The application of a self-designed microfluidic lung chip in the assessment for different inhalable aerosols

Electronic Supplementary Information

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Supplementary Experimental Section

Fabrication and assembling of the chip

Both the upper and lower layers of the chip were fabricated using polydimethylsiloxane (PDMS; Sylgard 184, DOW, Wiesbaden, Germany). The lower layer underwent processing via soft lithography, while the upper layer was created by pouring onto an aluminum mold (MesoBioSys Co., Ltd, Hubei, China). A polycarbonate (PC) membrane with a pore size of 5 μm (TMTP09030, Isopore, Carrigtwohill, Ireland) was sandwiched between the upper and lower layers. The polycarbonate (PC) microporous membrane underwent laser cutting into predetermined shapes using a CO_2 laser (VSL2.30, Universal, PA, USA), followed by rinsing three

times with deionized water and subsequent drying with nitrogen gas. Subsequently, the PC microporous membranes were subjected to continuous activation in an oxygen-filled plasma chamber (500 mTorr, 100 W) for 1 minute (DT-01, SuZhou OPS Plasma Technology Co., Jiangsu, China), after which they were immediately immersed in a 5% aqueous solution of 3-aminopropyltriethoxysilane (APTES; A3648, Sigma-Aldrich, MO, USA) at 80 ℃. After 20 min, the membrane was removed from the APTES aqueous solution and rinsed three times in deionized water. Concurrently, the fabricated lower PDMS layer underwent continuous activation in an oxygen-filled plasma chamber for 30 seconds (500 mTorr, 100 W). Subsequently, the wet membrane was gently contacted and aligned with the PDMS layer, rapidly dried with nitrogen gas, and then immediately placed in an oven at 80 ℃ for 2 h. To bond the membrane to another PDMS layer, forming the entire chip, the composite structure and the upper layer underwent activation in the same oxygen-filled plasma chamber. Subsequently, they were gently pressed together, and the sandwich structure was placed in an oven at 80 ℃. A 200 g metal block was carefully pressed onto it for 2 h.

Chip pretreatment and on-chip cell culture

Following assembly, the chips underwent sterilization via 2 h of UV irradiation. Subsequently, a sequential rinsing process was conducted using 75% alcohol followed by sterile water. Collagen I (150 μg/mL, 354249, Corning, USA) was then introduced into the chip's channels to enhance cell adhesion. The chip was subsequently incubated in a CO_2 cell culture incubator set at 5% CO_2, 37 ℃, and 95% humidity for 12 h

(HERAcell 240, Thermo Scientific, Germany). The normal human bronchial epithelial cell line, BEAS-2B, obtained from the American Type Culture Collection (ATCC, USA), was utilized for loading and culturing at the gas-liquid interface of the chip. Initially, BEAS-2B cells were cultured until reaching 80%-90% confluence in RPMI medium 1640 (11875-093, Gibco, USA) supplemented with 10% fetal bovine serum (10100147, Gibco, USA), 100 IU/ml penicillin, and 100 μg/ml streptomycin (15140-122, Gibco, USA). Upon reaching the desired confluence, the cells were harvested, adjusted to a cell density of 5×10^6 cells/mL, and introduced into the gas concentration gradient generator (GCGG) unit of the chip using a syringe pump (JSP-01-4A, JianMi Co., Ltd, China) at a flow rate of 80 μL/min. Following cell loading, the chip was placed in a cell culture incubator maintained at 5% CO_2, 37 ℃, and 95% humidity until cellular fusion occurred.

Detection of cell viability, reactive oxygen species content, nitric oxide content and caspase-3/7 activity level

The assessment of cell viability was conducted employing a Live/Dead cell viability assay Kit (L3224, Invitrogen, USA). Intracellular levels of reactive oxygen species (ROS) were measured utilizing the fluorescent probe DCFH-DA (R252, Dojindo, Japan), while intracellular nitric oxide (NO) content was assessed using the fluorescent probe DAF-FM-DA (D23844, Invitrogen, USA). Additionally, the activity of caspase-3/7 was determined using the caspase-3/7 fluorescence assay kit (KGAS037-100, KeyGEN BioTECH, China). These methodologies were executed

according to established protocols. Specifically, fresh working solutions were prepared by diluting individual stock solutions with Hank's balanced salt solution (HBSS; H1025, Solarbio, China), adhering to the manufacturer's instructions. Prior to introducing the prepared working solution into each channel, the channels underwent rinsing with HBSS. Following this, the chips loaded with the working solution were incubated for 20 min in a cell culture incubator. Subsequently, an inverted fluorescence microscope (IX71, Olympus, Japan) was utilized to capture images of the chip, after a final rinse with HBSS for 15 min.

