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
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
Institute facts and figures 2013/2014

- Founded in 1981
- Employees 292
- Total budget > €24 million
- Industrial income €9.6 million
- Investments more than €1.7 million
Our focus, our aim

**Our focus:** Lung and airways

**Our aim:** Prevention and therapy of diseases
# Alternative methods in inhalation toxicology

1. **P.R.I.T. ExpoCube: an innovative in-vitro exposure system**  
   Detlef Ritter

2. **Precision-cut lung slices: a translational ex-vivo technique**  
   Katherina Sewald

3. **Isolated perfused lung model: almost in vivo**  
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4. **Making sense out of data: a first step towards (q)IVIVE**  
   Annette Bitsch
P.R.I.T. ExpoCube: an innovative in-vitro exposure system

Detlef Ritter
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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
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?

No “one-for-all” solution

Large ”Toolbox”
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
g- 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**
Stagnation point flow

Exposure efficiency

Particle deposition

Thermophoresis

“Smarter” work, read-out

“All-in-one-plate” concept
Device-based refined ALI exposure procedure

ExpoCube

primary test atmosphere feeding line A
flow 10 – 1000 ml/min

primary test atmosphere feeding line B
flow 10 – 1000 ml/min

ALI cultures on filter or microporous supports, ~1 cm² growth area, in standard multiwell plate

test atmosphere source A

test atmosphere source B

exposure group A
non-exposed control cultures
exposure group B

flow 10 – 1000 ml/min

control unit

flow 10 – 1000 ml/min

cell exposure flows

temperature control

cell exposures line A
flows 1 – 10 ml/min each

cell exposures line B
flows 1 – 10 ml/min each

“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

Clean Air

Ozone

Online readings
Kinetic studies

mitochondrial membrane potential (% of start value)

exposure time [h:min]

Fraunhofer ITEM
**Conclusion**

<table>
<thead>
<tr>
<th>Focus</th>
<th>Status and perspectives</th>
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<td>Biological test systems</td>
<td>• Large toolbox / no one-for-all solution</td>
</tr>
<tr>
<td></td>
<td>Tailored setups</td>
</tr>
<tr>
<td>Cell exposures</td>
<td>• Gases/vapors: Efficient and relevant methods</td>
</tr>
<tr>
<td></td>
<td>• Aerosols: Thermophoresis as a promising approach</td>
</tr>
<tr>
<td></td>
<td>High deposition rates / less side effects</td>
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<tr>
<td>Read-out</td>
<td>• Common in-vitro endpoints</td>
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<td></td>
<td>• Online fluorescence read-out</td>
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<td>High content readings, reporter gene assays, kinetic studies...</td>
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<tr>
<td>Whole process</td>
<td>• Multiwell plates throughout the experiment</td>
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<td></td>
<td>Smart, more robust, repeated dose etc.</td>
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Precision-cut lung slices – a translational ex-vivo technique

Katherina Sewald
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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 responses
- For some substances lungs are main route but not main target

- Impaired lung function
- Organ injury
- Hyperplasia
- Fibrosis
- Respiratory allergy

K. Sullivan et al., 2014 ATS
Pre-clinical pharmacology and toxicology

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

From bench to living organisms

- **in vitro**
  - Single-cell culture, co-culture

- **ex vivo**
  - Ex vivo

- **ex vivo**
  - Isolated perfused lung

**Precision-cut lung slices**
Pre-clinical pharmacology and toxicology

Precision-cut lung slices are obtained from lungs

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

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

Foto: BASF
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
Precision-cut lung slices are viable
Macrophages in precision-cut lung slices
Mast cells in precision-cut lung slices
Airways in precision-cut lung slices

- SMA
- Keratin
- TO-Pro-3
Microanatomical organization

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

- Preparation
- Washing steps
- 2 PCLS/well
- Duplicates
- Post-incubation without chemicals
Study was performed in three independent labs
Repeated exposure to chemicals

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

- Preparation
- Washing steps
- 2 PCLS/well
- Duplicates
- Post-incubation without chemicals
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)
The isolated perfused rat lung (IPL) model – almost in vivo

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IPL system

*bolus application

*perfusate application

*aerosol application

ventilation and perfusion modules

pulmonary arterial pressure, respiratory flow, weight, pO₂, pCO₂, pH

periodic sinusoidal negative pressure

100% O₂

RR, P_{insp}, EEP, I:E

data analysis and calculation: tidal volume, resistance & dynamic compliance

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 cmH₂O

- **Ventilation:** Positive or negative pressure: inspiration -7-5 cmH₂O, end expiration -3.0 cmH₂O, deep inspiration every 5 min: -23 cmH₂O

- **Standard parameters:**
  - Breathing frequency: 80/min (insp. : exp.: 50 : 50)
  - Tidal volume: 1.2 – 3.0 ml
  - Resistance: 0.20 ± 0.02 cmH₂O/ml/sec
  - Compliance: 0.45 – 0.80 ml/cmH₂O
  - pO₂: 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 < PaO₂ / FiO₂ ≤ 200 mmHg

→ Testing of artificial lung surfactant
Kinetics

Model substance: caesium chloride
Transfer constant $k = 0.0202/\text{min}$

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<th>t (min)</th>
<th>CsCl (µg/100ml)</th>
<th>(%)$^*$</th>
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<tr>
<td>0</td>
<td>&lt;0.03</td>
<td></td>
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<tr>
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<tr>
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<td>210</td>
<td>8.75</td>
<td>6.6</td>
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</table>

($%)^* =$ 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

![Graphs showing changes in respiratory parameters over time](image-url)
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

- Repeated application
- Minimal changes in respiratory parameters
- No edema or atelectasis
Formulation with acute toxic effects

- Significant changes compared with control
- Distinct changes in respiratory parameters
- Partly collapsed areas to complete atelectasis
Macroscopic evaluation of acute lung toxicity

solvent

fluorine polymere

atelectasis

silane
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)

Bridging the gap

Exposure probability → IPL test → Substance exclusion → In-vivo trial
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 and Cosmetics regulation.
REACH and animal testing

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

Estimated animal needs

- 54 million vertebrate animals
  Hartung & Rovida (2009)*

- 2.6 million animals
  data estimated by ECHA


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/)
REACH and animal testing

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

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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
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
  - **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

  ![Diagram of AOP pathway](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
Paradigm shift in toxicological science

T. P. Pastoor et al. (2014)
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 – genotoxicity 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

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**How to fill these gaps?**

- There are methods that offer promising possibilities for bridging

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Inhalation toxicity

- *Almost in vivo*
- *Ex vivo*
- *In vitro*
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 immunohistochemical and cytotox parameters
Possibilities of alternative methods presented by ITEM

IPL
- allows control of lung parameters/function in continuous data acquisition
- allows observation of macroscopic 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
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

IPL
PCLS
P.R.I.T ALI
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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