PMI RESEARCH & DEVELOPMENT

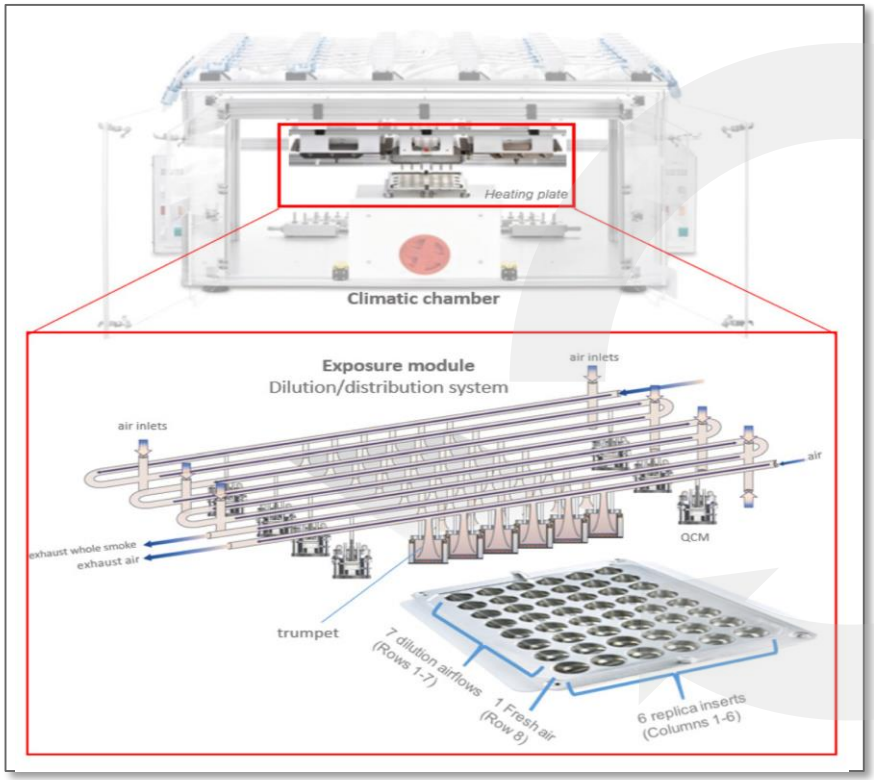
A new fluorescence based method for the characterization of *in vitro* aerosol exposure systems

Steiner, S., Majeed, S., Kleinhans, S., Nordlund, M., Frentzel, S., Mathis, C., Hoeng, J.

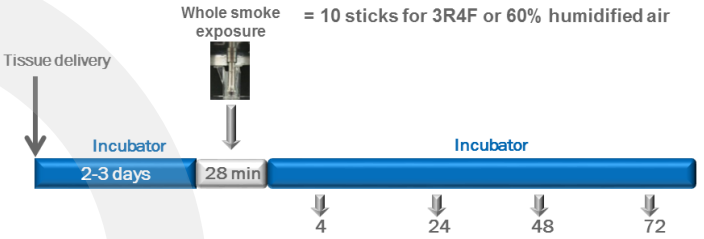
Philip Morris International R&D, Philip Morris Products S.A., Neuchâtel, Switzerland (part of Philip Morris International group of companies)

IIVS Exposure-Dosimetry workshop, April 2016

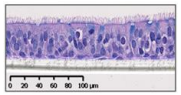
In vitro exposure studies utilizing the Vitrocell 24/48 system are a major component of our biological impact assessment of 3R4F reference cigarette smoke compared to aerosols from potential reduced risk products (RRP's)

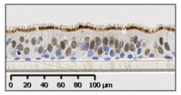


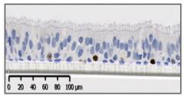
Experimental design

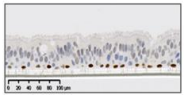


Endpoints	Post-exposure time (h)					
	Pre	0	4	24	48	72
Cytotoxicity (AK assay)			✓	✓	✓	✓
mRNA Microarray			✓	✓	✓	✓
Pro-inflammatory mediators				✓	✓	✓
Ciliary Beating Frequency	✓	✓		✓	✓	✓
Histology				✓	✓	✓

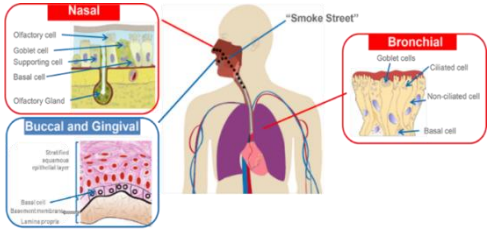

Hematoxylin Eosin/Alcian Blue


FoxJ1 (ciliated cells)

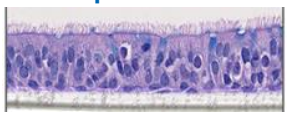

Ki-67 (proliferating cells)


p63 (basal cells)