RNA extraction, transcriptome analysis and real time RT-PCR

Following continuous on-chip dynamic incubation, phosphate buffered saline (PBS) was injected into the chips via a syringe pump. Subsequently, after removing the PBS, total RNA extraction was performed using the GenElute Mammalian Total RNA Miniprep Kit (RTN350, Sigma, USA). The on-column DNase digestion step (DNASE70, Sigma, USA) was employed as per the manufacturer's instructions to eliminate any genomic DNA contamination. Purity of RNA was determined by A_{260} to A_{280} ratio (NanoDrop, Thermo Scientific, USA).

Total RNA processing was performed by the mRNA enrichment method through Oligo(dT) magnetic beads. The purified mRNA was fragmented into small pieces to serve as templates. Subsequently, first-strand cDNA synthesis was performed using reverse transcriptase and random hexamer primers. The resulting cDNA was validated for quality control before constructing the final sequencing library (Agilent Bioanalyzer

4150 system, Agilent, USA).

The RNA-sequence data analysis was completed by BGI (The Beijing Genomics Institute, China). Differential expression analysis of these genes was performed using the DESeq2 R package (1.20.0). Adjusted *p*-values (*P*.adj) <0.05 and | log2FC | >1 was set as the threshold for significantly differential gene expression. To gain insights into phenotype changes, Gene Ontology (GO, http://www.geneontology.org/) and Kyoto Encyclopedia of Genes and Genomes (KEGG, https://www.kegg.jp/) enrichment analyses were conducted on the annotated differentially expressed genes using the Phyper tool (https://en.wikipedia.org/wiki/Hypergeometric_distribution) based on the hypergeometric test.

The purified RNA underwent reverse transcription using the Roche Transcriptor first strand cDNA synthesis kit (04896866001, Roche, Germany). Subsequently, the real-time RT-PCR assay was conducted using the FastStart Essential DNA Green Master kit (06924204001, Roche, Germany) following the manufacturer's instructions and performed on the LightCycler® 96 Instrument (Roche, Germany). The expression of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene served as an internal control to normalize the relative gene expression. The PCR conditions were as follows: initial denaturation at 95 ℃ for 10 min, followed by 45 cycles of denaturation at 95 ℃ for 10 s, annealing at 60 ℃ for 10 s, and extension at 72 ℃ for 10 s. Finally, the expression levels were normalized to the GAPDH level using the equation $2^{-\Delta\Delta Ct}$.

Immunocytochemistry

The immunocytochemistry procedures followed established protocols. BEAS-2B

cells cultured at the gas-liquid interface of the chip, after exposure to aerosols and continuous dynamic culture experiments, were briefly fixed with a 4% paraformaldehyde solution at room temperature for approximately 15 min. Subsequently, the fixed cells were blocked in PBS containing 0.25% Triton X-100 (A110694, Sangon Biotech, China) and 5% bovine serum albumin for about 20 min at room temperature. Following this, the cells were incubated overnight (approximately 12 h) with primary antibodies: rabbit anti-human FOSB (1:200; SN703, Invitrogen, USA) and rabbit anti-human KLF4 (1:200; ab215036, Abcam, USA) in the aforementioned PBS buffer supplemented with 5% bovine serum albumin. Specific visualization was achieved using secondary antibodies conjugated with goat anti-rabbit IgG H&L Alexa Fluor 488 (1:200; ab150077, Abcam, USA).

Table S1. Primer sequences for real time RT-PCR.

