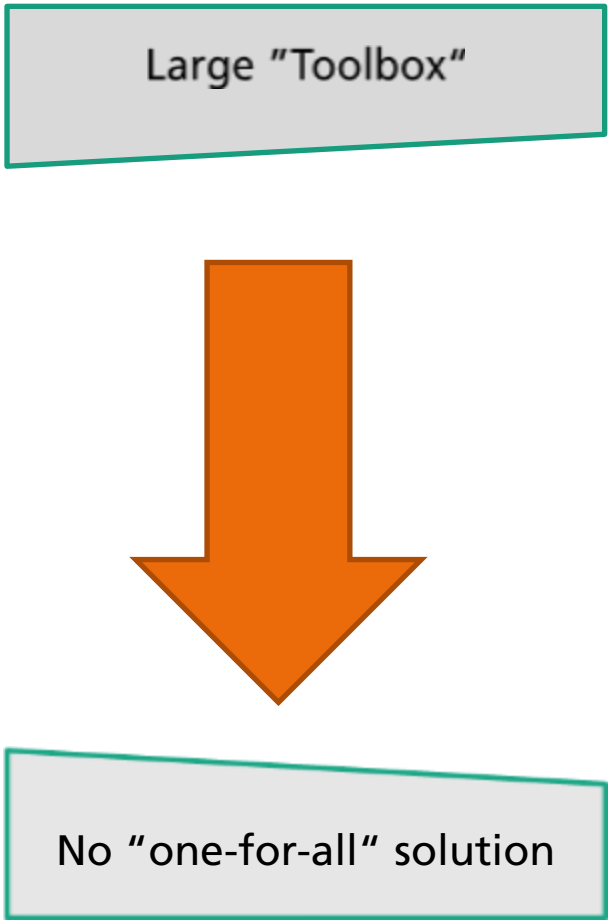
Air-lifted interface (ALI) cultures



- Human cell lines
- Primary cells
- Complex models
  - 3D-models
  - Ex-vivo models
    Precision-cut lung slices

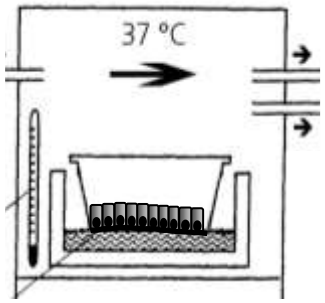
- *Competence…*
- *Coverage…*

- *Commercial availability…*
- *Costs…*

- *"Validated" model ?*

ALI exposure

"Incubator type"



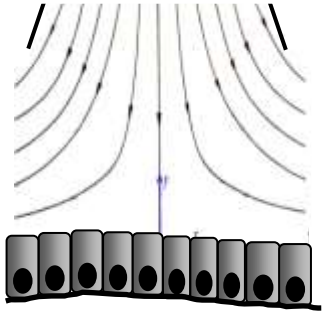
Pro

- "Easy-to-use" setup

Con

- Less effective exposure
- No single culture exposure

"Stagnation point flow"



Pro

- Single culture exposure
- Effective exposure

Con

- More elaborate setup necessary

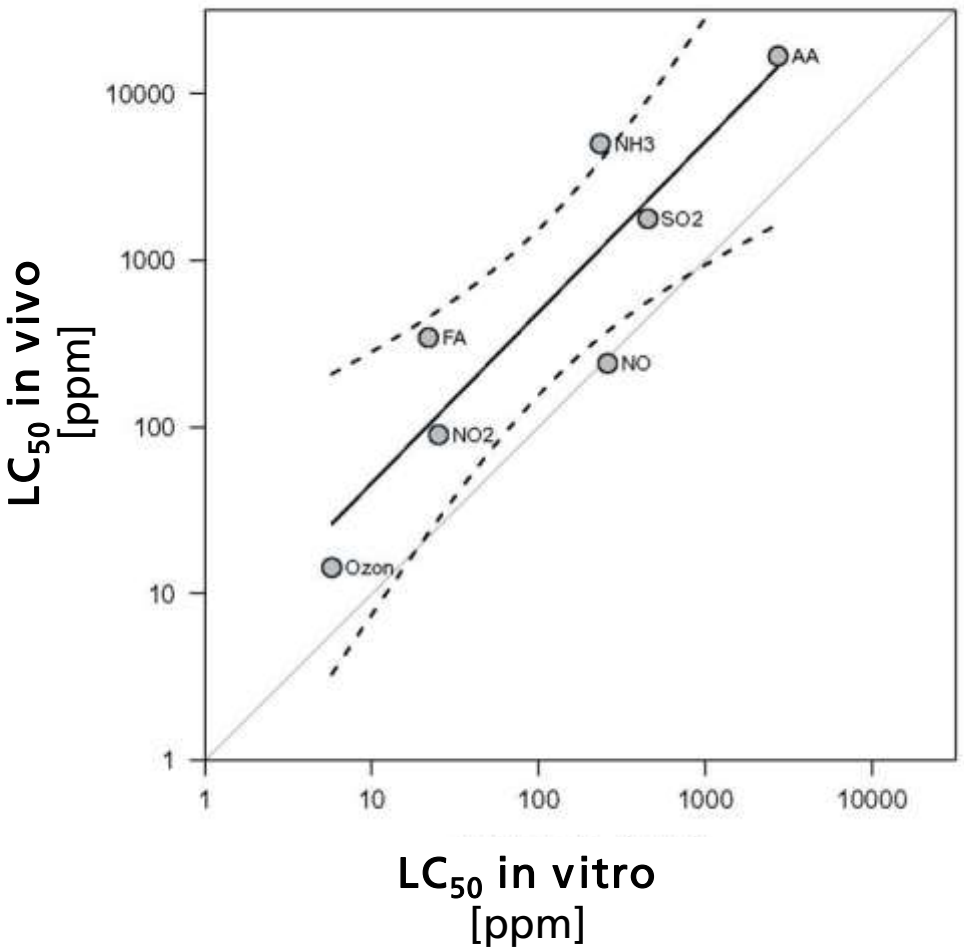
Chemical gases

- **(Pre-)validation study**

- 4 labs (Germany)
- "acute" tox
- A549 human lung cells
- 7 (highly) toxic chemical gases
- 3 non-toxic inert gases

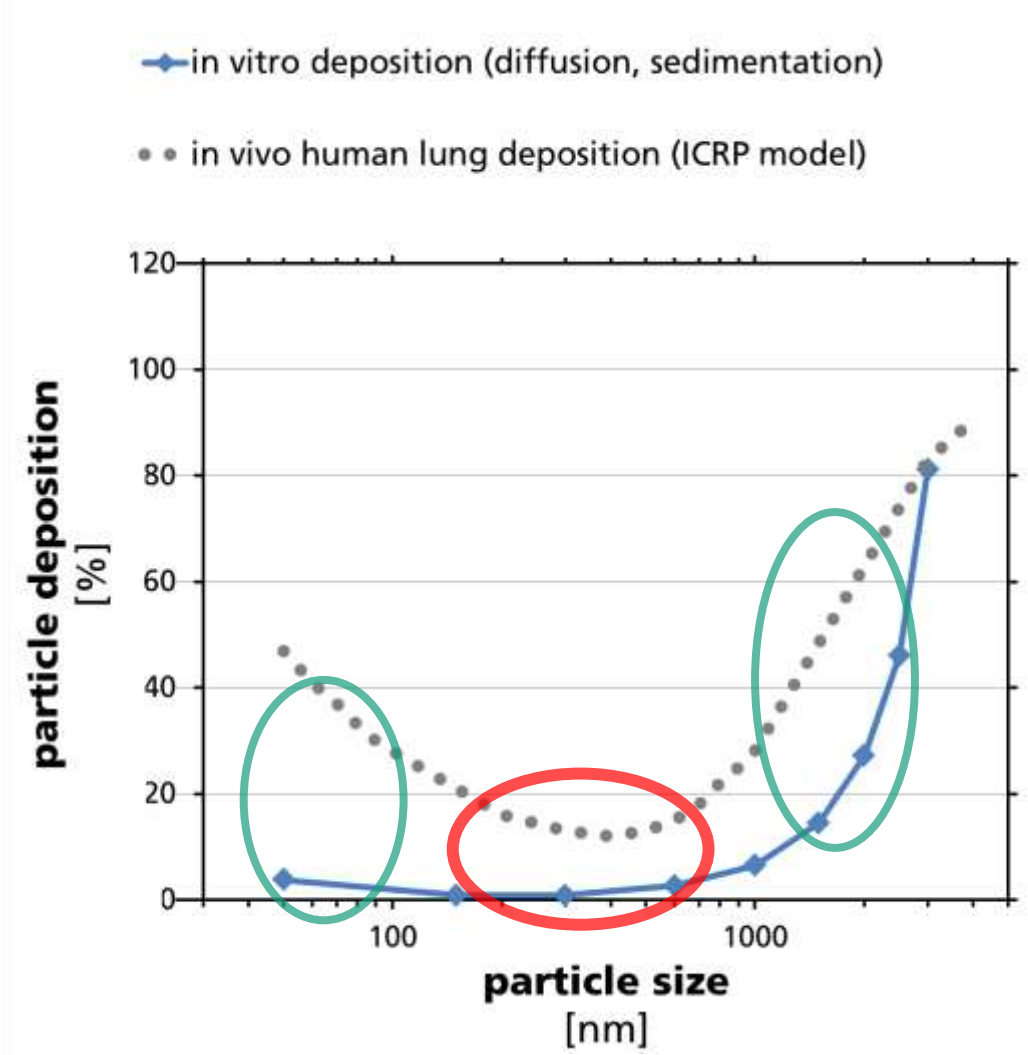
- good inter- and intra-lab reproducibility

- first **prediction model**
- **no false positives** detected



Pirow et al. 2015, in preparation

The "standard" ALI particle deposition scenery



+Similar relative particle deposition rates

! Particles < 1000 nm

! Low absolute particle deposition rates

- long, not realizable exposure times

Enhancement of particle deposition

Electrostatic deposition

- Aerosol charging
- Unipolar field
- Bipolar field

Effective method

- **Theory: 100%**
- **Lab: 4 – 47%**

Interactions between electrical forces and cell biology / mode of action? *)

**) Nanoparticle charge modifies toxicity (Schaeublin et al. 2011)*
Cellular uptake of nanoparticles is dependent on particle charge (Schrade et al. 2012)

Droplet deposition

- Nebulization of particle suspensions

Effective method

- **56% (liquid droplets)**

No native or dry particle aerosols

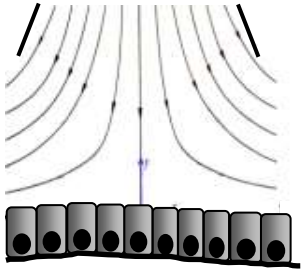
Thermophoresis

- Thermal gradient

- **No adverse effects on exposed cells**
- **Only minimal manipulation of aerosol**
- **Effective**