Model systems



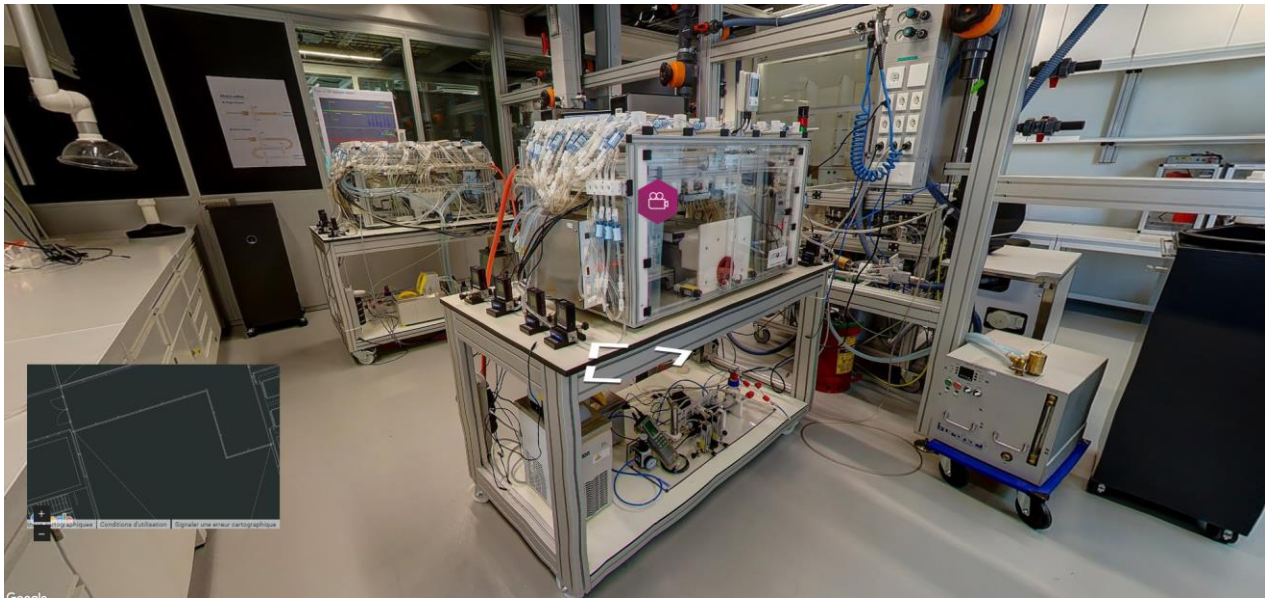
Air-liquid interface



3D-organotypics

Reduced-Risk Products ("RRPs") is the term the company uses to refer to products with the potential to reduce individual risk and population harm in comparison to smoking cigarettes. PMI's RRPs are in various stages of development and commercialization, and we are conducting extensive and rigorous scientific studies to determine whether we can support claims for such products of reduced exposure to harmful and potentially harmful constituents in smoke, and ultimately claims of reduced disease risk, when compared to smoking cigarettes. Before making any such claims, we will rigorously evaluate the full set of data from the relevant scientific studies to determine whether they substantiate reduced exposure or risk. Any such claims may also be subject to government review and authorization, as is the case in the United States today.

The *in vitro* smoke exposure laboratory at PMI – take a visit at the PMI Science website for a tour…



https://www.pmiscience.com/interactive-tour/

Recent communications:

Majeed *et al. Chemistry Central Journal* 2014, **8**:62
http://journal.chemistrycentral.com/content/8/1/62

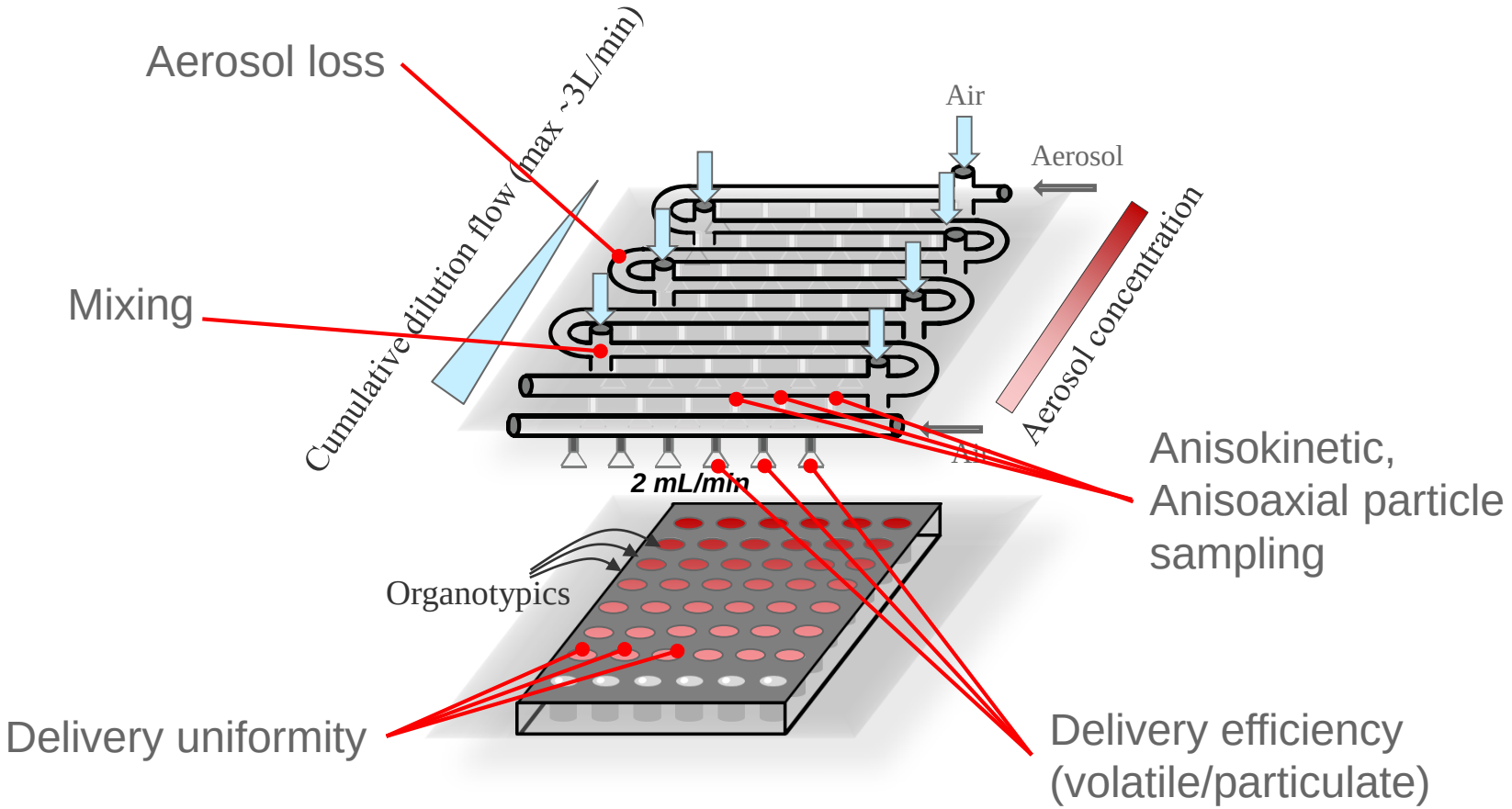
Chemistry Central Journal

RESEARCH ARTICLE Open Access

Characterization of the Vitrocell® 24/48 *in vitro* aerosol exposure system using mainstream cigarette smoke

