Electronic Supplementary Material (ESI) for Lab on a Chip.

Supplementary Information

Label-Free Optical Detection of Calcium Ion Influx in Cell-Derived Nanovesicles Using a Conical Au/PDMS Biosensor

Jisung Kwak[1,2,†], Woochul Kim[1,†], Hyerim Cho[1,3,†], Jiyun Han[4], Sang Jun Sim[2], Hyun Gyu Song[1], Yusin Pak[1]*, and Hyun Seok Song[1]**

[1]Sensor System Research Center, Korea Institute of Science and Technology (KIST), Seoul 02792, Republic of Korea

[2]Department of Chemical and Biological Engineering, Korea University, Seoul, 02841, Republic of Korea

[3]KU-KIST Graduate School of Converging Science and Technology, Korea University, Seoul, 02841, Republic of Korea

[4]Center of Water Cycle Research, Korea Institute of Science and Technology (KIST), Seoul, 02792, Republic of Korea

Figure S1. Schematic of fabrication method for molding the conical PDMS structure by using micropipette.

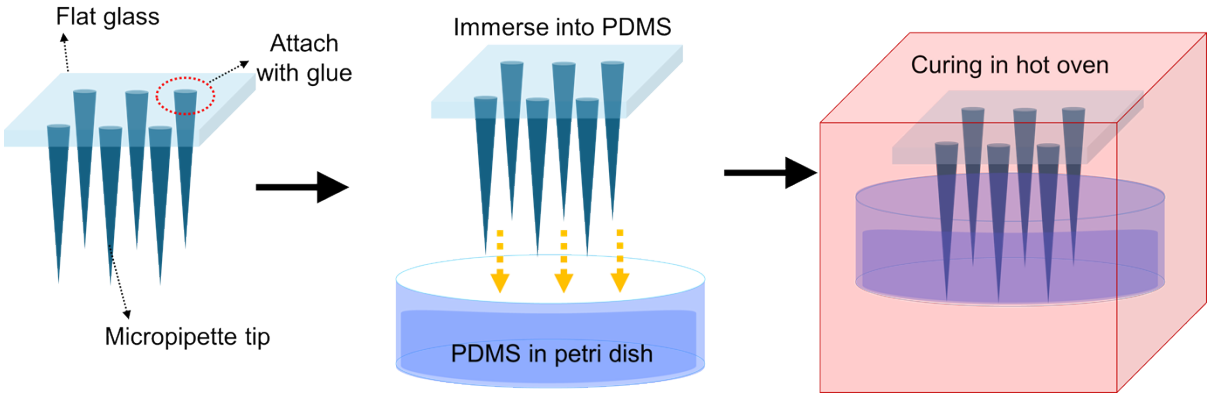

Figure S2. Dynamic light scattering measurement. The average diameter of A549–nanovesicle is ~ 360 nm, confirmed by dynamic light scattering measurement.

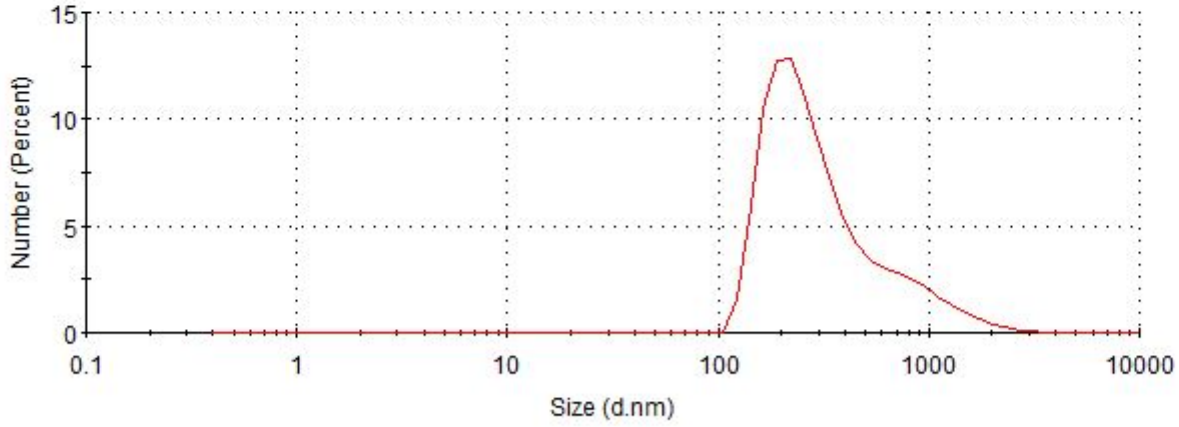

Figure S3. Optical microscope image of conical Au/PDMS sensor. The cross-sectional optical microscope image of conical Au/PDMS sensor at **a** top, **b** bottom hole, and **c** direction of cross-sectional view. The diameter of top and bottom hole is measured as 2.3 and 1.3 mm, respectively.