Particle deposition

Exposure efficiency



Stagnation point flow

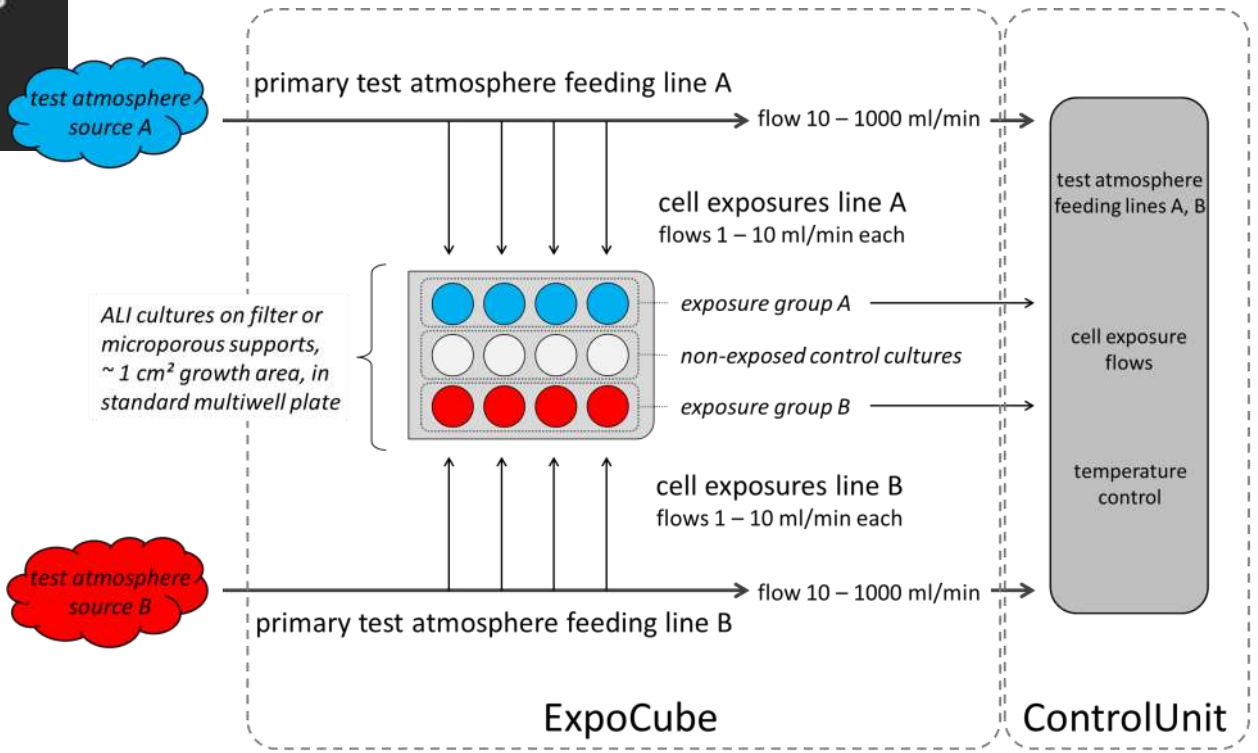
Thermophoresis

“Smarter” work, read-out

“All-in-one-plate” concept

PRIT ExpoCube®

Device-based refined ALI exposure procedure



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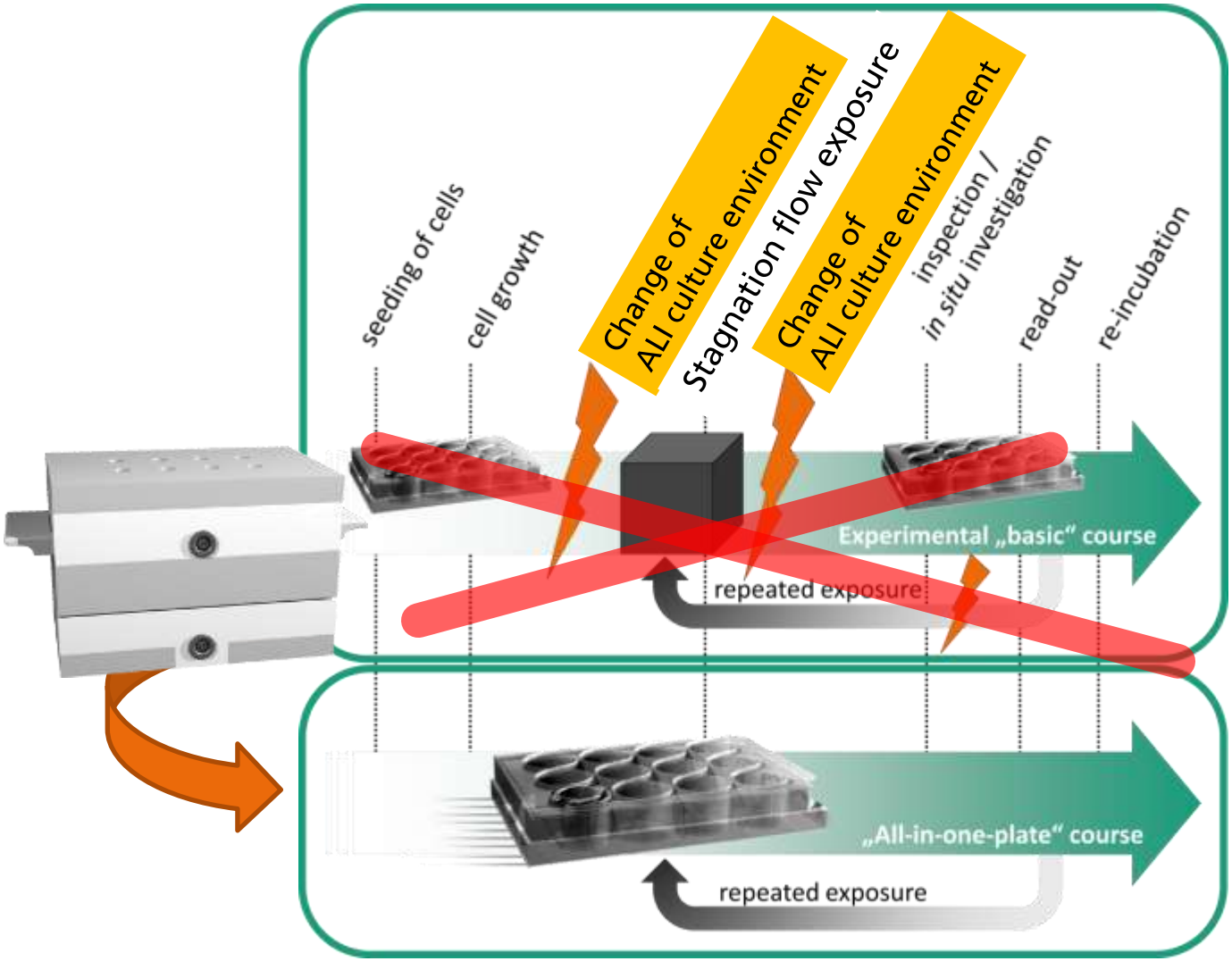
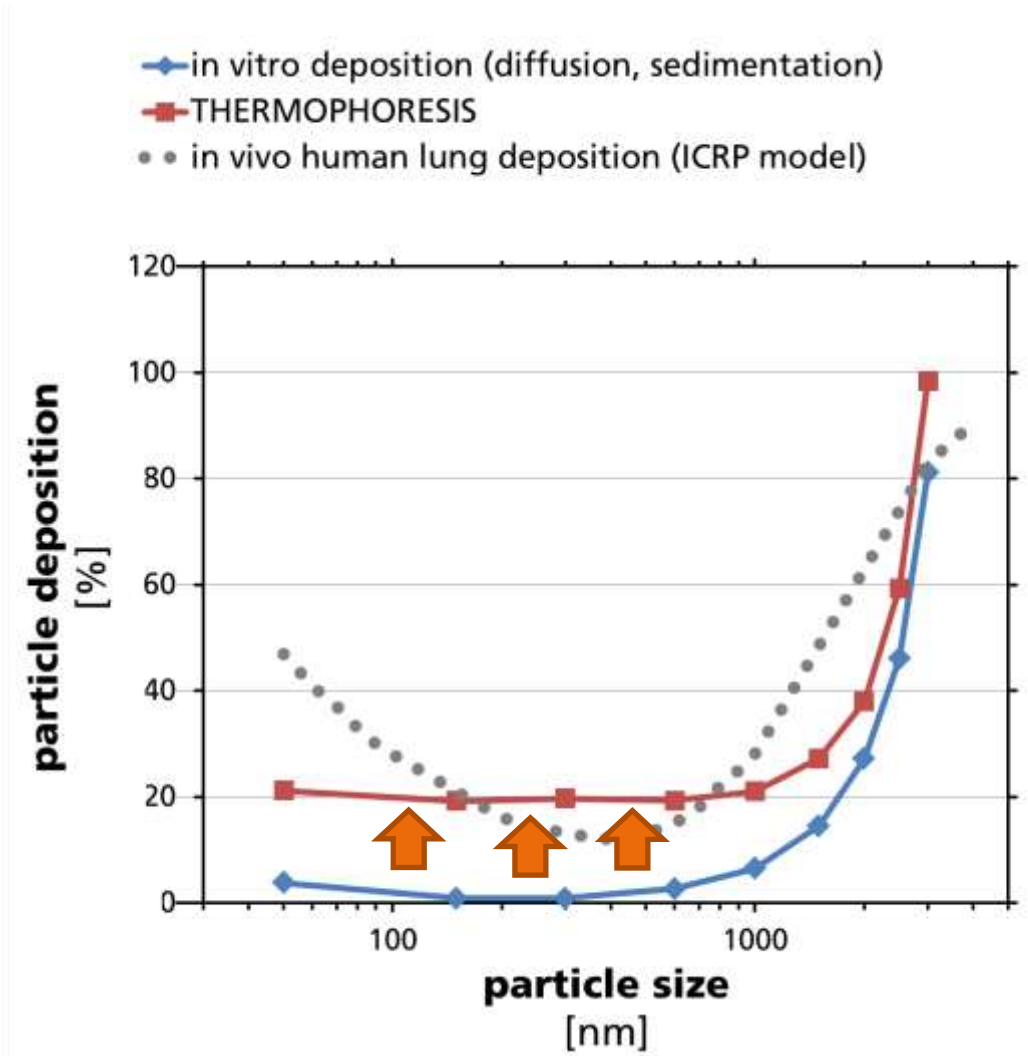
“Smarter working”



Enhancement of particle deposition by thermophoresis

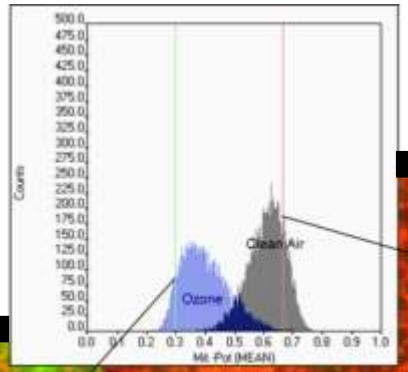


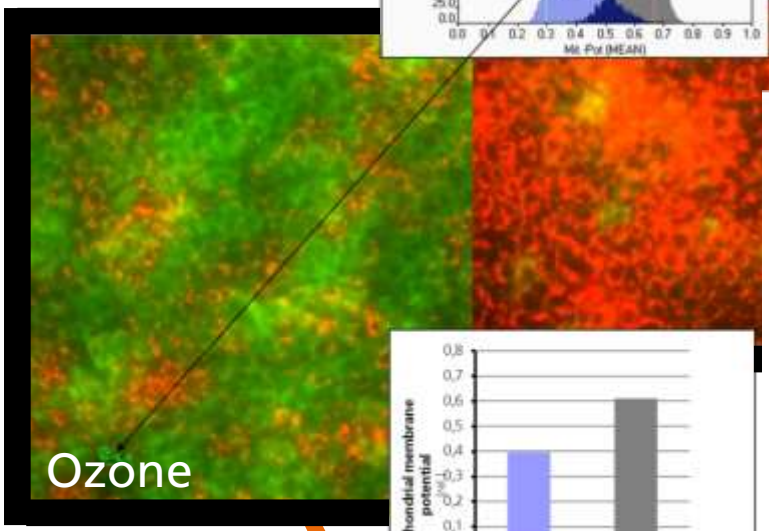
CFD Simulations

- Only minimal modification of test aerosol
- Preserved deposition characteristics for particles > 1 µm
- Enhancement to ~20% deposition rate

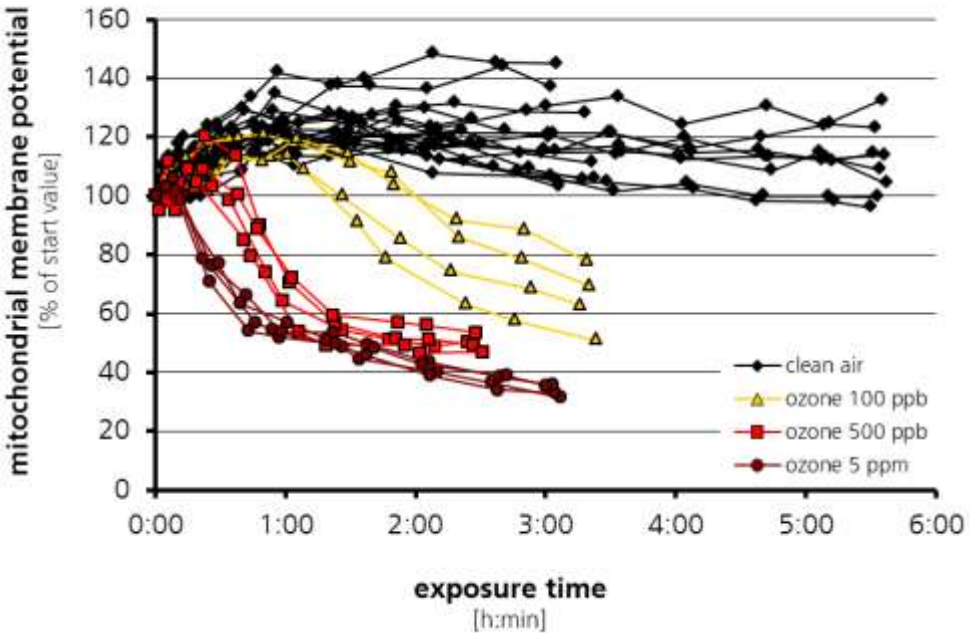
Online observation during cell exposure

Microscopic view
Single cell analysis





Online readings
Kinetic studies



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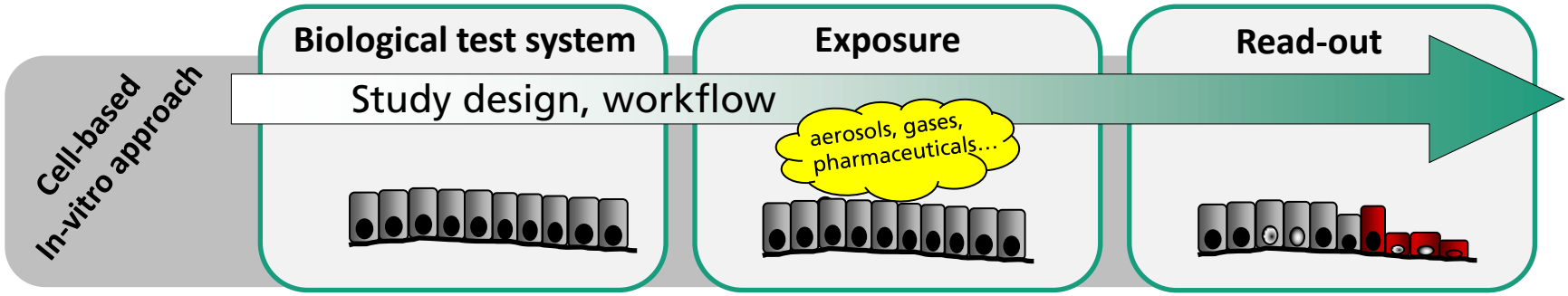
Conclusion



Focus	Status and perspectives
Biological test systems	• **Large toolbox /**no one-for-all solution **Tailored setups**
Cell exposures	• **Gases/vapors:** **Efficient and relevant methods** • **Aerosols:** Thermophoresis as a promising approach **High deposition rates/less side effects**
Read-out	• **Common in-vitro endpoints** • **Online fluorescence read-out** High content readings, reporter gene assays, kinetic studies…
Whole process	• **Multiwell plates** throughout the experiment **Smart, more robust, repeated dose etc.**

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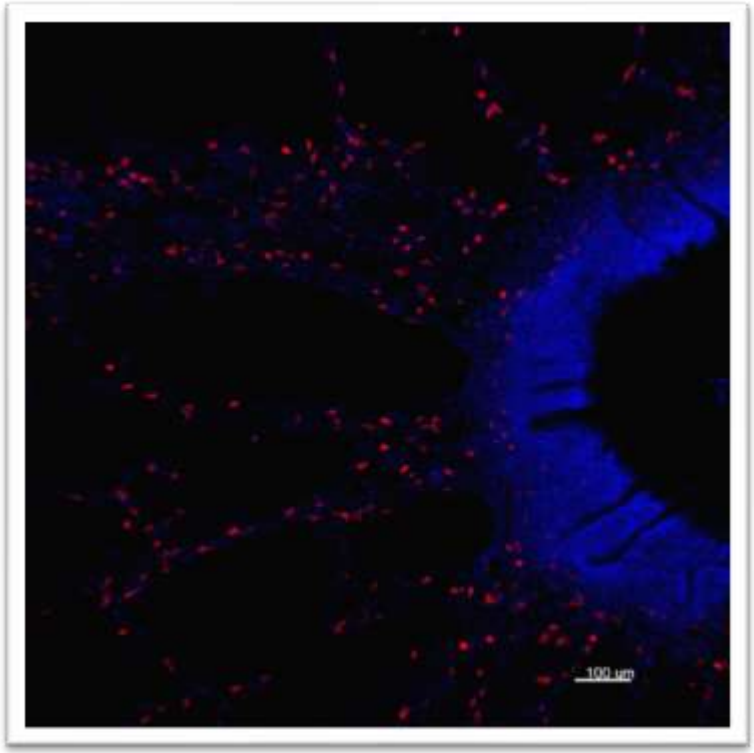
Precision-cut lung slices – a translational ex-vivo technique



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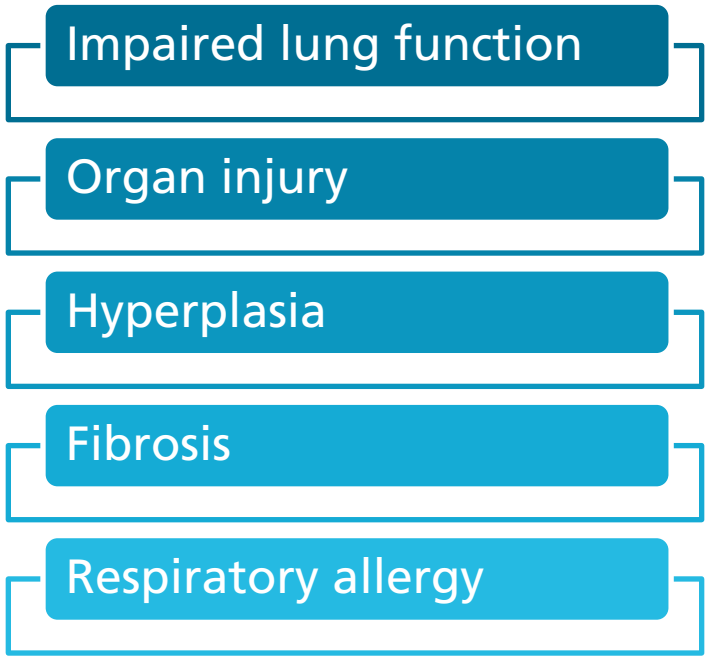
Need to breathe, want to breathe – but can't

Inhalation of harmful substances

- For many substances, inhalation is the most relevant route of exposure
- But regulatory application of alternatives has lagged behind
- Complexity of respiratory system
- Diversity of local and systemic respones
- For some substances lungs are main route but not main target