Shoaib Majeed[1*†], Stefan Frentzel[1†], Sandra Wagner[2], Diana Kuehn[1], Patrice Leroy[1], Philippe A Guy[1], Arno Knorr[1], Julia Hoeng[1] and Manuel C Peitsch[1]

The Vitrocell 24/48 exposure system



Vitrocell 24/48 aerosol exposure system characterization: Rationale

Exposure system characterization
= detailed description of aerosol delivery:

- Dosing accuracy / precision

- Generated *vs.* delivered aerosol

    - Chemical composition
    - Particles / volatiles
    - Particle size distribution

- Uniformity (replica positions)

- Reproducibility

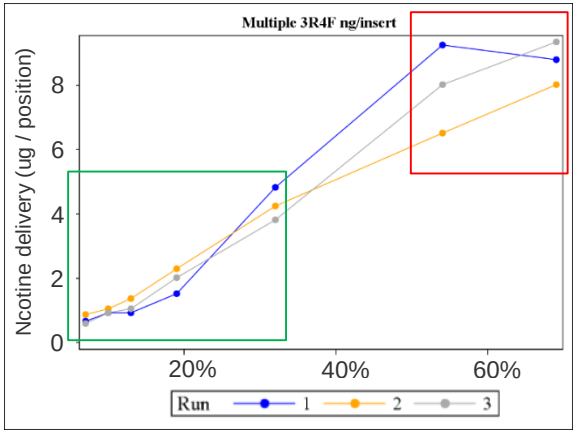
→ **Comprehensive interpretation of biological responses**

Vitrocell 24/48 aerosol exposure system characterization:
Exposure of PBS to 3R4F smoke

3R4F smoke, serial dilution (10 sticks, Health Canada)

cumulative dilution air-flow: 0.1, 0.2, 0.5, 1, 1.5, 2, 3 L/min
= 69, 54, 32, 19, 13, 10, 7% smoke

Nicotine quantification in WS exposed PBS (LC-MS)

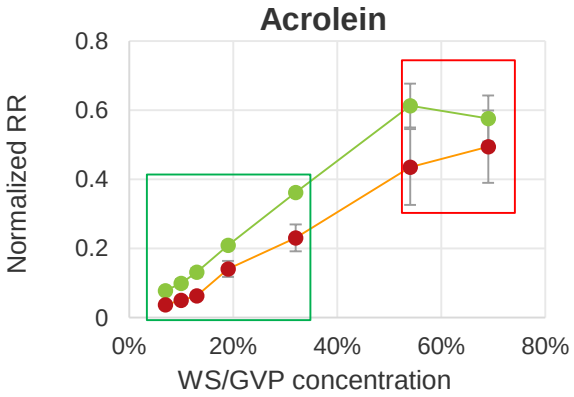


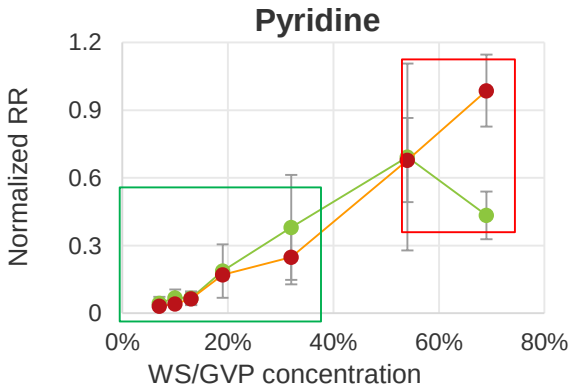
→ **Accurate relative dosing at 3R4F-relevant smoke concentrations**

But:

- **Inaccurate relative dosing at high concentrations**
- **Less pronounced for GVP**

Smoke constituents in whole smoke (WS) and **gas-vapor phase (GVP)** exposed PBS
(GC-TOF, > 50 identified volatile compounds)





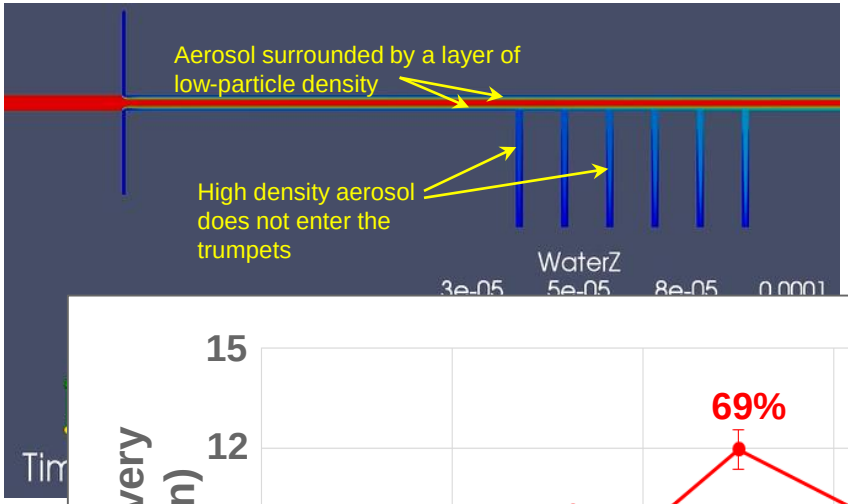
Averages ± SD,
n = 3

Vitrocell 24/48 aerosol exposure system characterization:
Exposure of PBS to 3R4F smoke