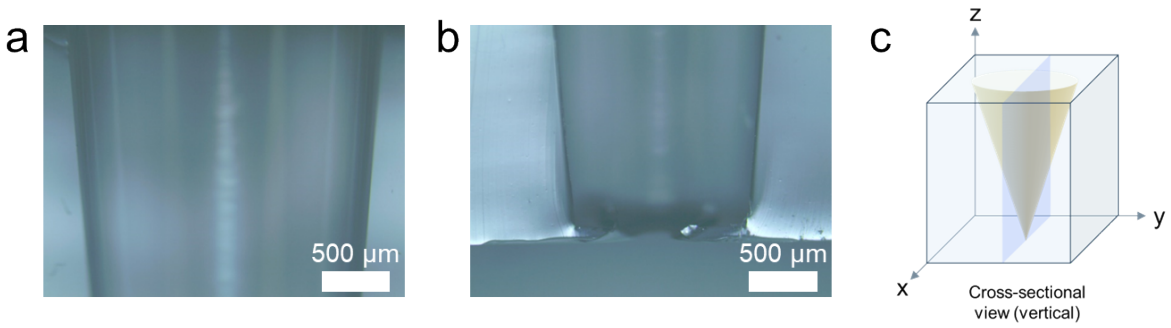

Figure S4. Direction of cross section view of conical Au-PDMS biosensor.