K. Sullivan et al., 2014 ATS

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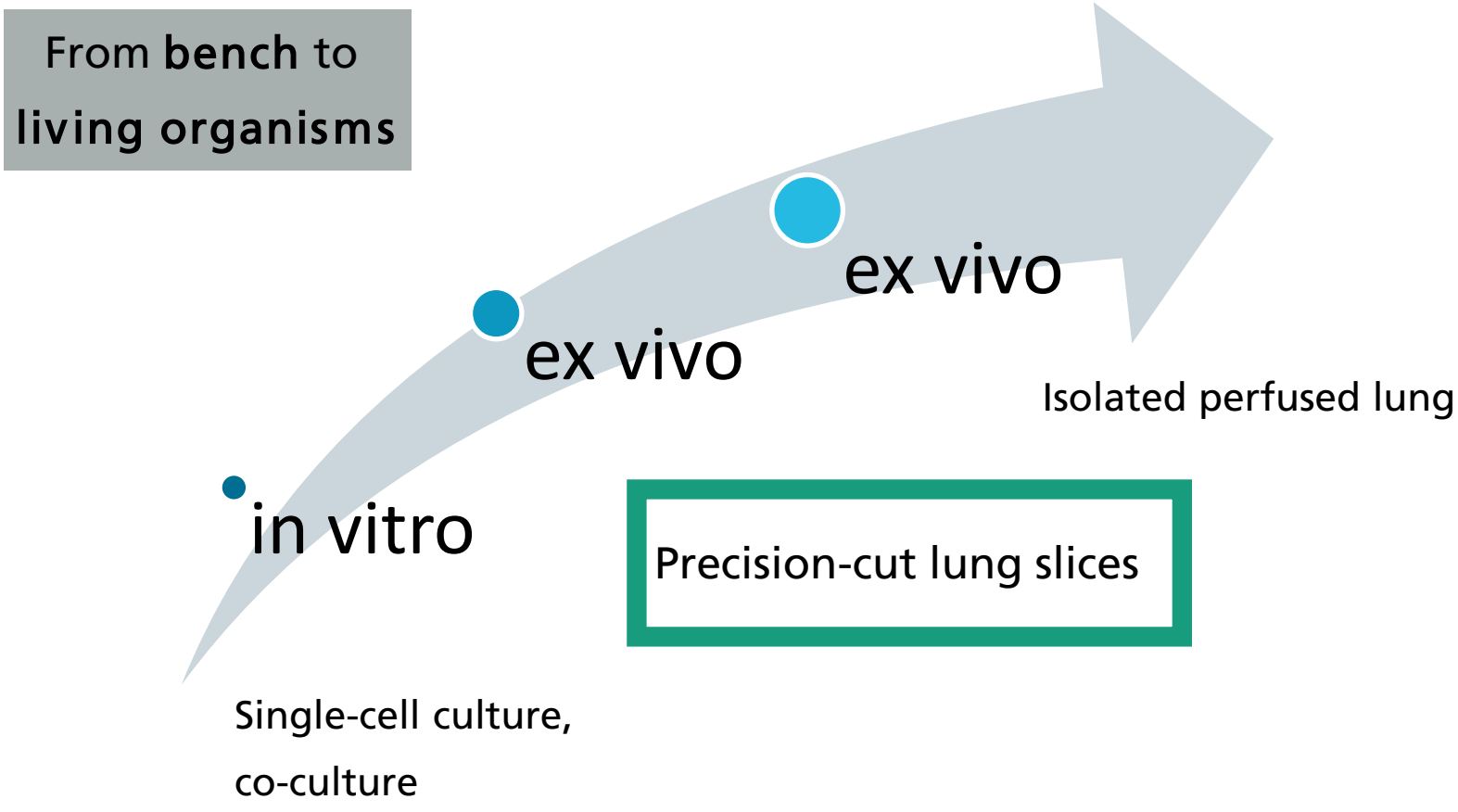
Pre-clinical pharmacology and toxicology

Precision-cut lung slices as bridge between in vitro and in vivo



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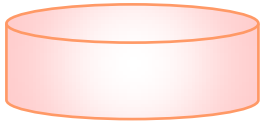
Pre-clinical pharmacology and toxicology

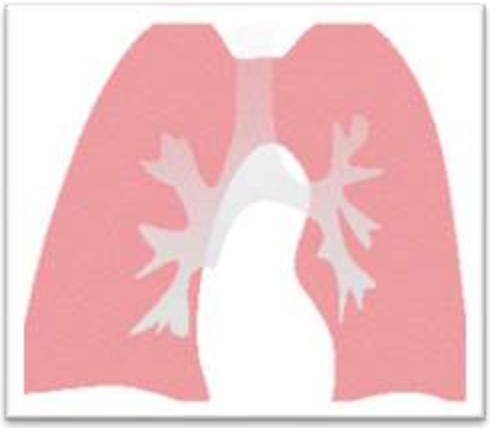
Precision-cut lung slices are obtained from lungs

Ø 8 mm



⌴ ~ 250 µm

Precision-cut lung slices are viable for days and can be exposed



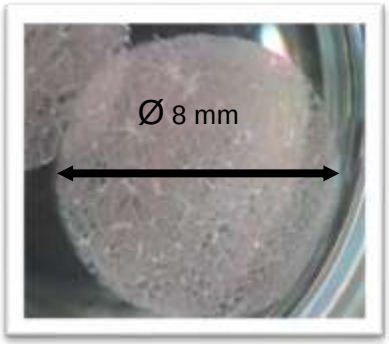


Foto: BASF

- Chemicals
- Lipopolysaccharides
- Bronchoconstricting agents
- Disease-related proteins

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Pre-clinical pharmacology and toxicology

Features of precision-cut lung slices

Precision-cut lung slices are:

- Tissue sections of the lung
- Vital
- Three-dimensional
- Composed of epithelial cells, endothelial cells, smooth muscle cells, fibroblasts, mast cells and a lot more

Species:

- Mouse, rat, guinea pig
- Non human primates (cynomolgus, marmoset, rhesus)
- Human

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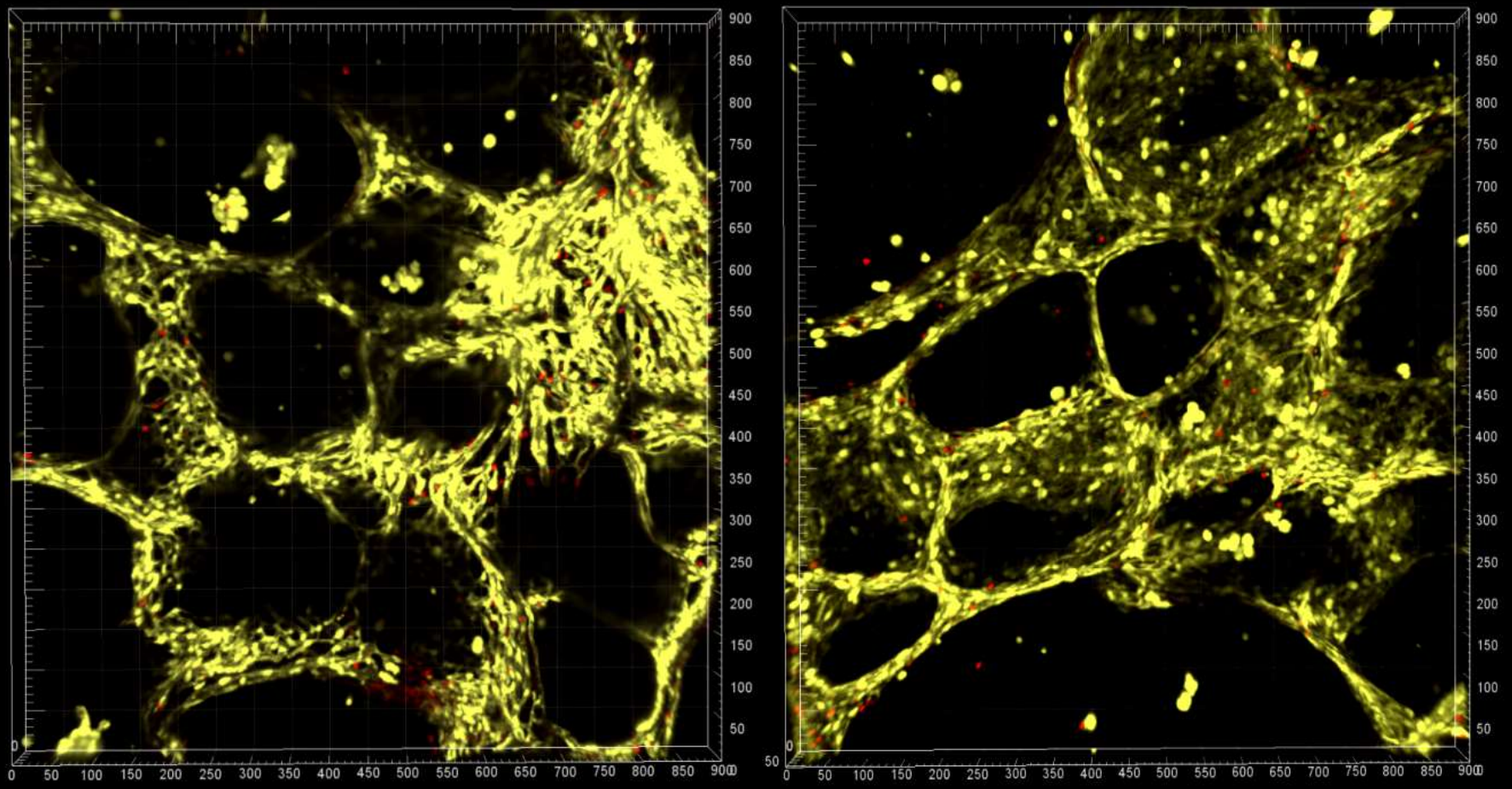
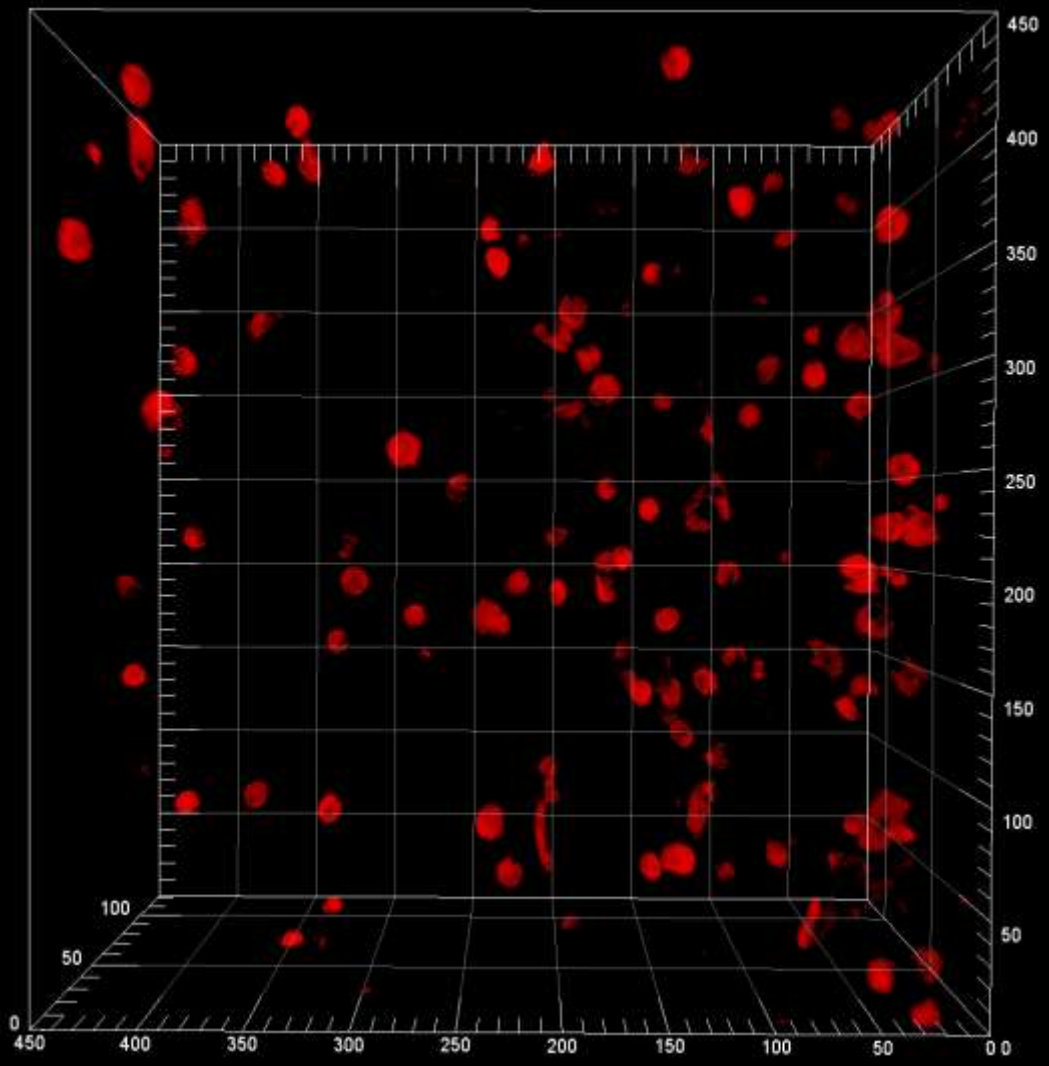
Precision-cut lung slices are viable