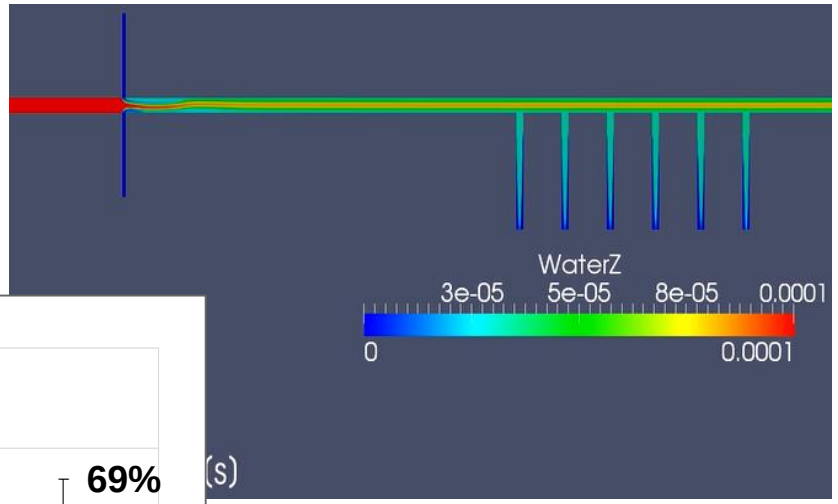
Prediction from computational fluid dynamics:

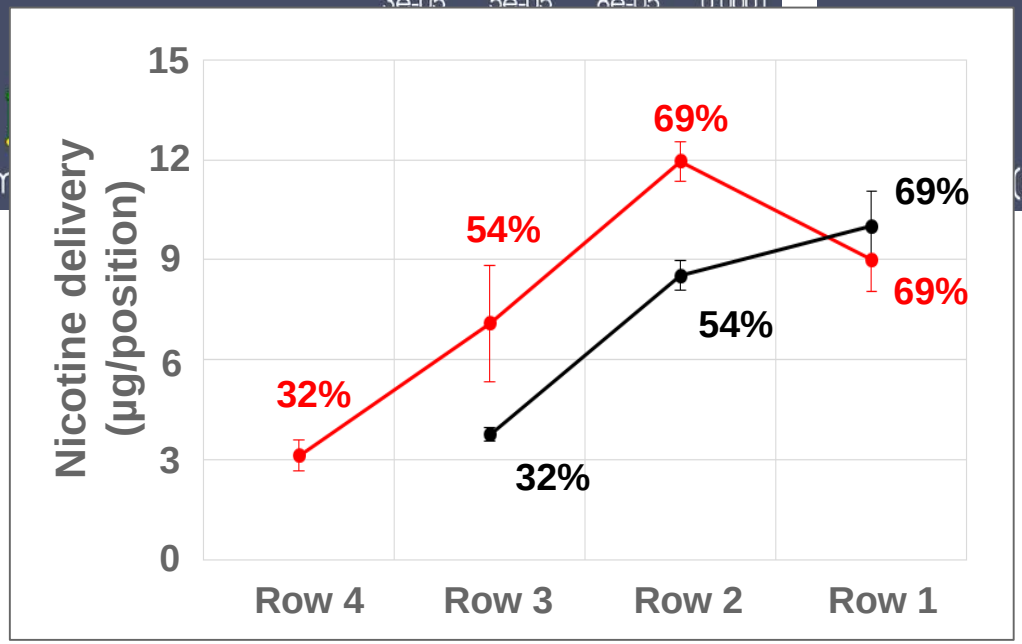
Inefficient aerosol sampling at trumpet inlets due to inefficient aerosol mixing at low dilution air flows

0.1 L/min dilution airflow (100 → 69%)



0.2 L/min dilution airflow (100 → 54%)





Increased travelling distance (= mixing time) improves aerosol sampling at trumpet inlets

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Expanding our efforts towards the Vitrocell 24/48 characterization:

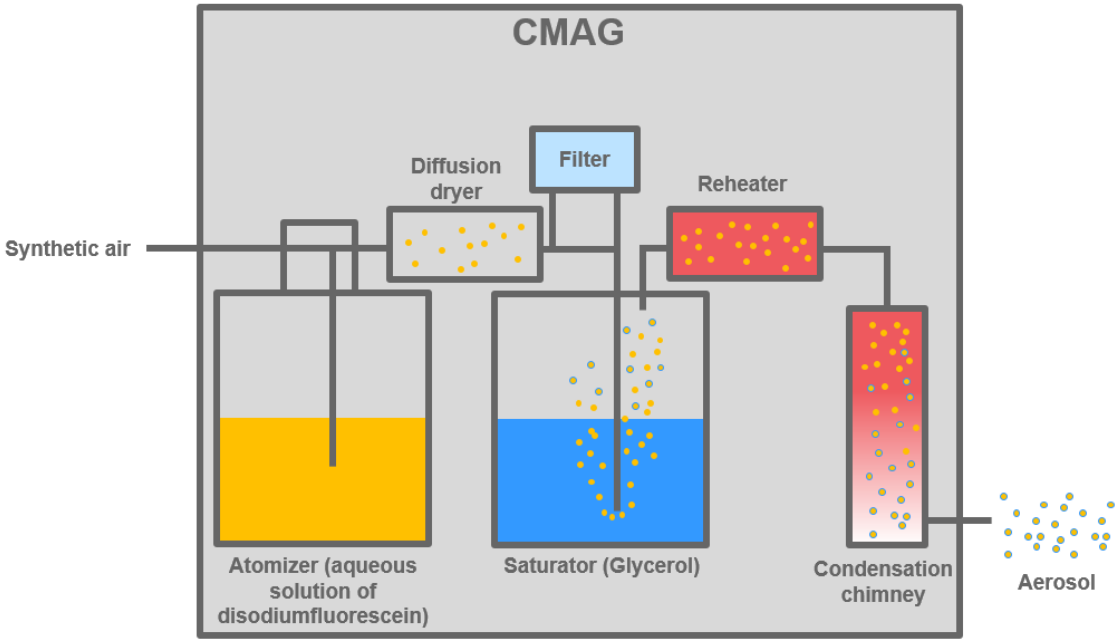
Mechanistic insight into aerosol dynamics

- Further investigation of mixing effects with focus on particulate fraction
- Particle size specific
- Focusing on liquid aerosols
- Quantitative retrieval of deposited material from any internal system part
- Direct, robust and fast method for quantification