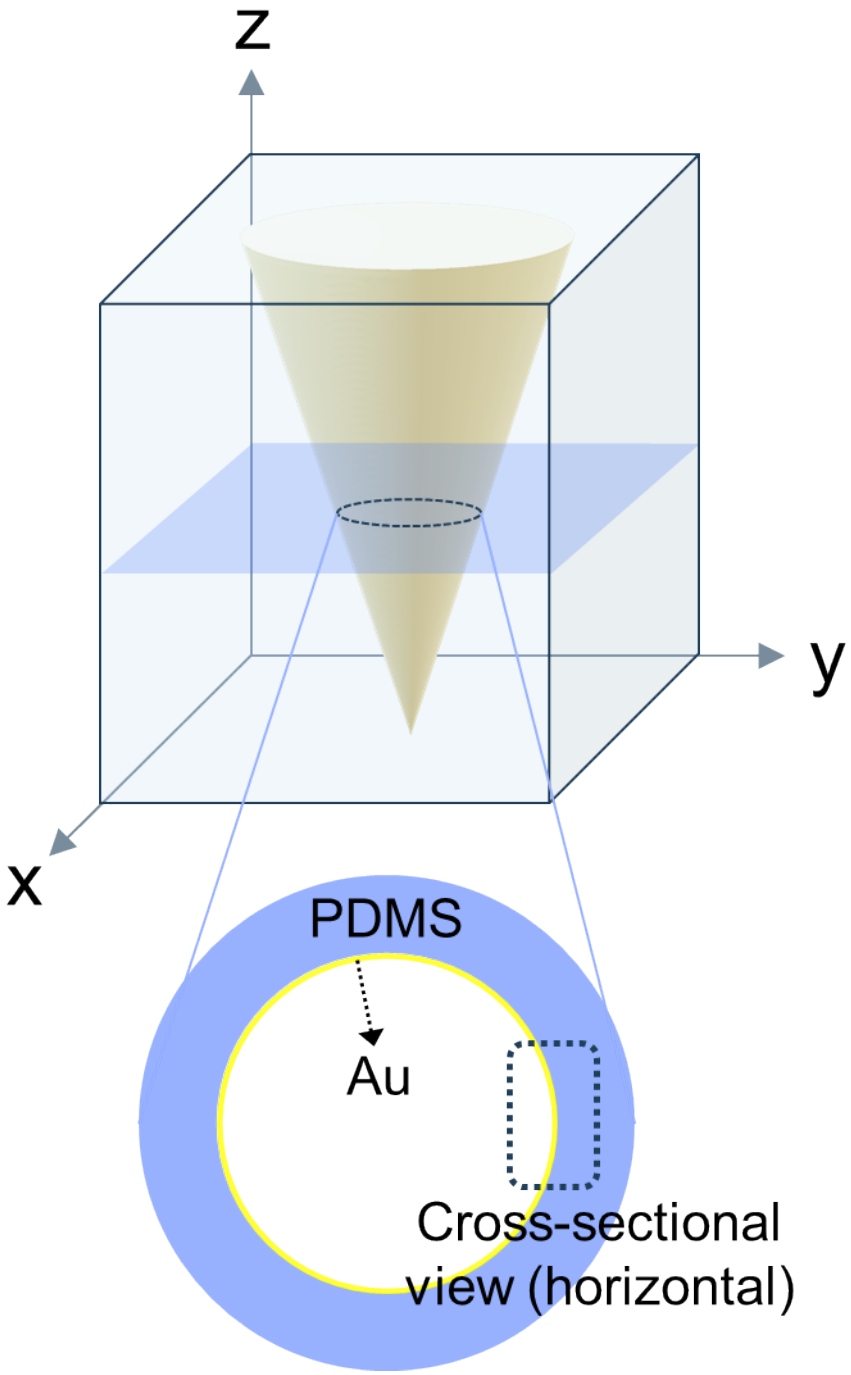

Figure S5. Fluorescence microscope image of A549 cell before and after application of the agonist AITC and cinnamaldehyde. Fluorescence image of A549 cell **a** before and **b** after exposure to cinnamaldehyde with 10 X magnification. Fluorescence image of A549 cell **c** before and **d** after exposure to AITC with 10 X magnification.

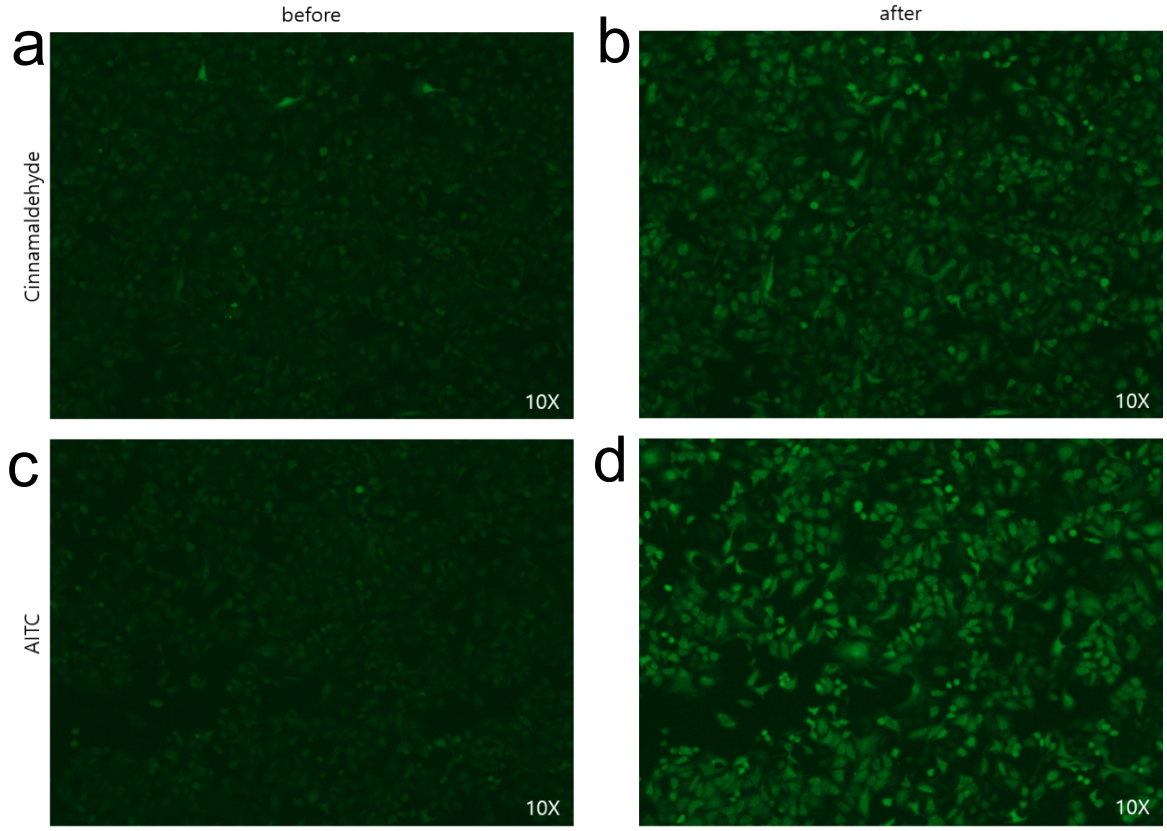

Figure S6. Comparison of ΔT at 447 nm of TRPA1 NV (w Ca^{2+}), TRPA1 knockdown (KD) NV (w Ca^{2+}), and TRPA1 NV (w/o Ca^{2+})