Calcein

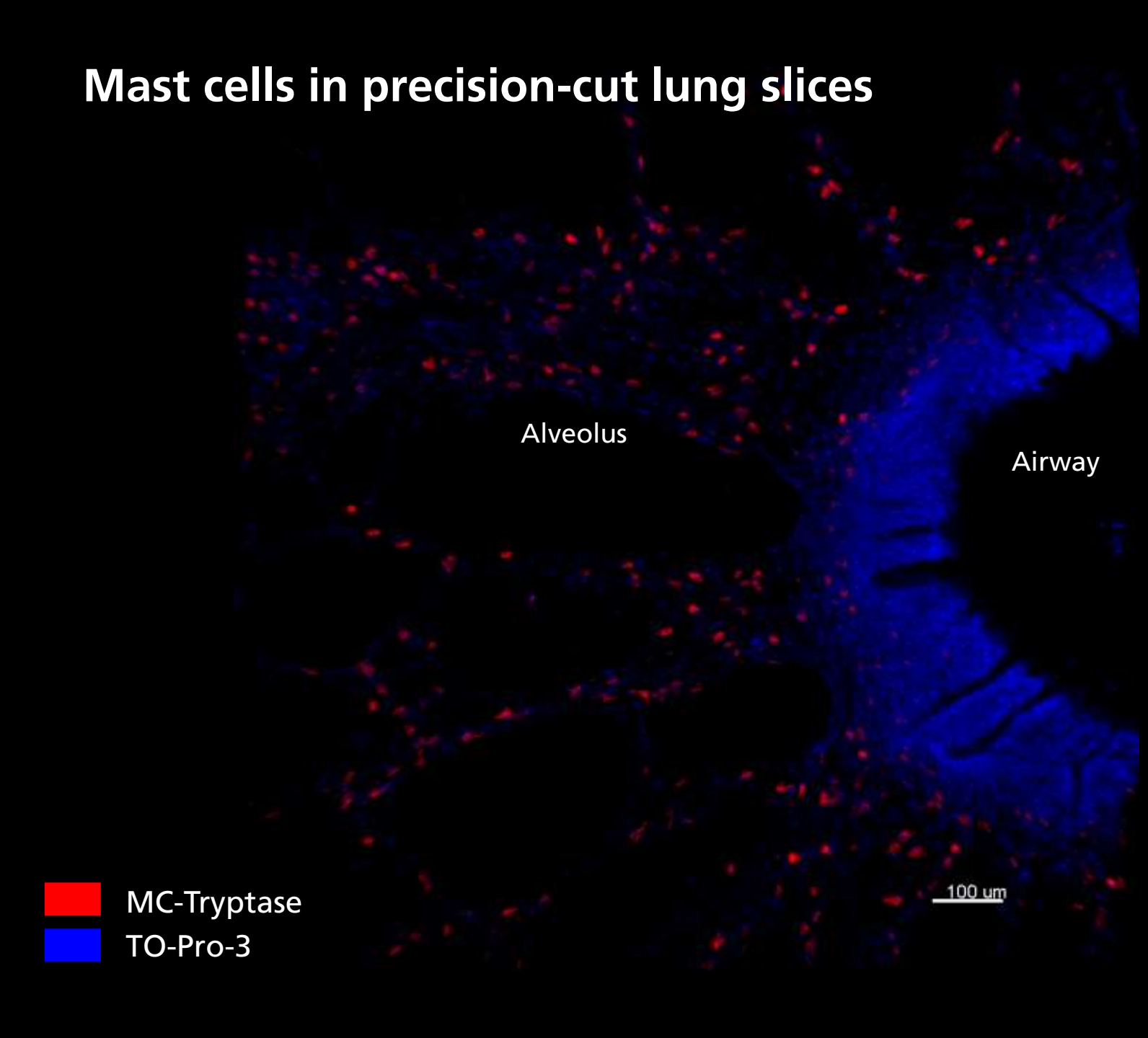
EthD-1

Macrophages in precision-cut lung slices

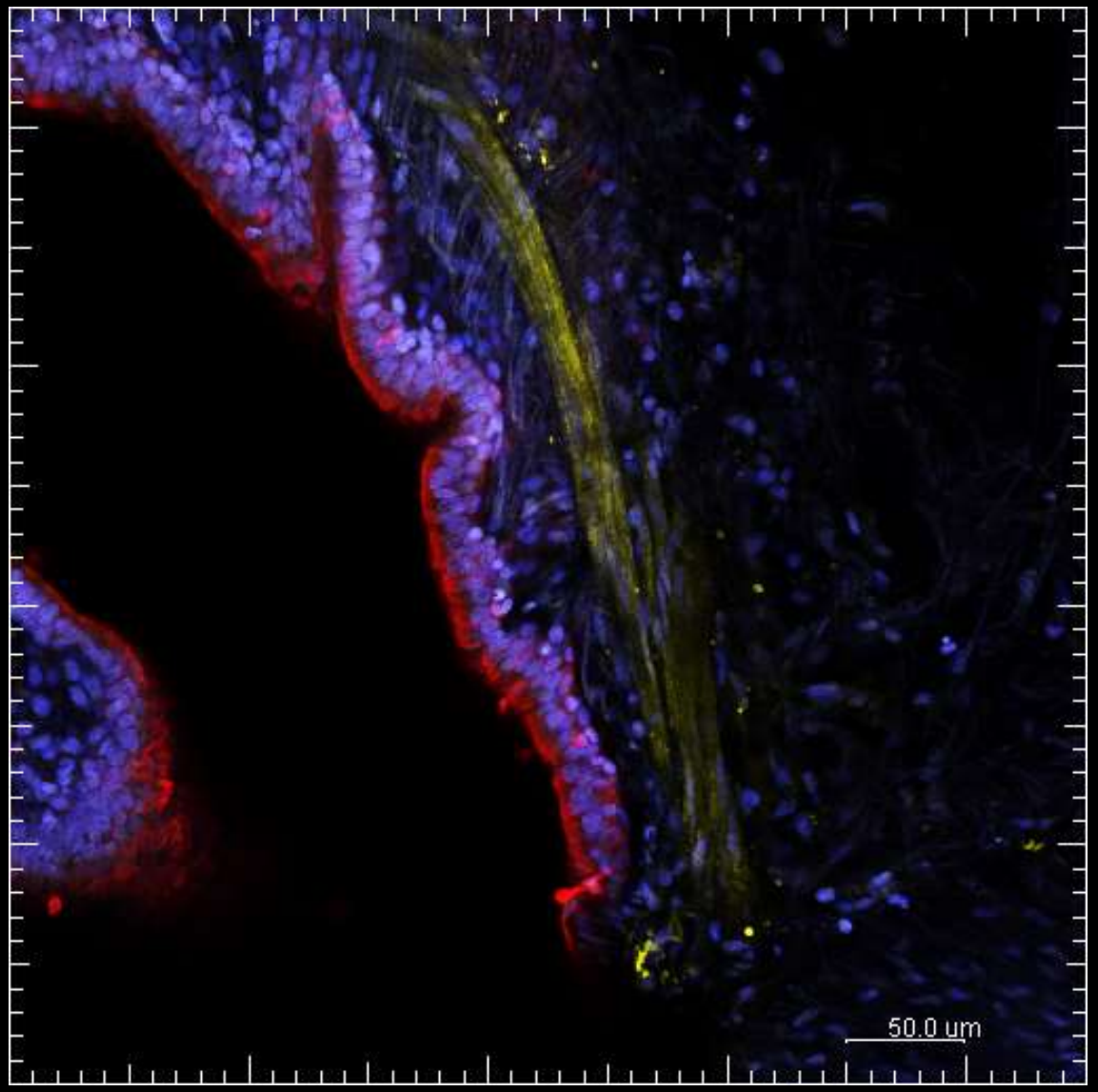


CD68

Mast cells in precision-cut lung slices

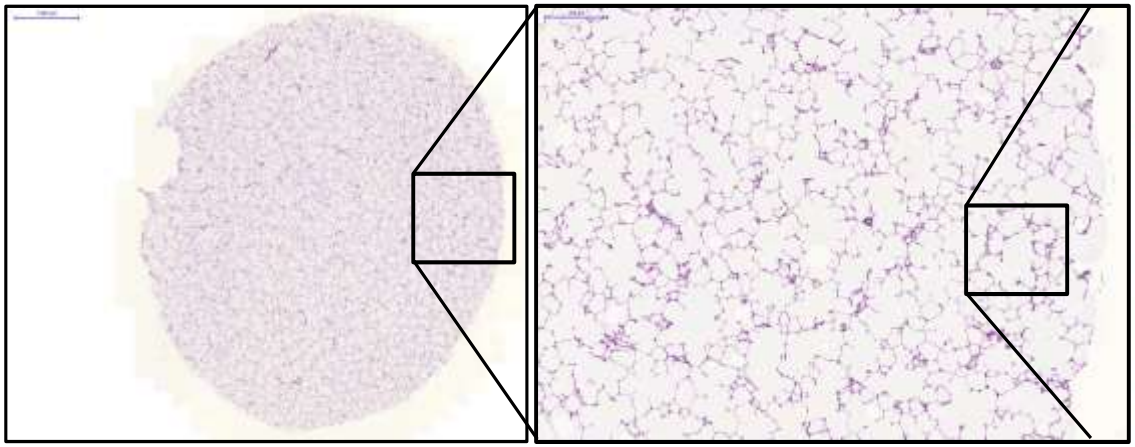


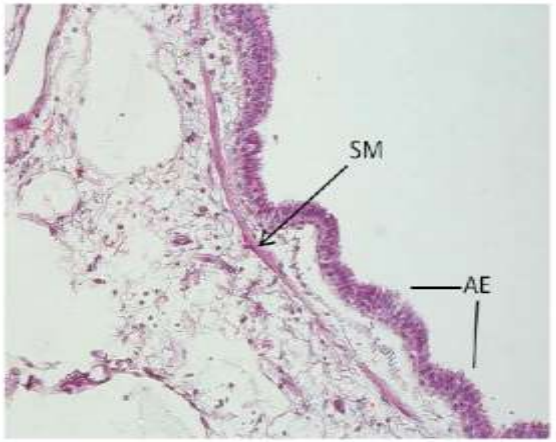
Airways in precision-cut lung slices



SMA
Keratin
TO-Pro-3

Microanatomical organization





H&E

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Reliable 3D model for all your alternative needs

- Precision-cut lung slices are:
  - Robust
  - Reliable
  - Relevant
- A large range of applications:
  - Cytotoxicity
  - Cytokine release
  - Bronchoconstriction
  - Tumor invasion

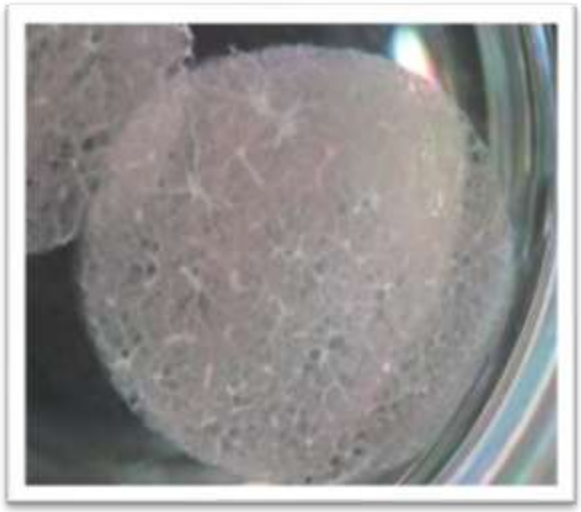

Foto: BASF

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Pre-clinical pharmacology and toxicology

Toxicity testing of chemicals, nanomaterial, pharmaceuticals

Precision-cut lung slices are offered for testing:

- From bench to in vivo:
  - Testing of substances before in-vivo inhalation studies
  - Prediction of safe doses in animals
- From cells to organs to living organisms:
  - Efficacy testing in the most complex tissue model before in vivo
- From mouse to human:
  - Translational testing of substances in mouse, rat, non-human primate, and human
  - Selection of appropriate species for further pre-clinical testing

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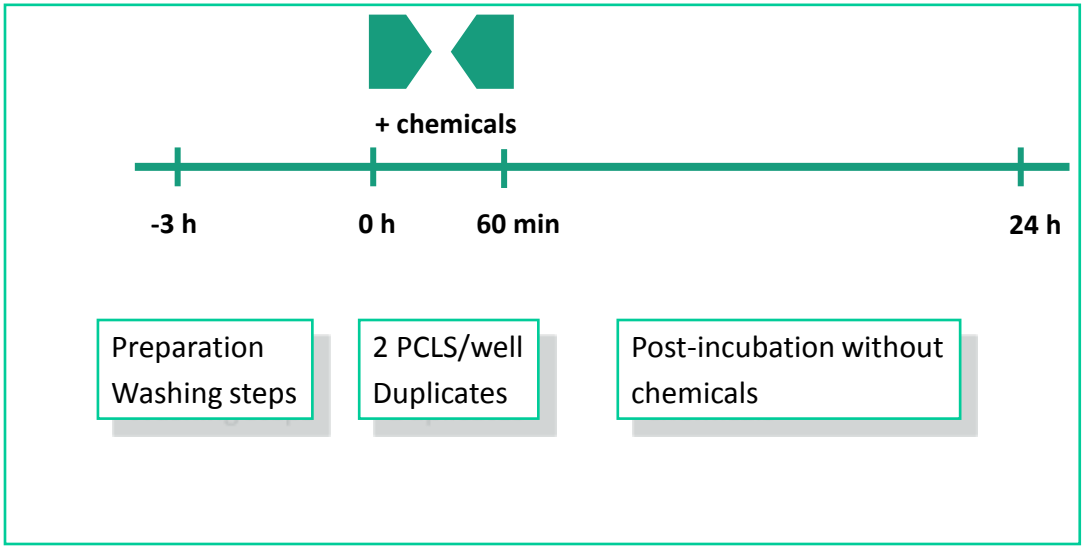
Acute exposure of precision cut lung slices – prevalidation for prediction of respiratory toxicity



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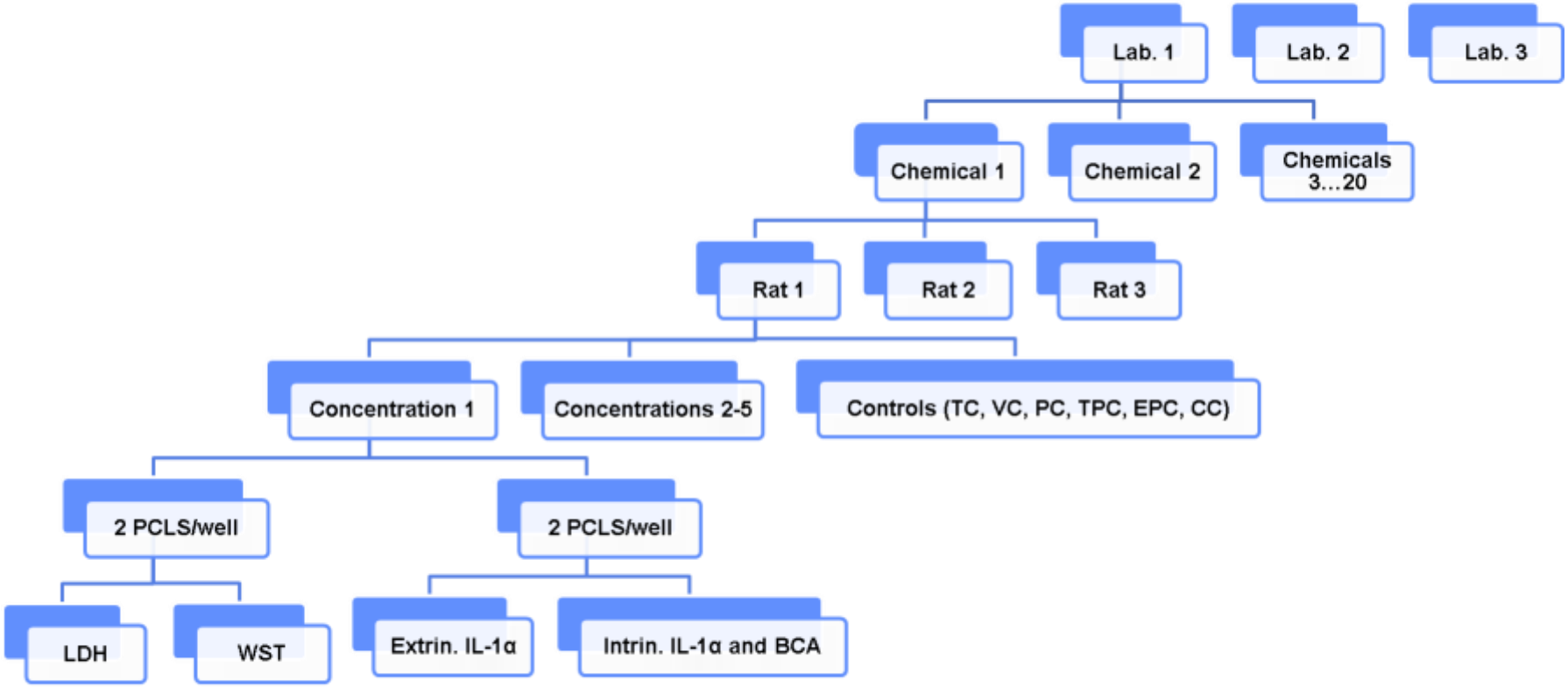
Study was performed in three independent labs