→ **Development of labeled model aerosols**

Fluorescent liquid aerosols of tunable mean particle size:
Aerosol generation

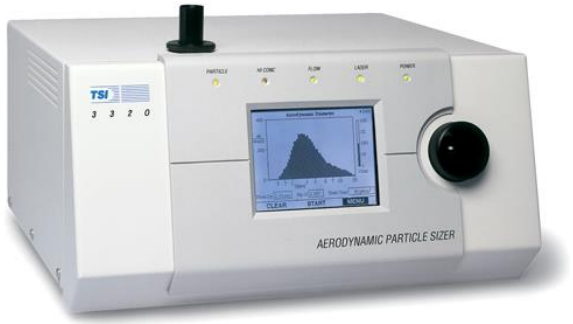
Use of a Condensation Monodisperse Aerosol Generator (CMAG)



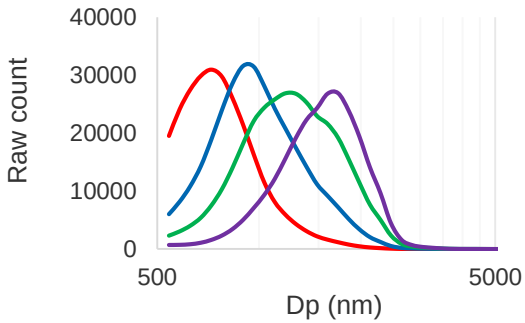
- Glycerol is condensed on disodium fluorescein nuclei
- Particle size depends on the number of nuclei provided and the amount of glycerol vapor used
- Continuous, stable generation of aerosols with geometric standard deviations of 1.3 – 1.4 and a wide range of mean particle sizes can be achieved

Fluorescent liquid aerosols of adjustable mean particle size:
Aerosol characterization

Particle number–size distribution



Aerodynamic particle sizer (APS)



- Mean size
- Mode
- GSD
- Total conc.

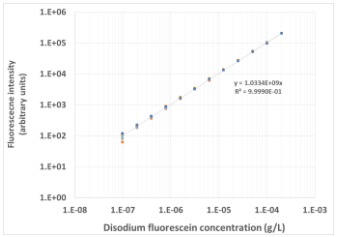
Aerosol mass flow rate and fluorescence

1) Aerosol trapping on quartz fiber filter

2) Aerosol mass flow rate determination

$$\frac{Aerosol\ mass}{time}$$

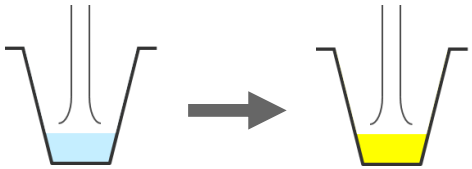
3) Elution and measurement of fluorescence



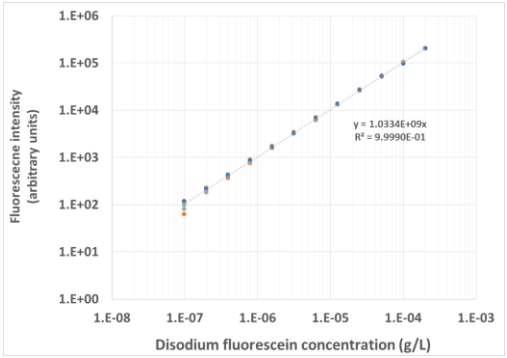
$$\frac{Disodium\ fluorescein\ mass}{time}$$

$$\frac{Disodium\ fluorescein\ mass}{Aerosol\ mass}$$

Fluorescent liquid aerosols of adjustable mean particle size:
Quantification of aerosol delivery



Cell culture insert containing 100µl PBS (pH9)



Robust linear range of Disodium fluorescein fluorescence in PBS, pH 9:

2×10^{-7} – 2×10^{-3} g/L

Primary readout:

Disodium fluorescein concentration in the exposed PBS
→ Direct measure for relative deposition
→ Relative dosing accuracy
→ Delivery uniformity

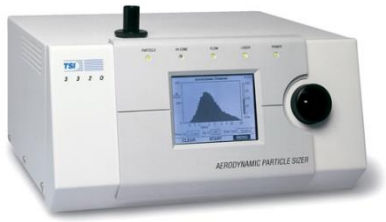
Inclusion of aerosol parameters:

$$Delivery\ efficiency = \frac{delivered\ disodium\ fluorescein}{disodium\ fluorescein\ mass\ flow\ rate\ x\ exposure\ time}$$

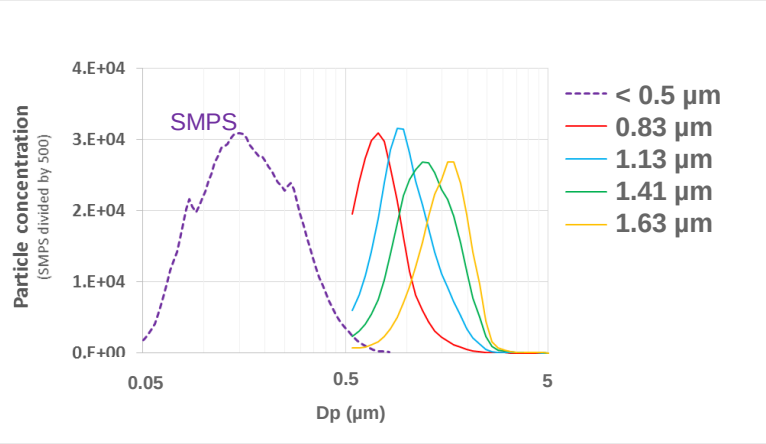
$$Absolute\ mass\ deposition = \frac{delivered\ disodium\ fluorescein\ mass}{\left(\frac{disodium\ fluorescein\ mass}{aerosol\ mass}\right)}$$

→ Reproducibility
→ Absolute dosing

Fluorescent liquid aerosols of adjustable mean particle size:
Reproducibility of aerosol generation



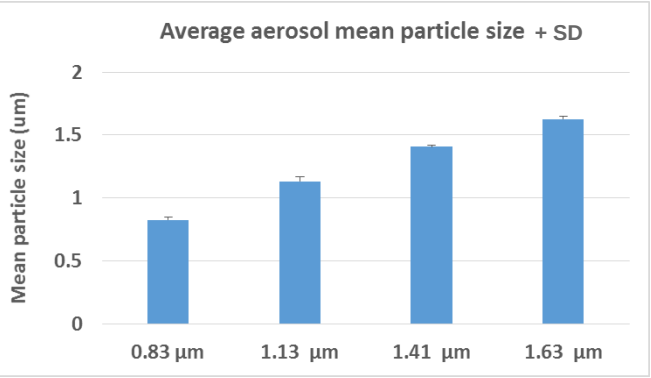
Five aerosols selected for method evaluation:
particle size distribution