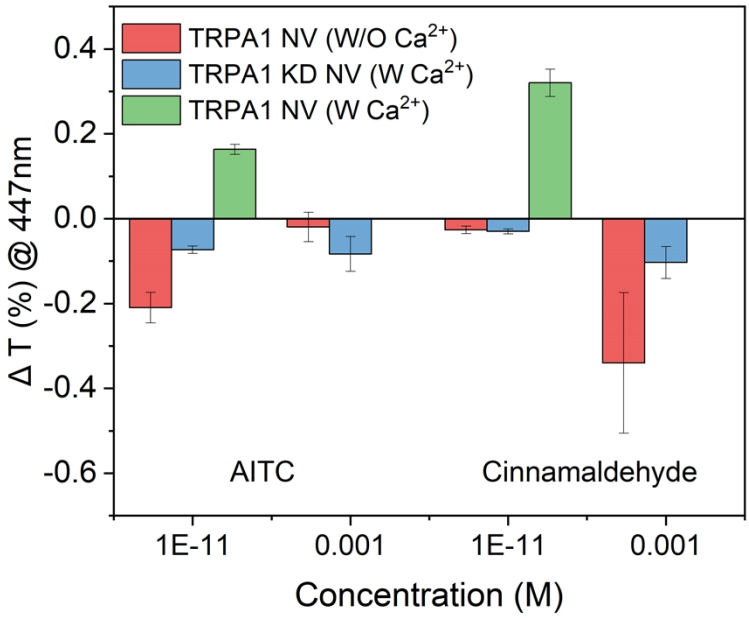

Figure S7. Relative transmittance change (ΔT) of non-nanovesicle contained samples depending on the agonist molecule.

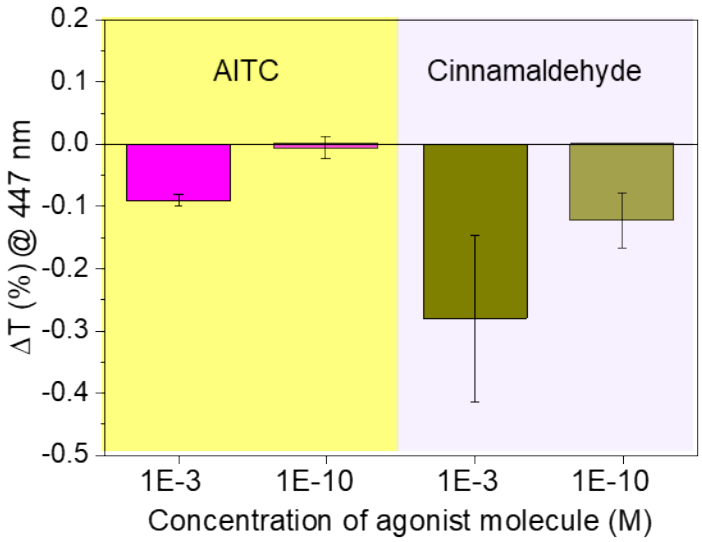

Figure S8. Response time of negative–controlled sample. Normalized transmittance at a wavelength of 447 nm of negative–controlled sample under **a** AITC and **b** cinnamaldehyde introduction. The response time is measured as 2.6 and 18 s for AITC and cinnamaldehyde introduction, respectively.