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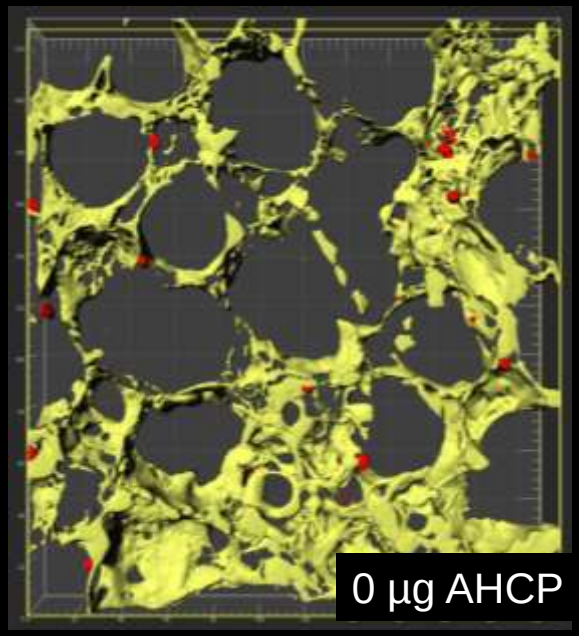
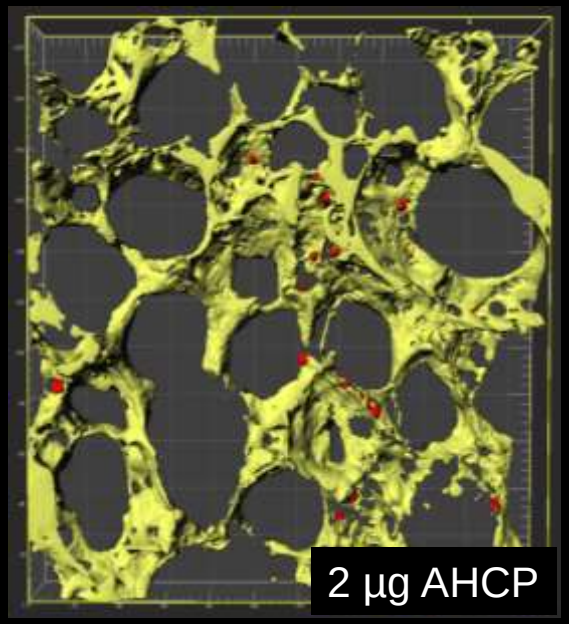
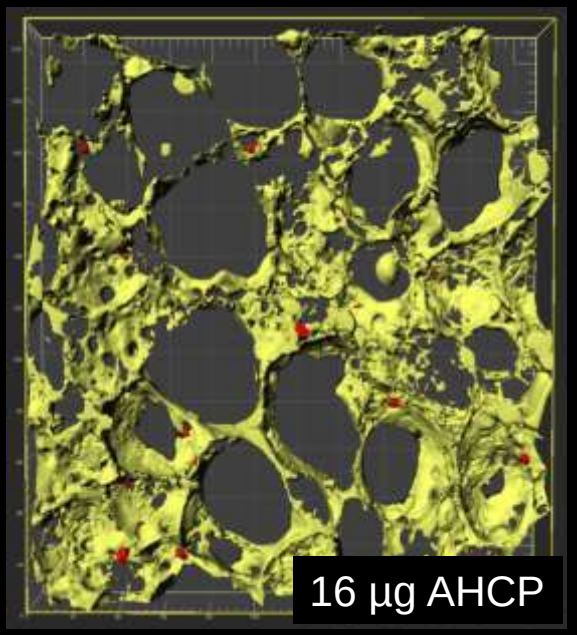
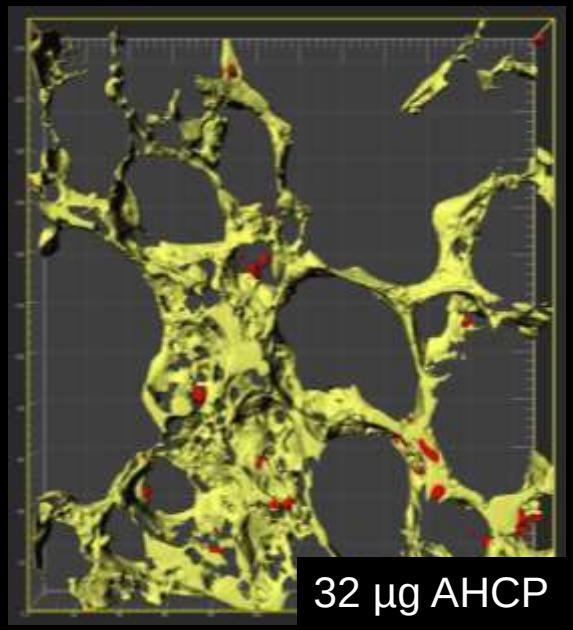
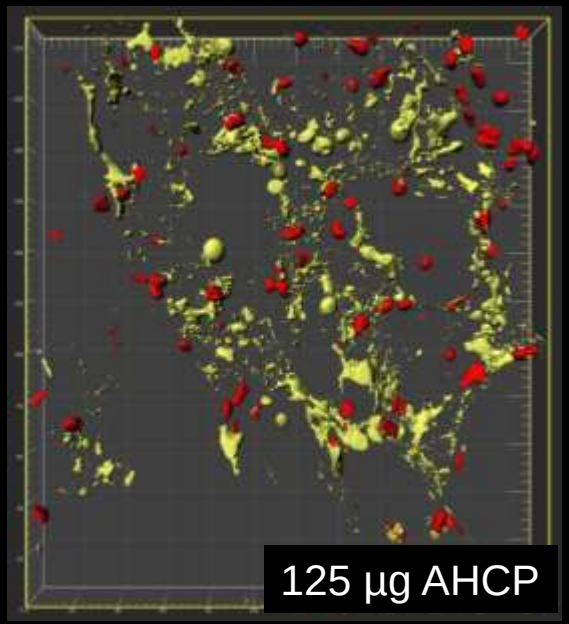
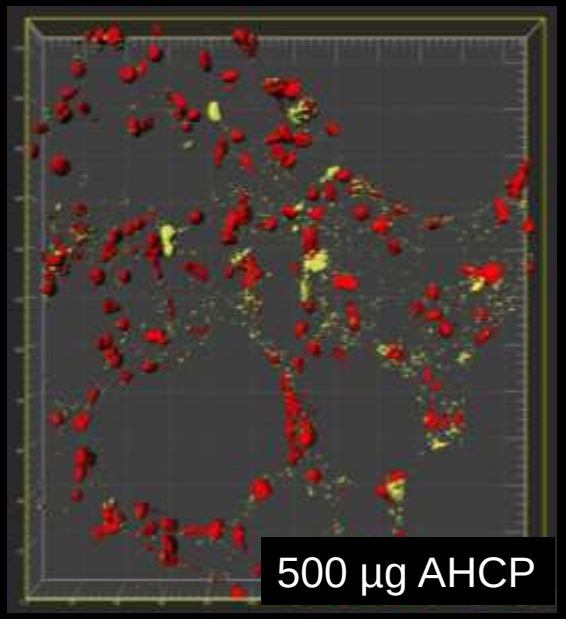

0 µg AHCP


2 µg AHCP


16 µg AHCP

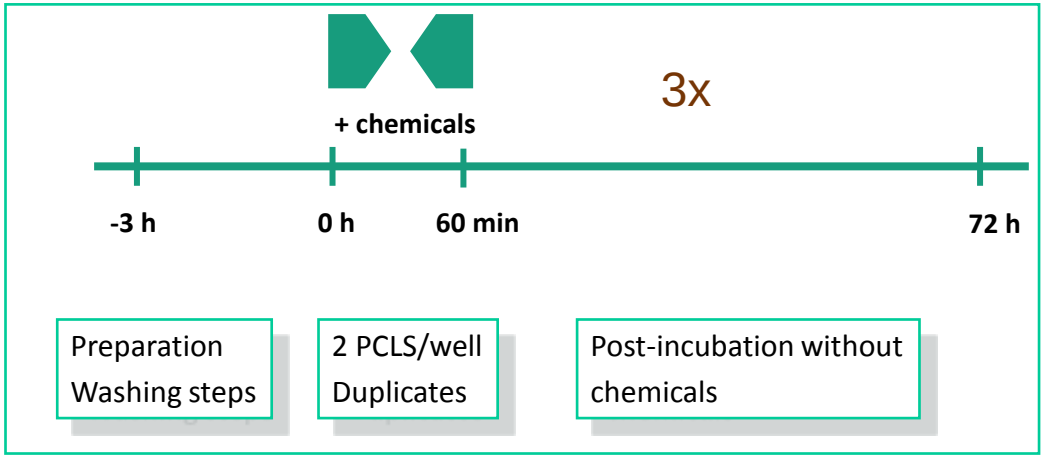

32 µg AHCP


125 µg AHCP


500 µg AHCP

Repeated exposure to chemicals

Precision-cut lung slices are exposed to selected chemicals for three days



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Summary

- PCLS is available at Fraunhofer ITEM
- Fraunhofer ITEM standardized and pre-validated PCLS with partners
- PCLS can be used to assess respiratory toxicity of
  - Soluble compounds (e.g. chemicals, chemical mixtures, pharmaceuticals, biopharmaceuticals)
    - Advantage: DRC of >1 chemical/biological donor
    - Limitation: acute responses; nanoparticles; highly reactive compounds
  - Gaseous compounds (e.g. irritant gases, aerosols)
  - Acute vs. repeated exposure
- Translation of findings from laboratory animals to humans
- Other (disease-related) endpoints can also be offered (e.g. inflammation, bronchoconstriction, changes in histology)

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The isolated perfused rat lung (IPL) model – almost in vivo

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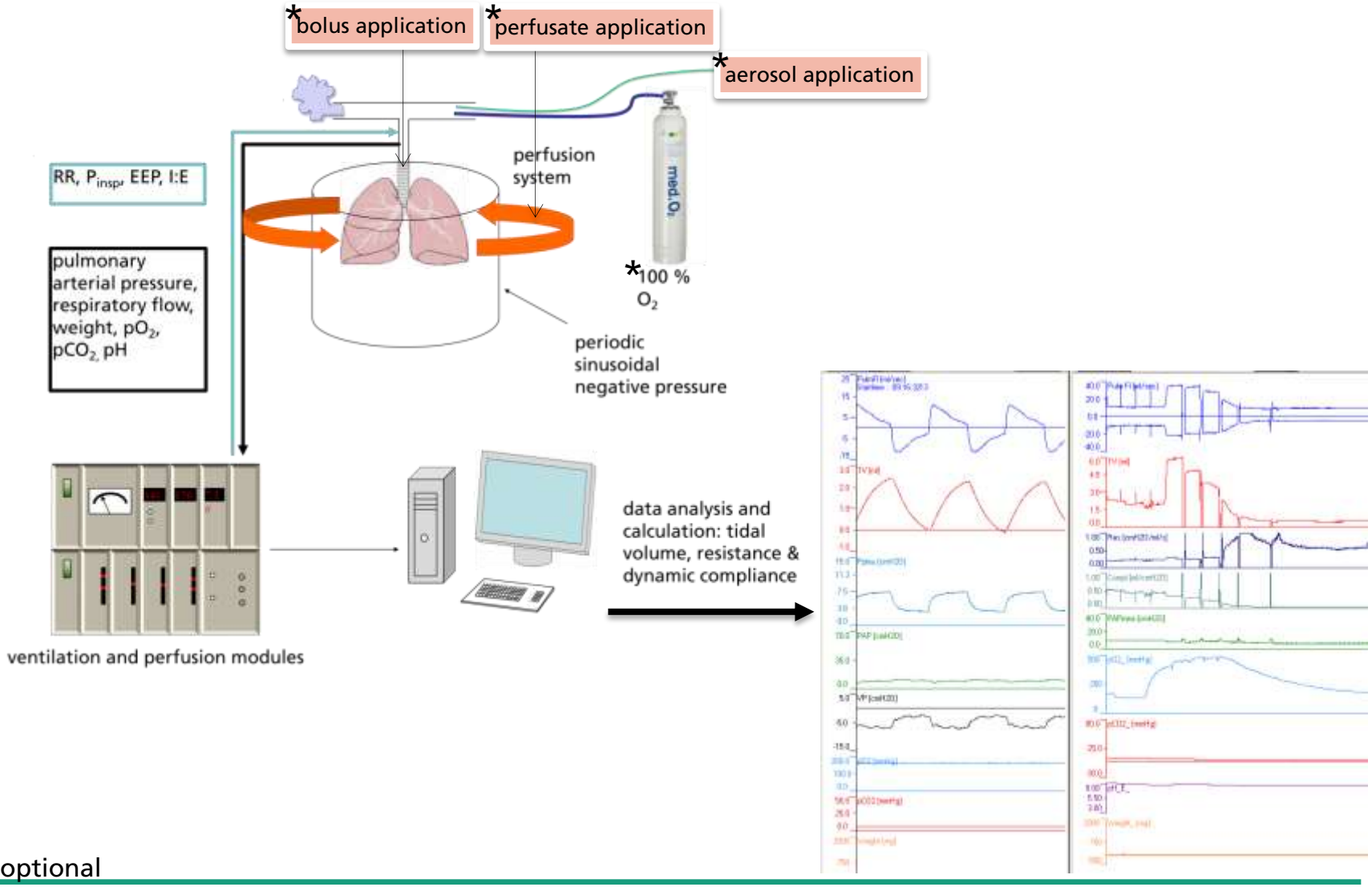
IPL system



*optional

Characterization IPL model

- **Rat** (170 – 550 g)
- **Perfusion:** Krebs-Henseleit buffer (4% albumin, pH 7.35), constant flow or PAP-controlled flow (10 - 20 ml/min), PAP < 15 cmH_2O
- **Ventilation**: Positive or negative pressure: inspiration -7-5 cmH_2O, end expiration -3.0 cmH_2O, deep inspiration every 5 min: -23 cmH_2O

- **Standard parameters:**

Breathing frequency:	80/min (insp. : exp.: 50 : 50)
Tidal volume:	1.2 – 3.0 ml
Resistance:	0.20 ± 0.02 cmH_2O/ml/sec
Compliance:	0.45 – 0.80 ml/cmH_2O
pO_2:	400 – 600 mmHg (100% oxygen)

Analysis

- **Lung**:
  - Respiratory parameters
  - Weight
  - Histology
  - Electron microscopy (deposition)

- **Perfusate/BAL**
  - Blood gases
  - Mediators
  - Substance kinetics, metabolites
  - Genetic analysis

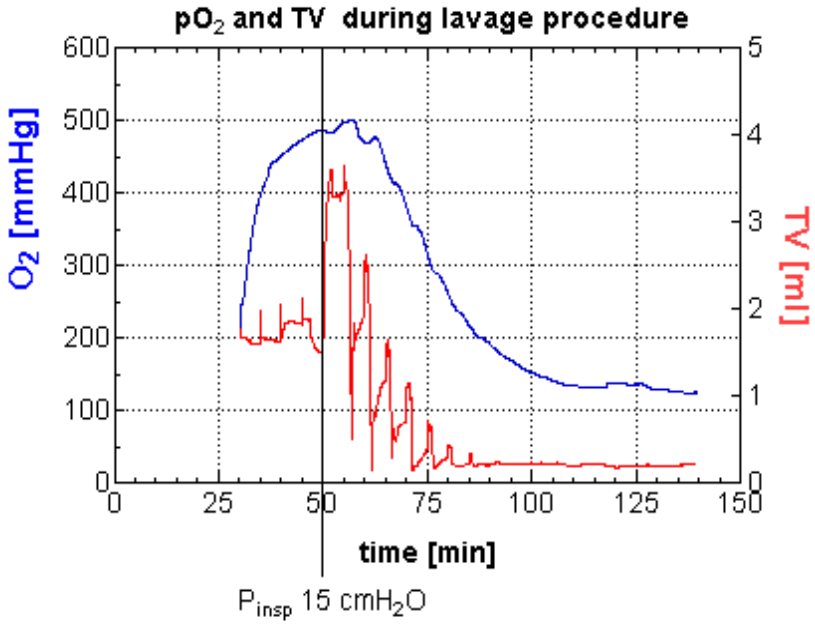
Application fields

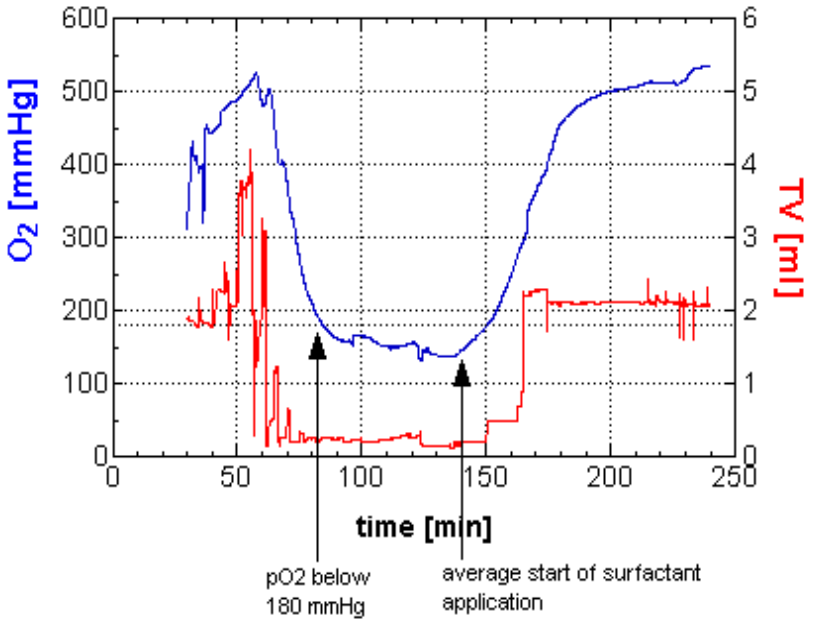
- **Lung injury**:
  - ARDS (injury model, medication)
  - Tumors (distribution and accumulation of chemotherapeutics)
- **Kinetics**:
  - Absorption, distribution, metabolism, excretion
- **New substance effects**:
  - Vasoactive, acute toxic, mediator release
- **Environmental pollutants**:
  - Absorption and distribution of diesel particles

ARDS imitation– lung-active medication

- Imitation of oxygenation status of moderate acute respiratory distress syndrome (ARDS) 100 mmHg < PaO_2 / FiO_2 ≤ 200 mmHg