Gene	Sequence
CXCL8	F: ACTGAGAGTGATTGAGAGTGGAC
	R: AACCCTCTGCACCCAGTTTTC
FOSB	F: GGAACCAGCTACTCCACACC
	R: CGAGTTCAGGGGATCGGAAG
KLF4	F: ACCCTGGGTCTTGAGGAAGT
	R: CATGTGTAAGGCGAGGTGGT
GAPDH	F: GGAGCGAGATCCCTCCAAAAT
	R: GGCTGTTGTCATACTTCTCATGG

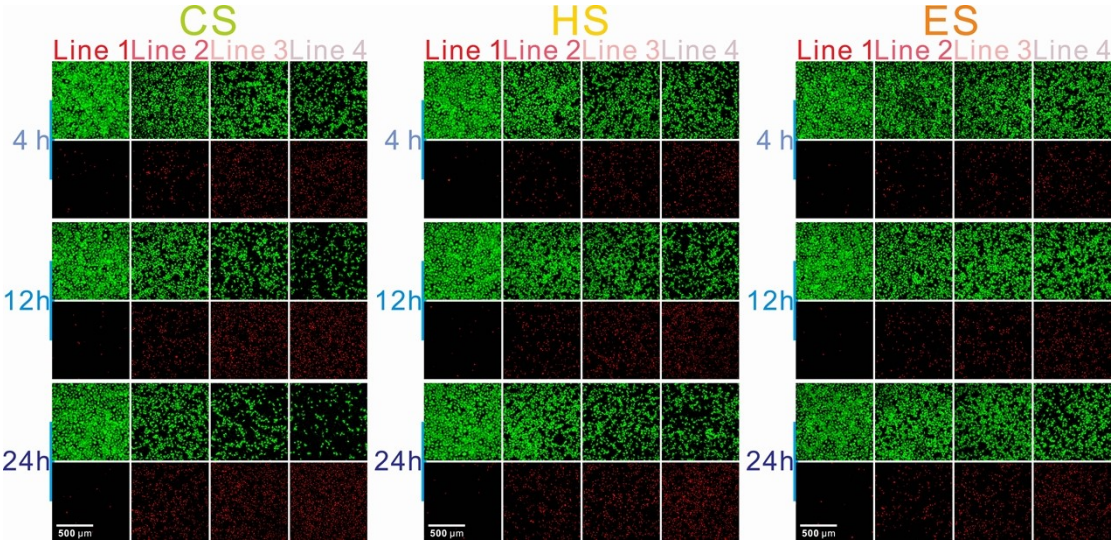

Figure S1. Fluorescence images of on-chip cell viability at 4, 12, 24 h after aerosol exposure experiments by Live/Dead assay (green: live cell, red: dead cell; CS, exposed to cigarette smoke aerosol; HS, exposed to heated tobacco product smoke aerosol; ES, exposed to electronic cigarette smoke aerosol).

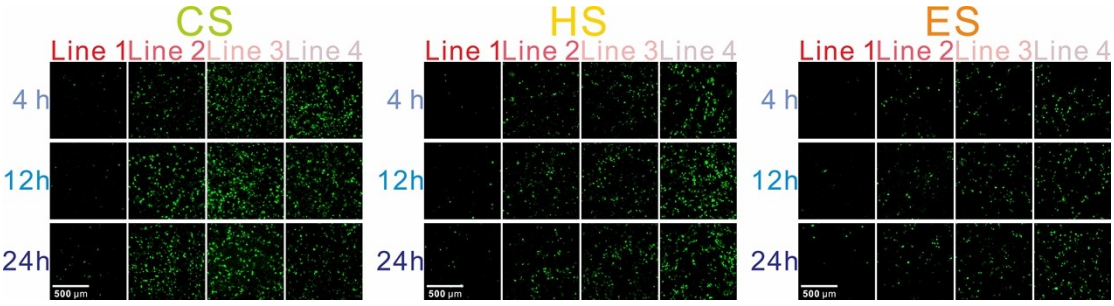

Figure S2. Fluorescence images of on-chip ROS content at 4, 12, 24 h after aerosol exposure experiments by ROS assay kit.