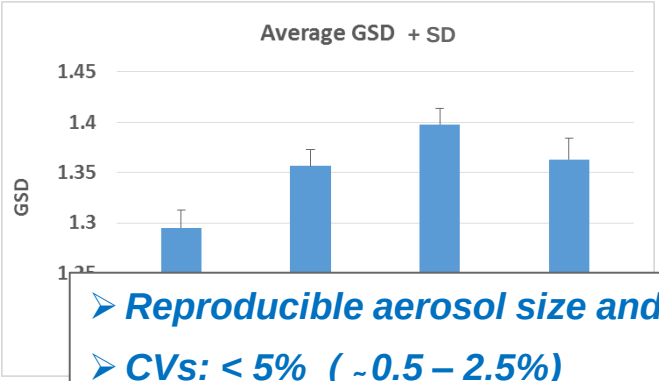


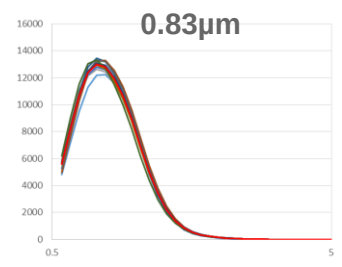
Applied CMAG settings

Mean particle size	Total flow rate (Scale)	Saturator flow rate (Scale)	Screen flow rate (Scale)	Saturator temp. (°C)	Reheater temp. (°C)
<0.5µm	8	0	0	160	340
0.83µm	8	1.5 - 2	0	160	340
1.13µm	8	3.75 - 4.25	0	160	340
1.41µm	8	6.75 - 7.25	0	160	340
1.63µm	8	7.75 - 8.25	3.5 - 4.5	160	340

APS measurements performed during 5 independent exposure runs (Average ± SD, 15 data points)
(<0.5 µm: below cut-off of APS, not measured)

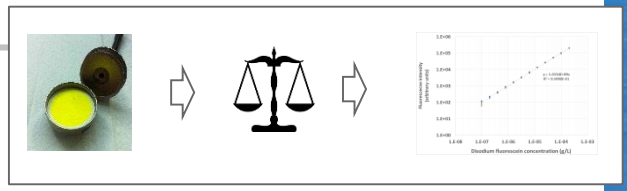






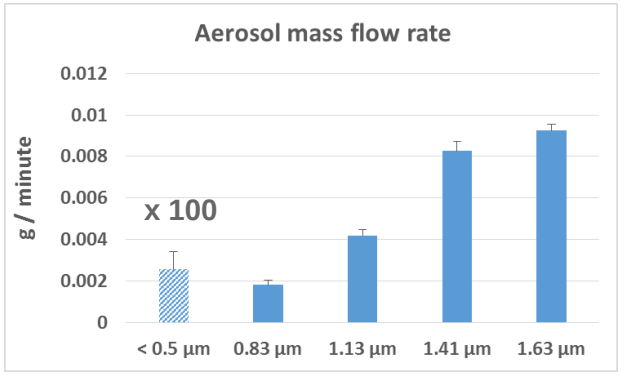
- ***Reproducible aerosol size and size distribution***
- ***CVs: < 5% (~0.5 – 2.5%)***

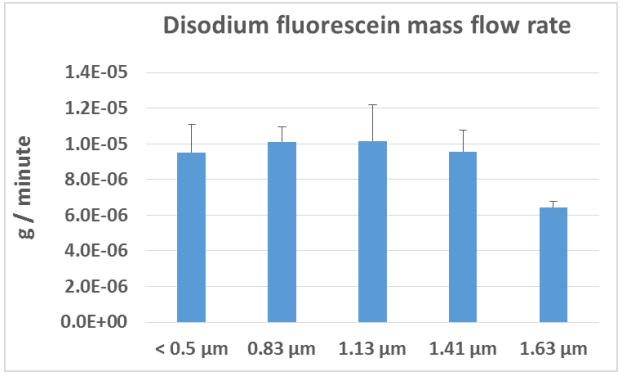
Fluorescent liquid aerosols of adjustable mean particle size:
Reproducibility of aerosol generation

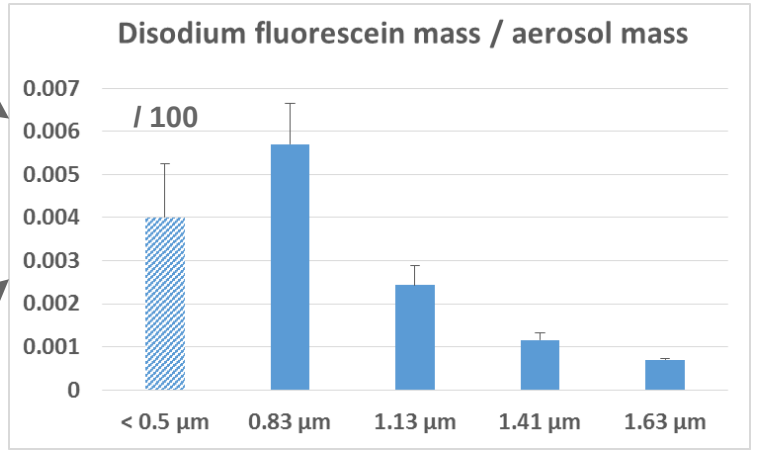


Five aerosols selected for method evaluation:
mass flow rates and fluorescence

5 independent exposure runs (Average + SD, 15 data points)