→ Testing of artificial lung surfactant

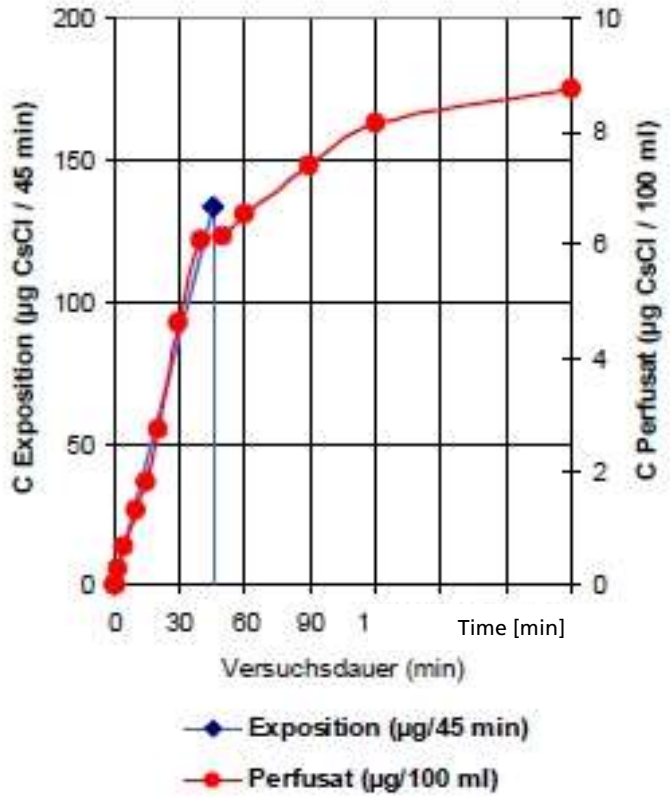




Kinetics

Model substance: caesium chloride

Transfer constant k = 0.0202/min

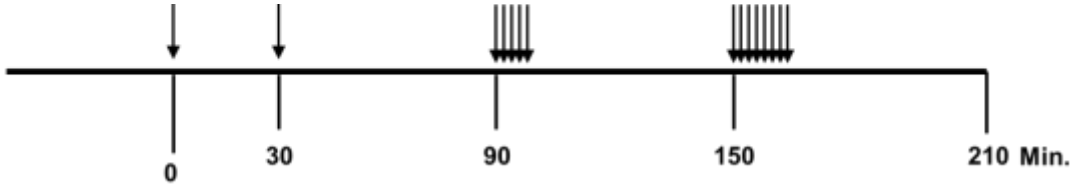


t	CsCl	
(min)	(µg/100ml)	(%)*
0	<0,03	
1	<0,03	
2	0,29	0,2
5	0,68	0,5
10	1,31	1,0
15	1,82	1,4
20	2,76	2,1
30	4,64	3,5
40	6,10	4,6
50	6,15	4,6
60	6,53	4,9
90	7,39	5,5
120	8,13	6,1
210	8,75	6,6

(%)* = Anteil CsCl gelöst / Gesamt

Application

- **Aerosol generation** (sonication, micro pump nebulizer etc.)
  - Gases, liquids, solid material
  - Native, fluorescence-labeled
- **Single/repeated or continuous**

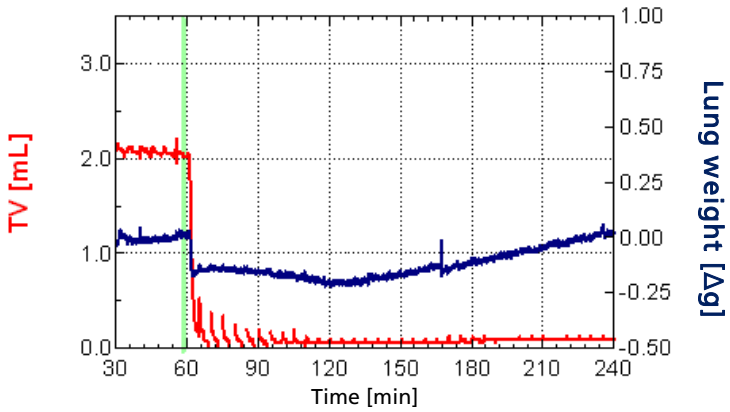


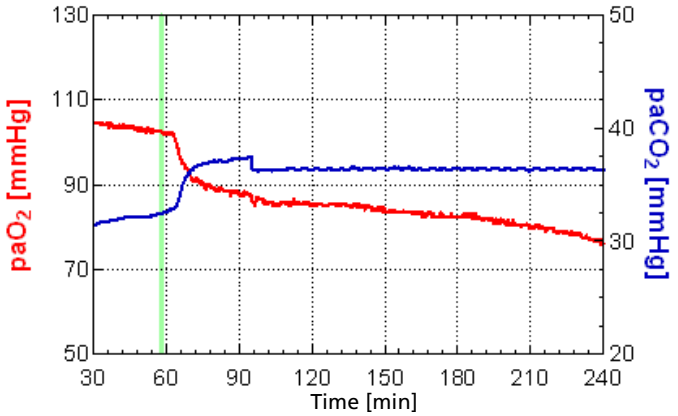
- **Routes**
  - Bolus
  - Perfusate
  - Aerosol (extrapolation of particle sizes)
  - Gases

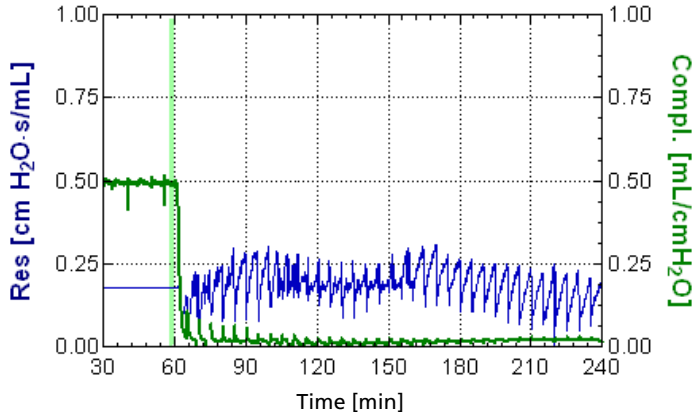
Impregnating agent

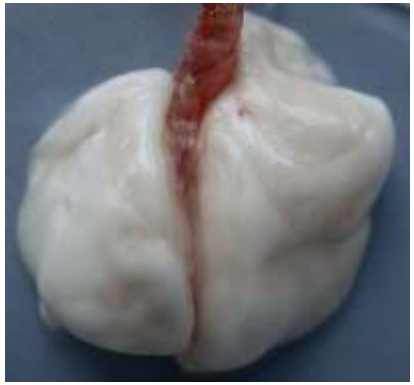
- Several case reports with severe lung edema formation
- Aerosol exposure: 0.1% agent solution, single application

  → Significant change in all respiratory parameters

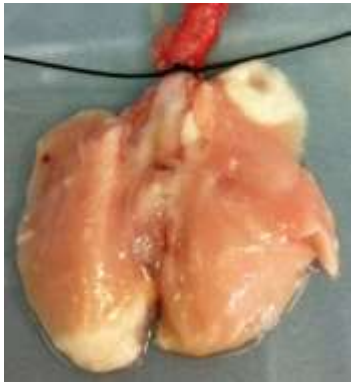








Control



0.1% Impregnating agent

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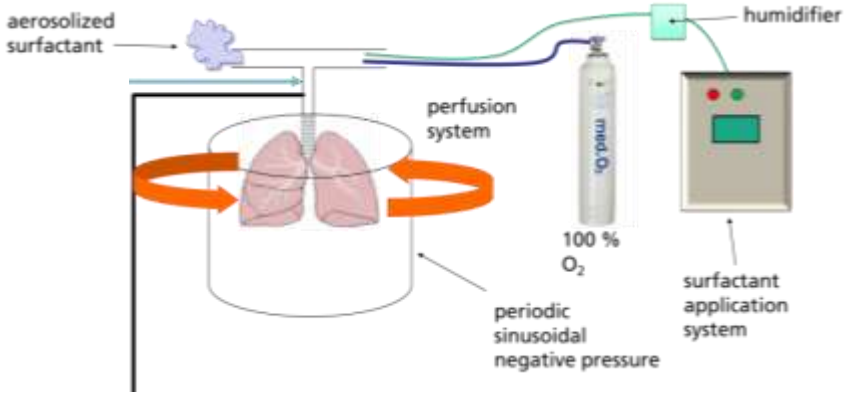
Impregnating agent

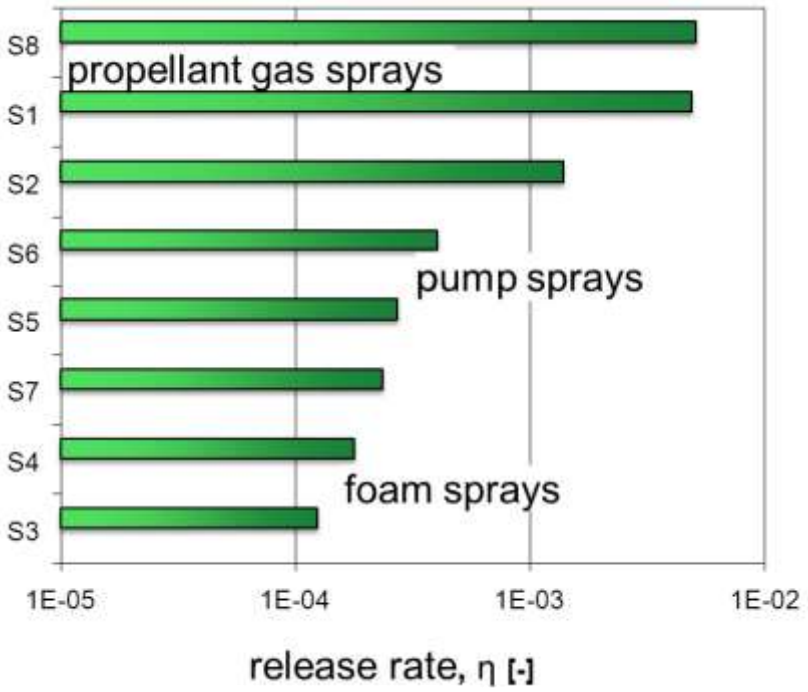
- **Reversibility of atelectasis** by artificial lung surfactant

- **Lung improvement**:
  - pO_2
  - Tidal volume
  - Compliance



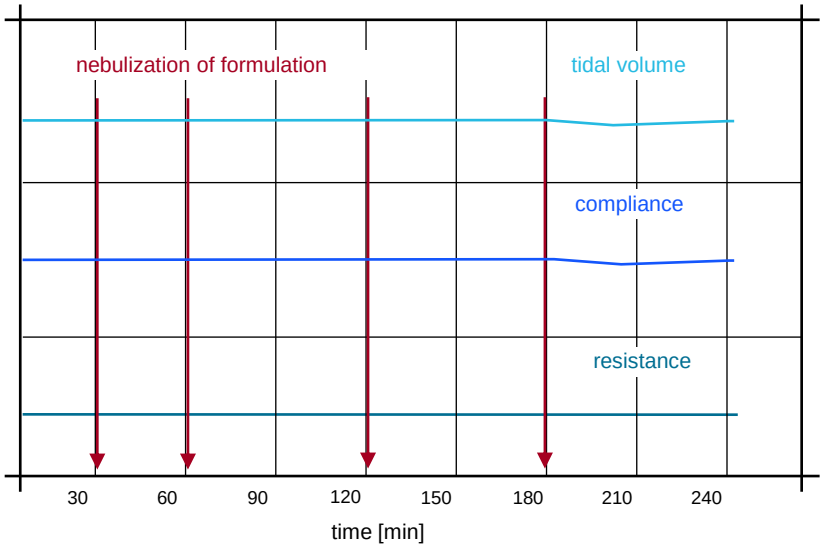
Acute toxicity testing ex vivo

- **Test scenario**:
  - Aerosolization of diluted spray formulation
  - Solvent: heptane
  - 0.1% active substance
  - MMAD 1.1 µm
  - Increasing dose

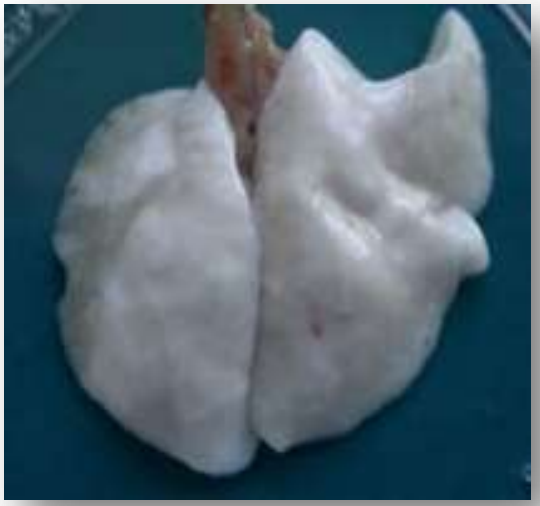


Formulation w/o acute toxic effects

during exposure



- **Repeated application**
- **Minimal changes in respiratory parameters**
- **No edema or atelectasis**



after exposure

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Formulation with acute toxic effects


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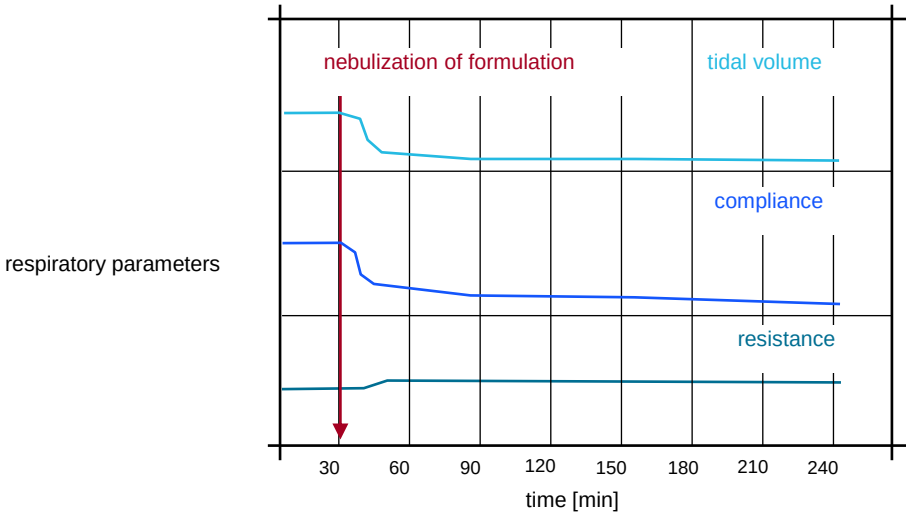
during exposure



- **Significant changes compared with control**
- **Distinct changes in respiratory parameters**
- **Partly collapsed areas to complete atelectasis**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after exposure

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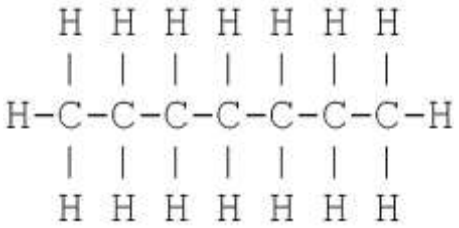
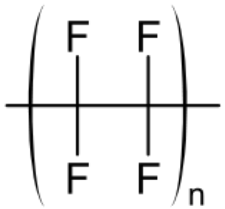
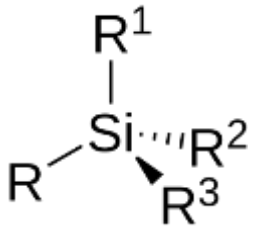
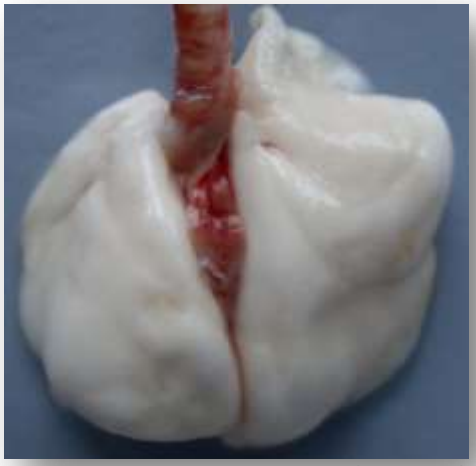
Macroscopic evaluation of acute lung toxicity