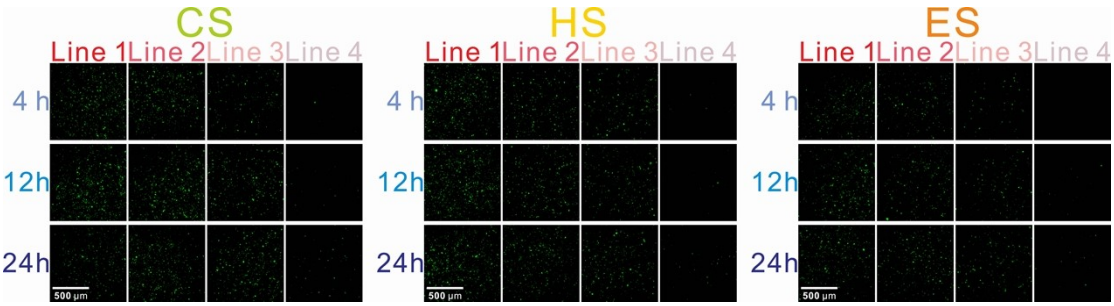

Figure S3. Fluorescence images of on-chip NO content at 4, 12, 24 h after aerosol exposure experiments by fluorescent probe DAF-FM-DA.

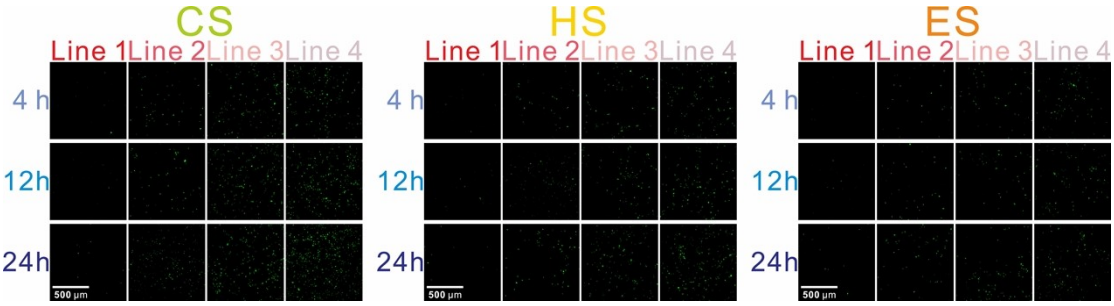

Figure S4. Fluorescence images of on-chip caspase-3/7 activity level at 4, 12, 24 h after aerosol exposure experiments by caspase-3/7 fluorescence assay kit.

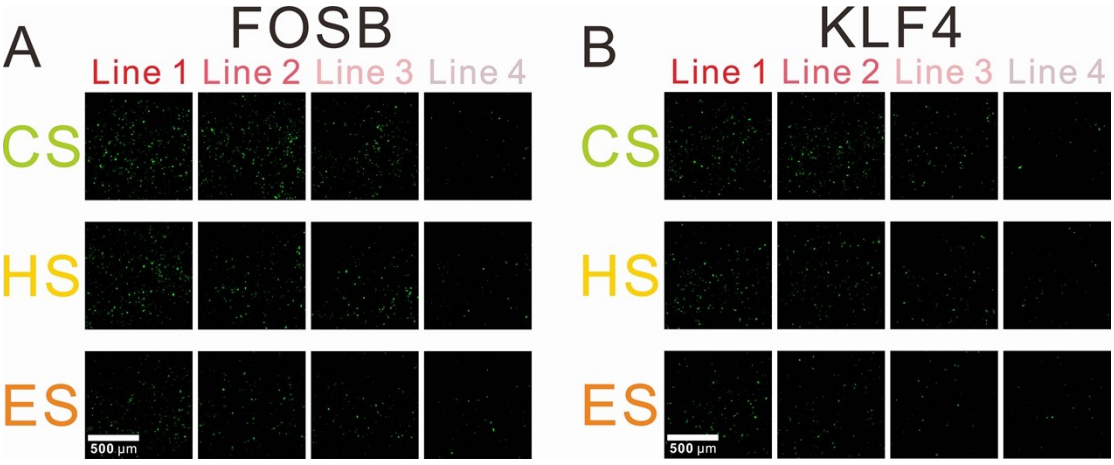

Figure S5. Immunofluorescence analysis of FOSB and KLF4 in different channels of the chip after aerosol exposure experiments.