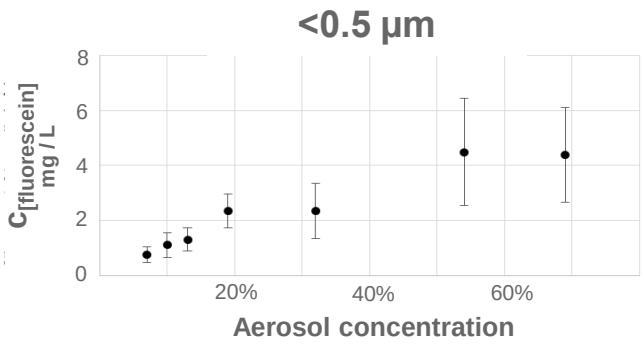


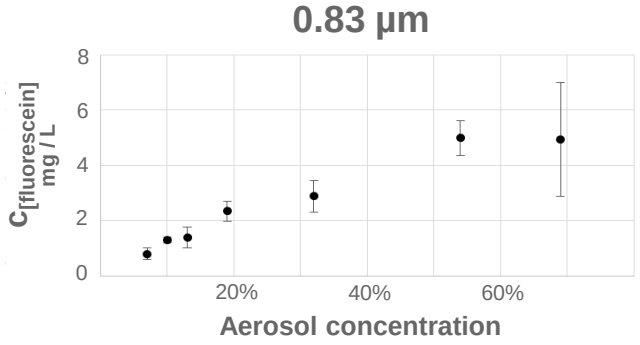
- ***Reproducible aerosol mass flow rates***
- ***More stable values for larger aerosols (1.63µm: all CVs < 5.5)***
- ***LLOQ: 50 ng aerosol (1.63µm aerosol)***

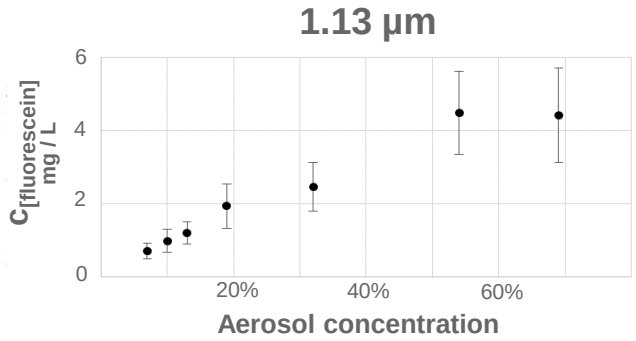
Steps towards Vitrocell system characterization using fluorescent glycerol aerosols: Aerosol dilution / dosing accuracy

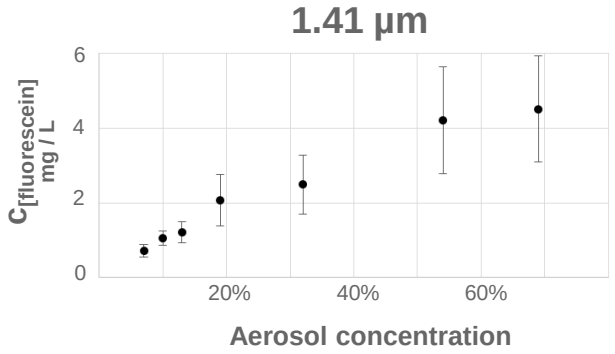
Detected disodium fluorescein concentrations in exposed PBS

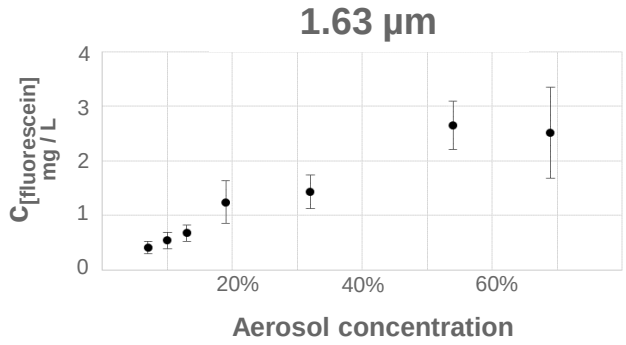
(7, 10, 13, 19, 32, 54, 69% aerosol, average per row/dilution over 5 runs)





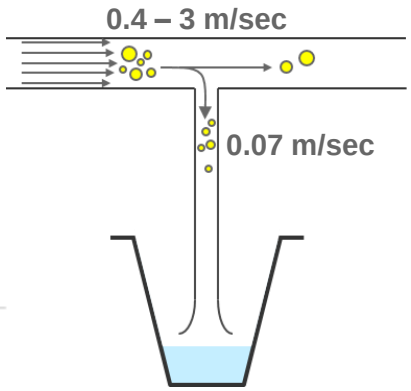






- ***All measurements above LLOQ***
- ***Sensitivity sufficient for position specific quantification***
- ***Plateaus at 69 → 54% (analogous to 3R4F)***
- ***Additional plateau at 32 → 19% aerosol***
  - ***Aerosol specific?***

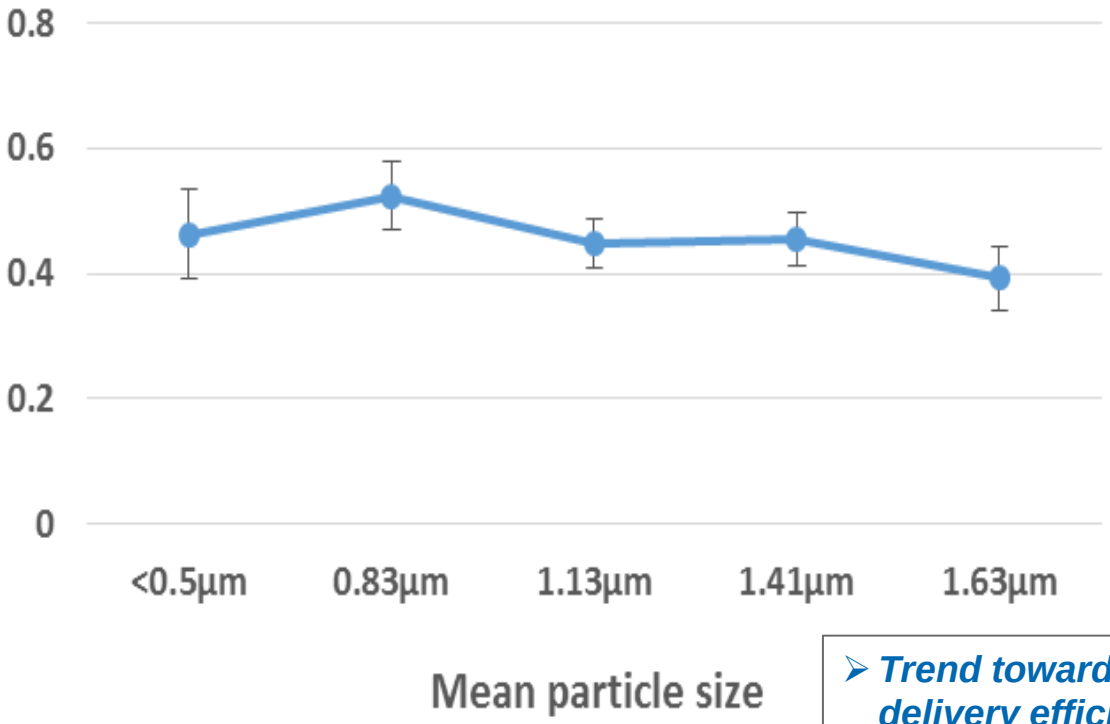
Steps towards Vitrocell system characterization using fluorescent glycerol aerosols: Effect of particle size