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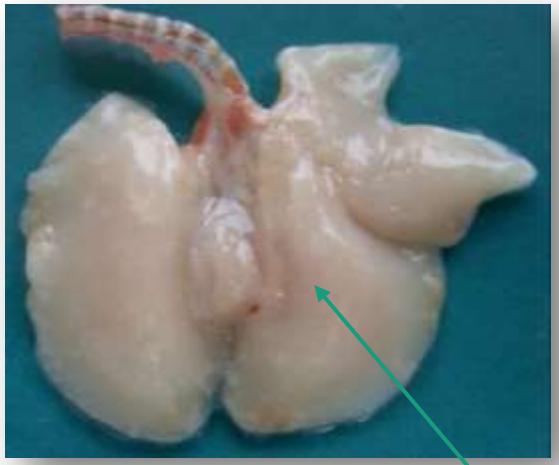
solvent



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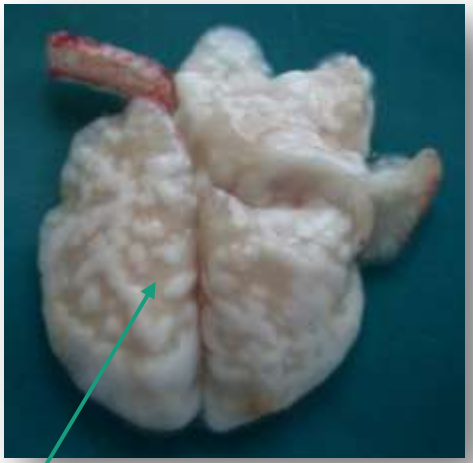
fluorine polymere



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silane

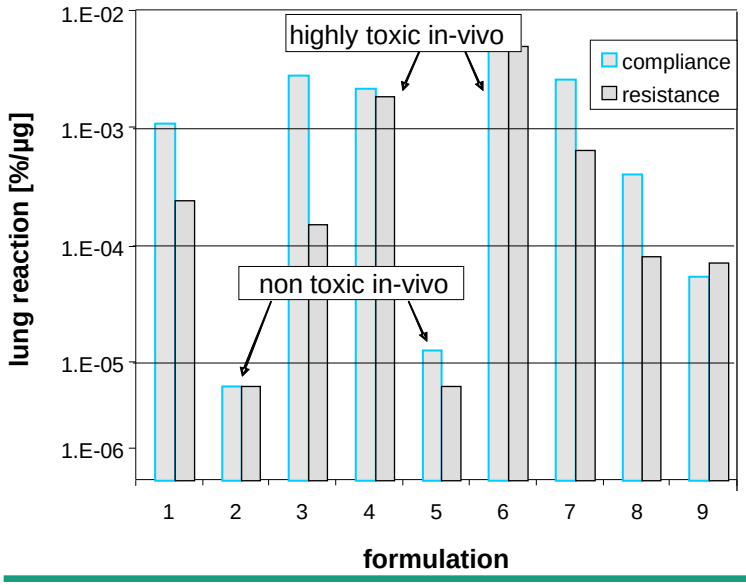
atelectasis

Correlation ex vivo vs. in vivo

- Standardization of effects to inhaled dose
- Comparison with in-vivo trials

→ Moderate to severe reactions in the IPL correlate with moderate to severe acute toxicity in vivo

→ NOAEL (µg/lung)



Substance #	IPL Test	Acute Inhalation Toxicity (OECD TG 403 - Limit-test)	Results fit together
		Target limit concentration [20 mg/L]	
	Atelectasis	Breathing pattern	
Control	-	-	+++
5	+	+	+++
3	++	++	+++
4	+++	+++	+++
6	+++	+++	+++
1	++	+++	+++
7	++	++	++
11	+++	+++	++
9	+++	++	++/+
8	+	+++	+
10	-	++	+
2	-	++	-
12	+++	+	-

Fischer M. et al., Altern Lab Anim. 2012 Sep;40(4):199-209.

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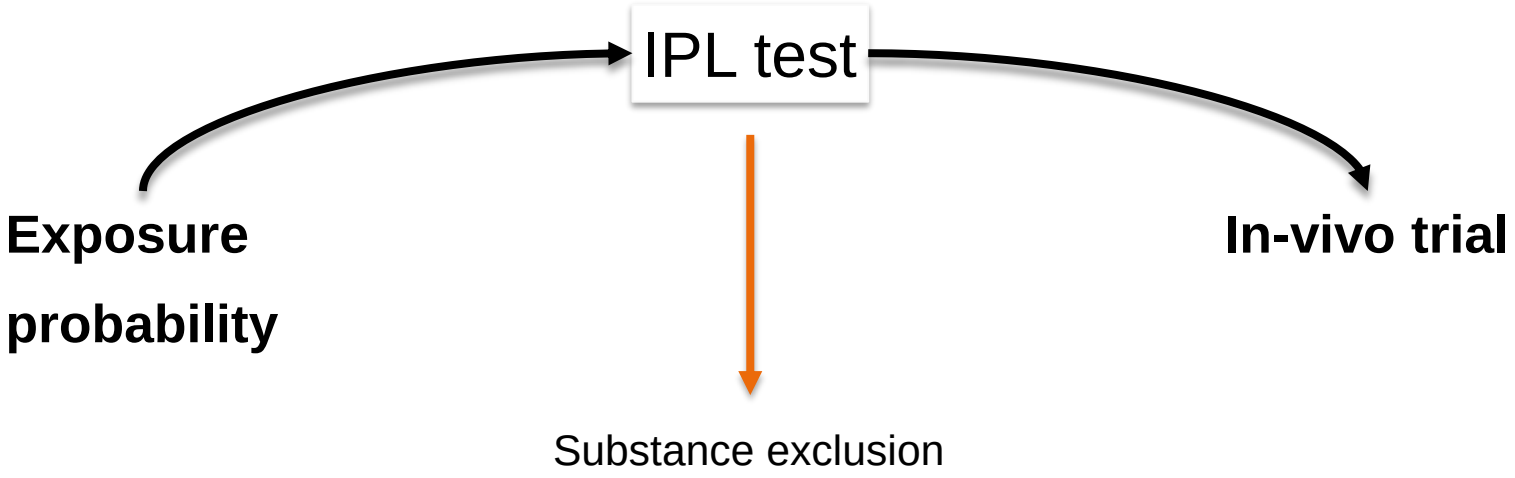
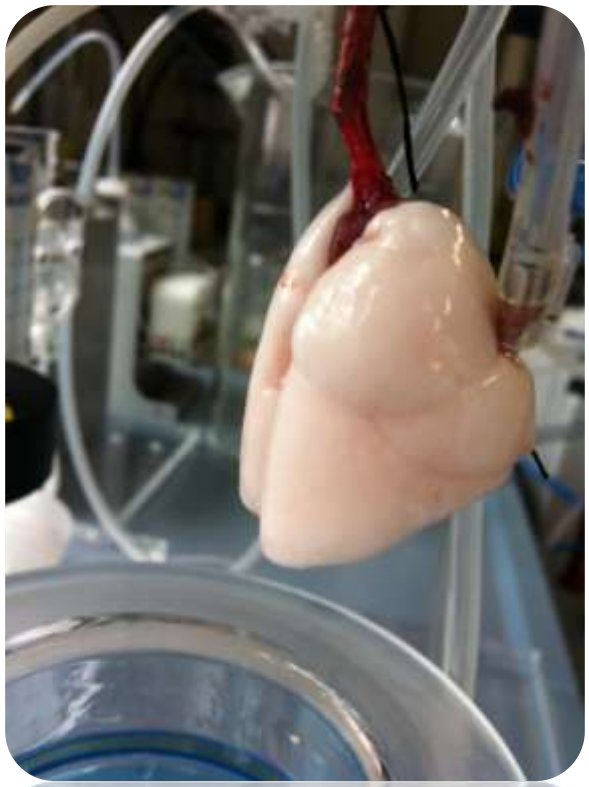
Bridging the gap



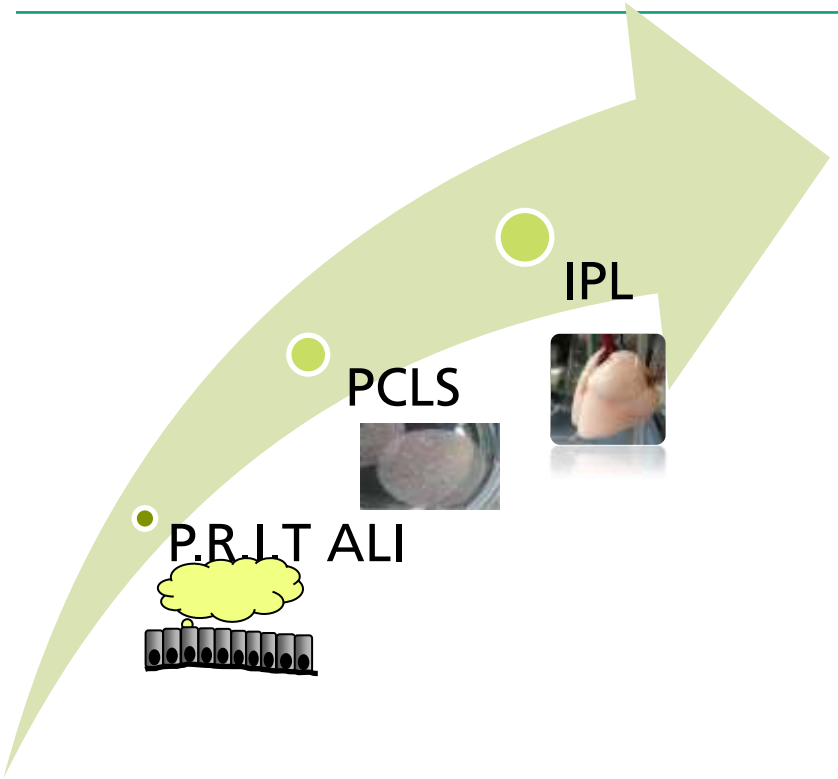
IPL benefits

- **More parameters than in vivo, const. data acquisition**
  - Tidal volume (TV)
  - Dynamic compliance
  - Resistance (bronchoconstriction)
  - pO_2, pCO_2, pH
- **Complete lung structure**
  - Pathologic changes (edema, atelectasis)
- **Kinetic analysis**
  - Systemic uptake
  - Mediators
  - Inflammatory markers
- **Identification of substances with acute toxic effects after inhalation**
  - Nebulization of solid and liquid compounds



Making sense out of data: a first step towards (q)IVIVE

Alternative methods in regulatory contexts



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Complexity of regulatory framework: examples from EU

Chemicals	
■ industrial chemicals (REACH)	EC Regulation 1907/2006
■ pesticides	Regulation (EC) No 1107/2009
■ biocides	Regulation (EU) No 528/2012
■ cosmetics	Regulation (EC) No 1223/2009
Pharmaceuticals	
■ veterinary drugs	EC Regulation 2377/90 (MRL)
■ human pharmaceuticals	
■ medical devices	
Feed and food additives etc.	EC Directives 70/524/EEC & 89/107/EEC etc.
Others	EC Directives 67/548/EEC & 99/45/EC (C&L)

Regulations show a high diversity for the requirements of animal data

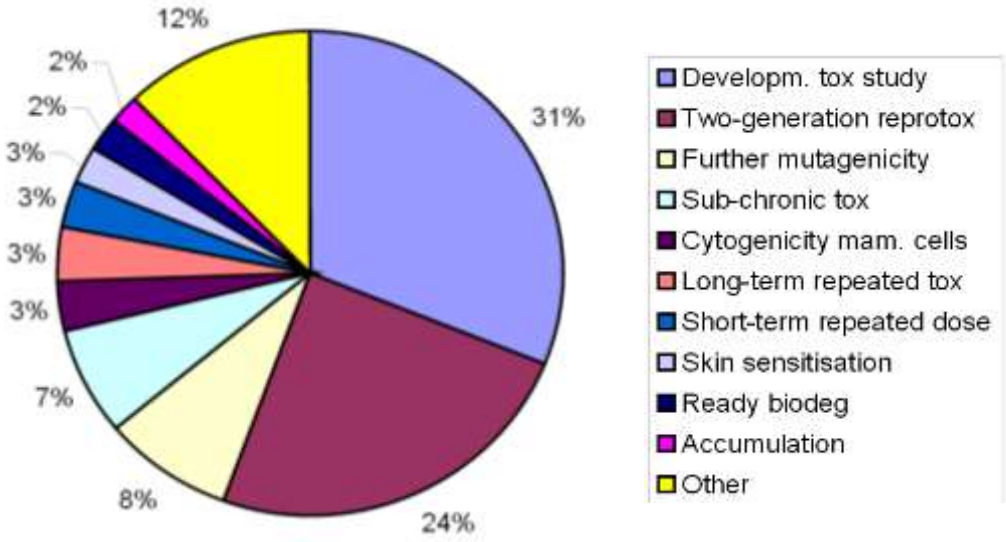
Most striking examples are REACH ⟷ Cosmetics regulation

REACH and animal testing

Animal toxicity studies to assess chemical safety:
a controversely discussed topic

Estimated animal needs

- 54 million vertebrate animals
  Hartung & Rovida (2009)*
- 2.6 million animals
  data estimated by ECHA



Data taken from: T. Hartung & C. Rovida (2009) *Chemical regulators have overreached.* *Nature* **460**, 1080-1081

Figure taken from: F. Pedersen, J. de Bruijn, S. Munn & K. van Leeuwen (2003) *Assessment of additional testing needs under REACH (*http://ihcp.jrc.cec.eu.int/)

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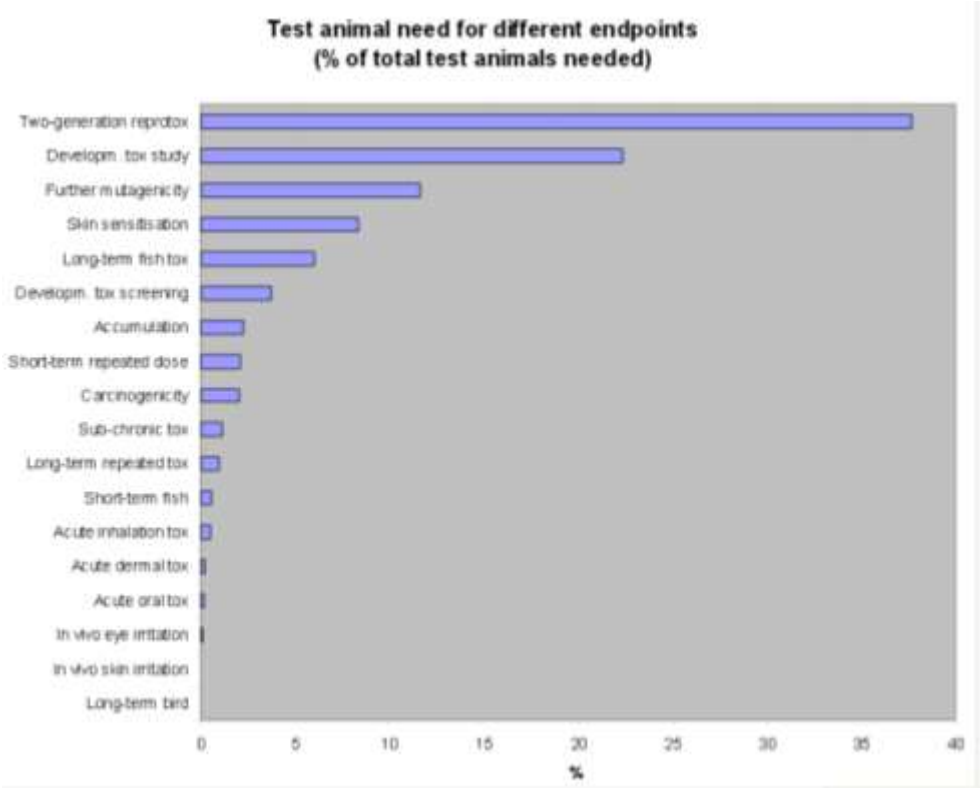

Data taken from: T. Hartung & C. Rovida (2009) *Chemical regulators have overreached.* *Nature* **460**, 1080-1081

Figure taken from: K. van der Jagt , S. Munn, J. Tørsløv & J. de Bruijn (2004) *Alternative approaches can reduce the use of test animals under REACH.* EUR 21405 EN

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Statements about the use of alternative testing methods

- **Biocides**

  "Although the new Regulation will not ban animal testing completely, it attempts to minimise …"

  "…testing may be waived …information may be provided using: … QSAR; in-vitro methods; or grouping or read across approaches…"

- **REACH**

  "….promotion of alternative methods to animal testing is among the objectives of the REACH Regulation. …"

  "Under REACH, animal testing is to be avoided in favour of alternative methods … tests involving the use of animals as a last resort…"

- **US HPV Challenge Program**

  "…EPA is committed to examining alternative test methods and whenever possible… replace animals in testing with validated in-vitro …test systems"

Efforts for alternative methods

- ICCVAM: US Interagency Coordinating Committee for the Validation of Alternative Methods
- ECVAM: European Centre for the Validation of Alternative Methods
- TSAR: a tracking system for in-vitro methods (http://tsar.jrc.ec.europa.eu/) includes a color guide for their status
  - **green** *already in the EU legislation or other regulatory use*
  - **orange** *undergoing process to be incorporated in the EU regulatory context*
  - **purple** *no regulatory use identified*
- QSAR: approaches at JRC and OECD to
  - -- give guidance for development and validation of QSARs
  - -- provide a list of existing models
  - -- develop a transparent reporting format for its use (QRMF)
- AOP: approaches at US EPA, JRC and OECD
- Further activities i.e. on read-across approaches

Short explanation of new approaches: AOP & (q)IVIVE

- AOP — adverse outcome pathway

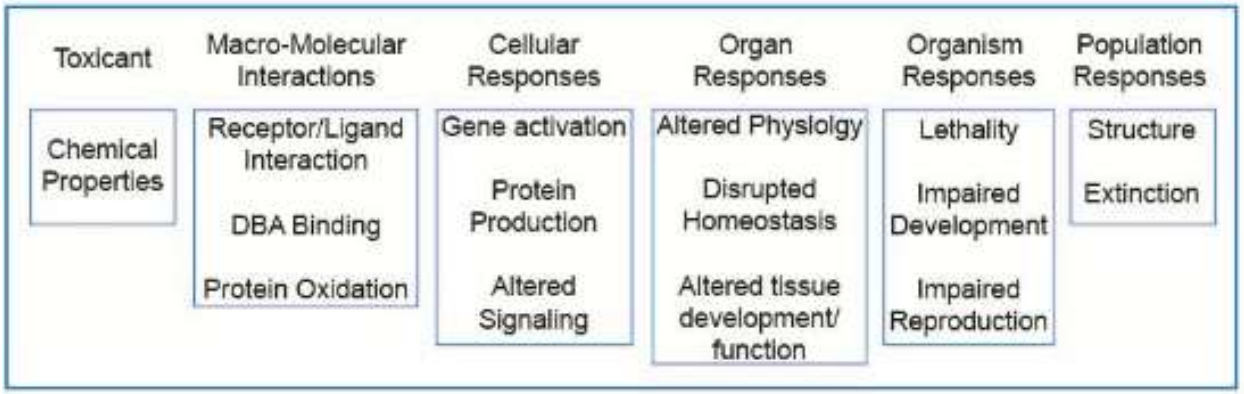
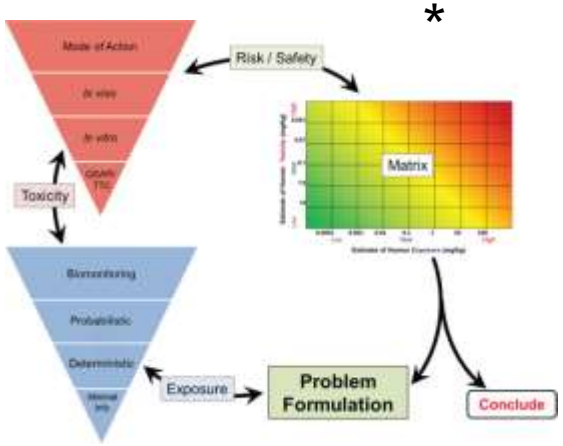


Figure taken from OECD (http://www.oecd.org/chemicalsafety/testing/adverse-outcome-pathways-molecular-screening-and-toxicogenomics.htm)

- (q)IVIVE — In-vitro – in-vivo extrapolation of quantitative data, i.e. predict in-vivo kinetics based on QSAR and in-vitro metabolism

Paradigm shift in toxicological science



*

- US: ToxCast™ & TOX 21
  ILSI: RISK21 Dose-Response Subteam
- EU: SEURAT-1 cluster and its followers in HORIZON 2020

All are focussing on a more appropriate prediction of human toxicity via alternative methods by:

- gain of mechanistic knowledge
- disclosure of adverse outcome pathways
- establishment of biomarkers at different levels
- Combination of computational and in-vitro methods

* Figure taken from T. P. Pastoor et al. (2014) Crit Rev Toxicol; 44(S3): 1–5

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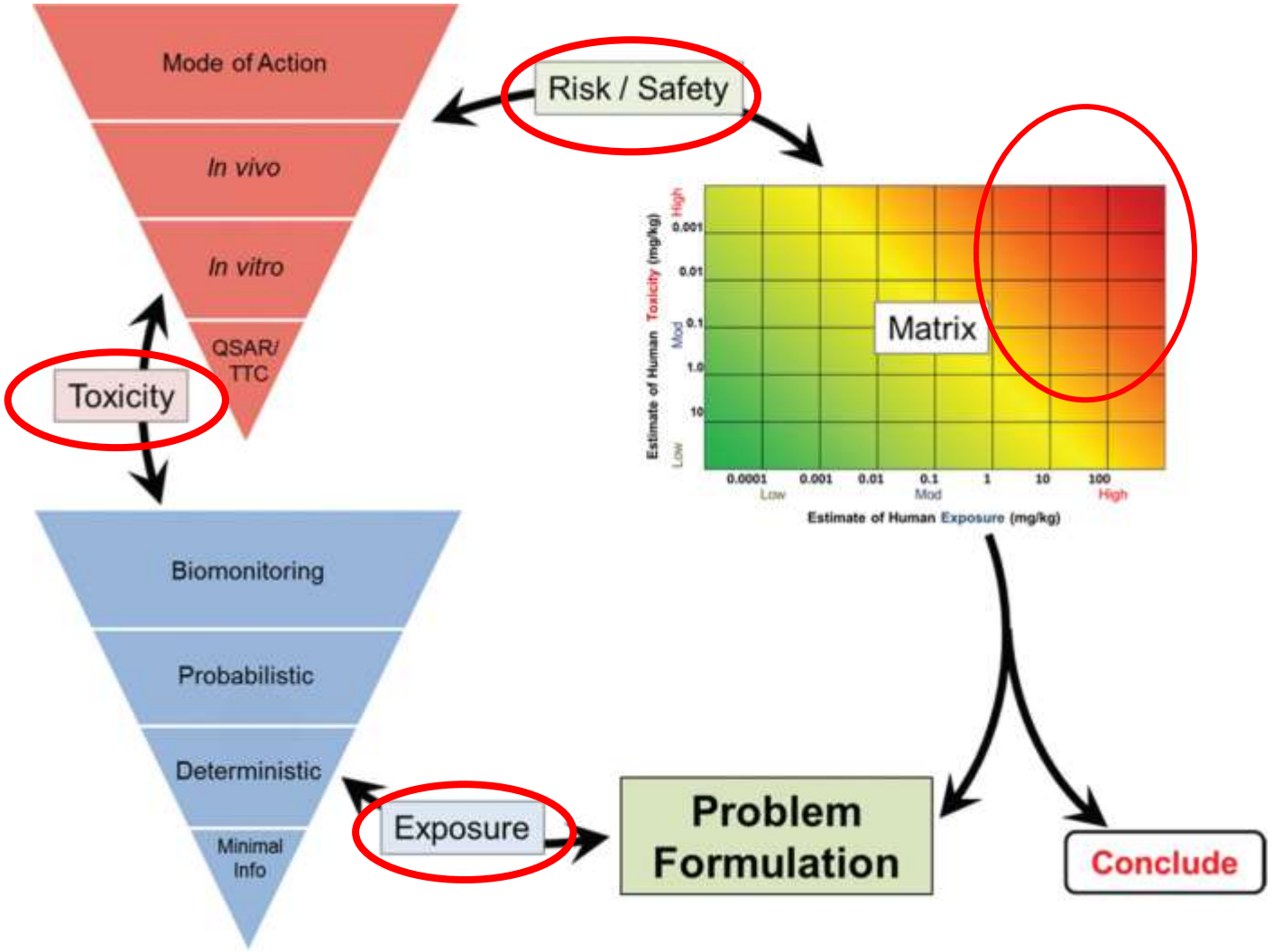
Paradigm shift in toxicological science



T. P. Pastoor et al. (2014)
A 21st century roadmap for human health risk assessment. Crit Rev Toxicol; 44(S3): 1–5

But:

---- a normal regulatory course within REACH----

- 394 testing proposals have already been evaluated
- A screening of (the first) 120 chemicals with evaluated testing proposals gives the following picture:

  201 tests in mammalians proposed

  9 – genotoxicty in vivo
  68 – repeated-dose toxicity
  82 – developmental toxicity
  42 – reprotoxicity mainly 2-G

- All in-vivo studies except the two-generation reprotoxicity studies were requested by ECHA – sometimes the study outline was changed
- Proposals such as QSAR, exposure-based waiving (TTC) and in-vitro tests submitted by third parties have been considered to be **not sufficient**

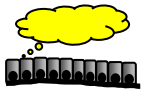
ECHA's reasons to reject alternative proposals

- QSARs:
  - A decision toxic/non-toxic is not sufficient
  - The applicability domain is not clear
  - The transparency and the reporting format are not sufficient
- In vitro:
  - Unclear toxicokinetics
  - No metabolizing activity
  - No dose-response given
  - In-vitro data cannot be translated to in vivo
    - → the problem of (q)IVIVE

How to fill these gaps ?

- There are methods that offer promising possibilities for bridging

Possibilities of alternative methods presented by ITEM



P.R.I.T ALI

- offers a "toolbox" for cell-based in-vitro testing of inhalable compounds
- allows exposure to gases/vapors & in refinement for aerosols
- special properties: online fluorescence and repeated application possible

PCLS

- is a test system at tissue level (organ structure largely maintained)
- allow testing of toxicity to the lung tissue under cell culture conditions
- possible read-outs include immunhistochemical and cytotox parameters

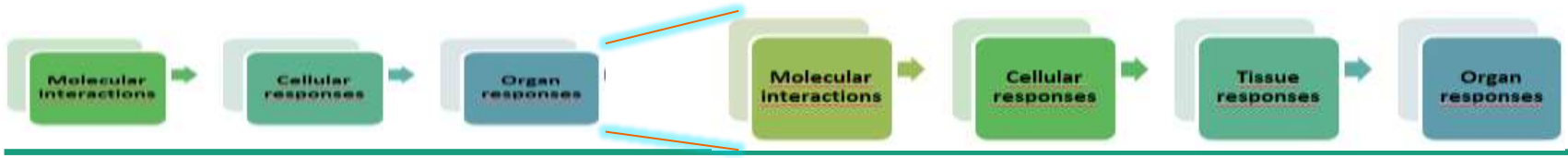
Possibilities of alternative methods presented by ITEM

IPL

- allows control of lung parameters/function in continous data acquisition
- allows observation of macroscopical and histopathological changes
- opens up the possibility to analyze kinetic parameters

A combination of these three test systems allows toxicological testing at different levels of differentiation (cell, tissue and organ level)

A verification of toxicological effects and dose responses is possible between the systems



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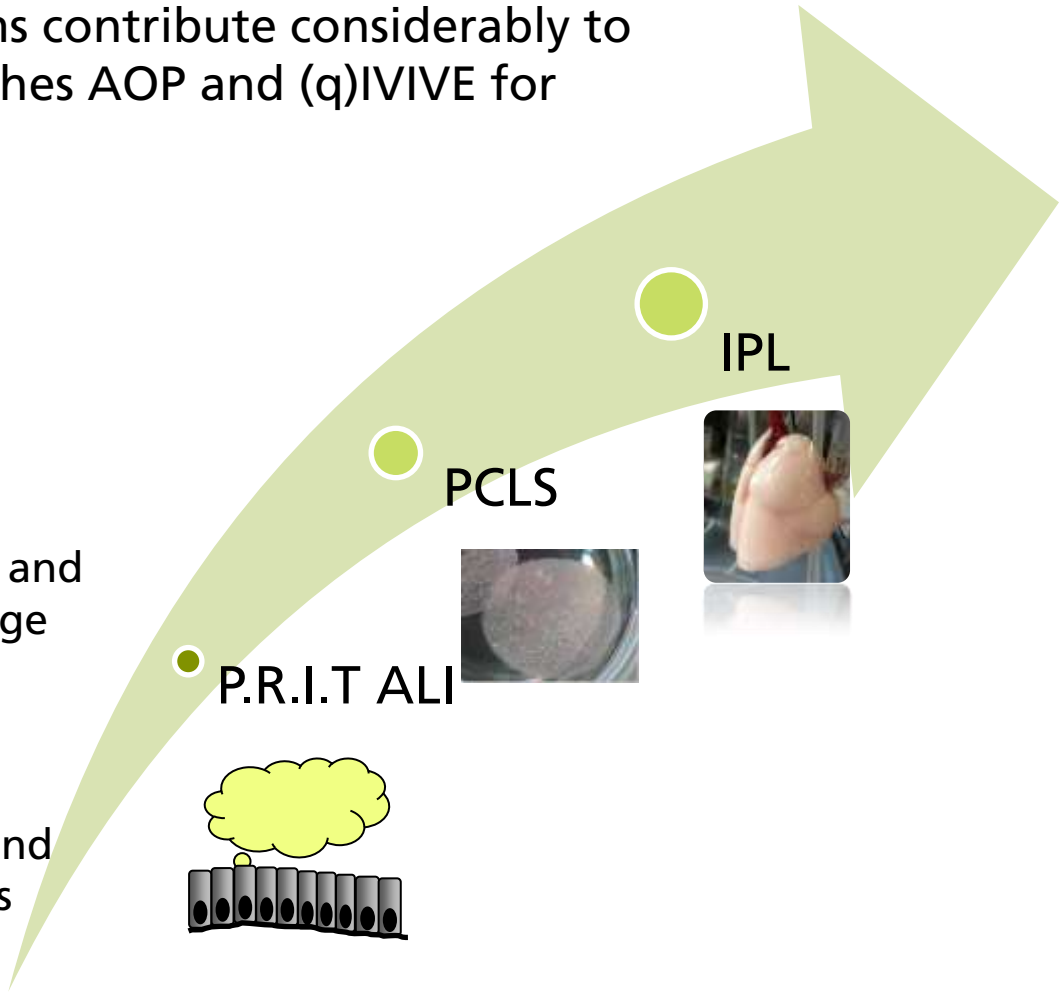
Making sense out of the data

The presented alternative systems contribute considerably to the “new” toxicological approaches AOP and (q)IVIVE for inhalation exposure

- They ensure that airborne substances reach the cell
- They cover three relevant differentiation stages for the detection of effects and markers and the gain of mechanistic knowledge (key events)
- They allow in parts (q)IVIVE by extrapolation of dose response and by comparison of relevant effects between the systems



Do not hesitate to contact us

We will be pleased to help you find answers to any questions you might have or solutions you are looking for.

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