Delivered aerosol fraction as function of aerosol size

Relative to aerosol passing exposure trumpets

(average over 5 repetitions ± SD, dilutions merged)



- ***Trend towards lower particle delivery efficiency with increasing particle size***
- ***Unexpectedly weak effect***

Summary and conclusions, outlook

- **Robust particle size specific generation of disodium fluorescein labelled glycerol aerosols is possible**
- **Robust, fast, sensitive quantification of aerosol deposition**
- **Suitability for exposure system characterization could be demonstrated**

→ **A valuable tool to study particle dynamics/delivery**

Next steps

- **Detailed description of Vitrocell exposures:**
  - Reproducibility
  - Deposition uniformity across replica positions
  - Dilution/mixing effects
  - Aerosol losses
  - Hygroscopic particle growth
  - *Optimization of system operation*

Acknowledgments

Thank you for your attention

https://www.pmiscience.com/interactive-tour/

Contributors

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Director Systems Toxicology
Julia Hoeng

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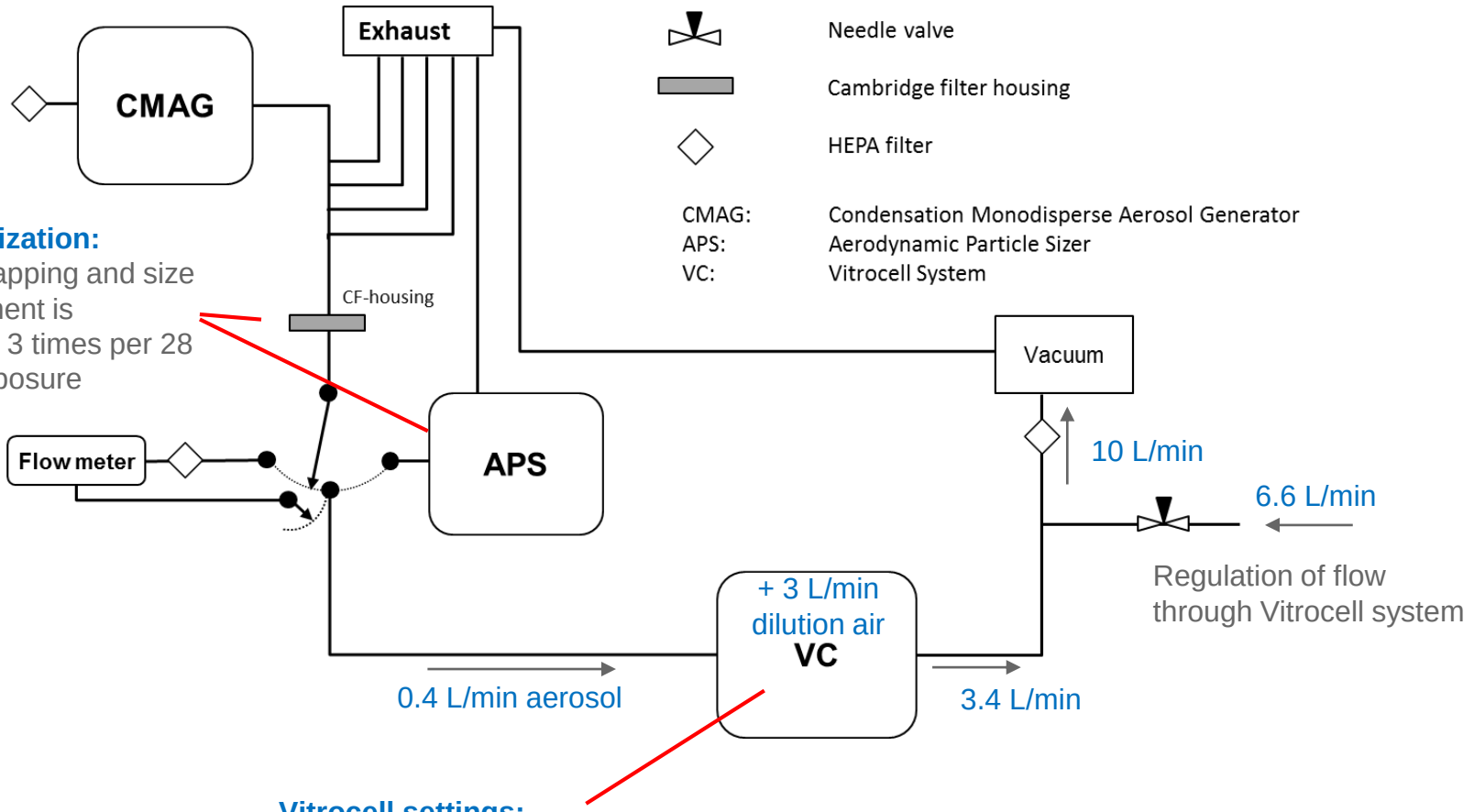
Fluorescent liquid aerosols of tunable mean particle size:
Experimental setup



Aerosol characterization:
Aerosol trapping and size measurement is performed 3 times per 28 minute exposure

Vitrocell settings:

- 28 minutes exposure of PBS samples in cell culture inserts
- 412 ml aerosol / minute
- 69, 54, 32, 19, 13, 10, 7% aerosol