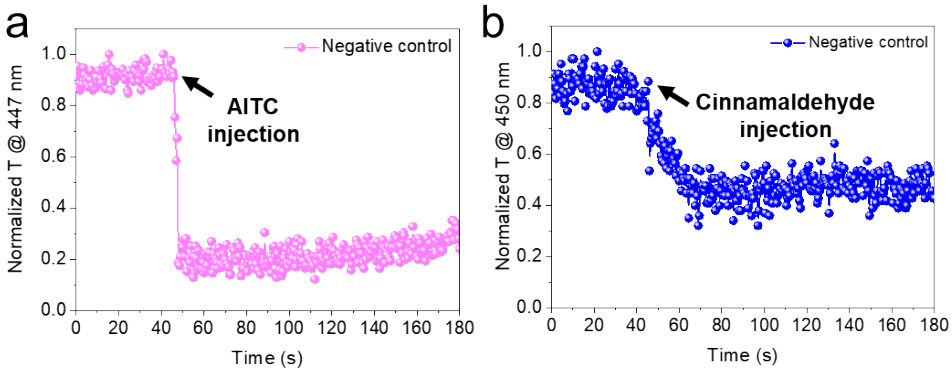

Figure S9. Response time of knockdown–controlled sample. Normalized transmittance at a wavelength of 447 nm of knockdown–controlled sample under **a** AITC and **b** cinnamaldehyde introduction. The response time is measured as 10 and 11.5 s for AITC and cinnamaldehyde introduction, respectively.

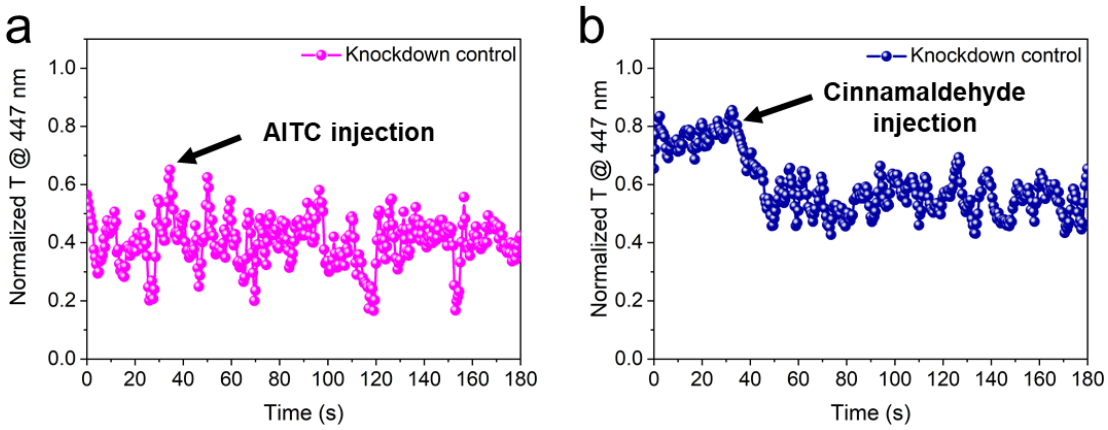

Figure S10. Optical microscope image of conical Au/PDMS sensor. a The angle of conical Au/PDMS sensor is measured as 78 degrees. **b** Direction of cross-sectional view.

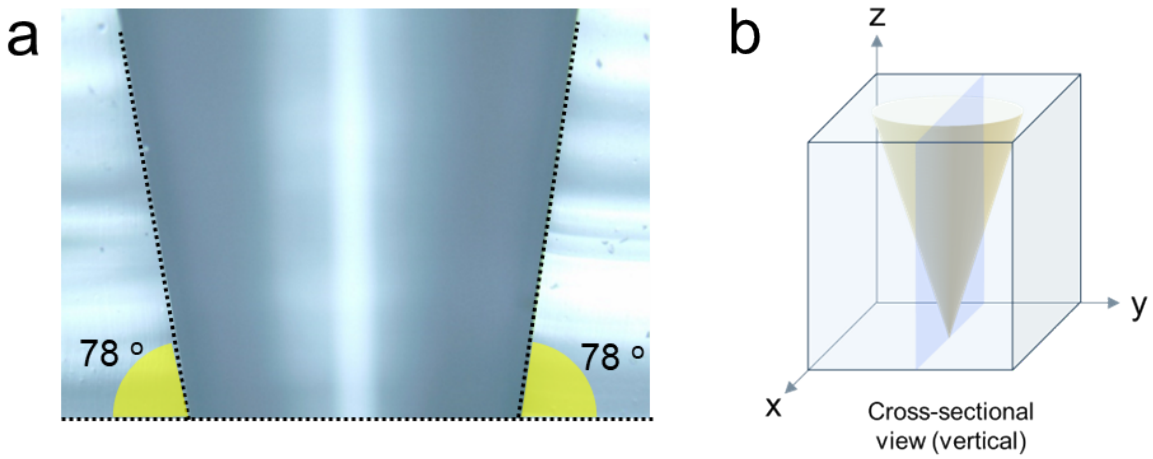

Figure S11. Data of FDTD simulation in relation to the angle between the conical structure and illuminated light.

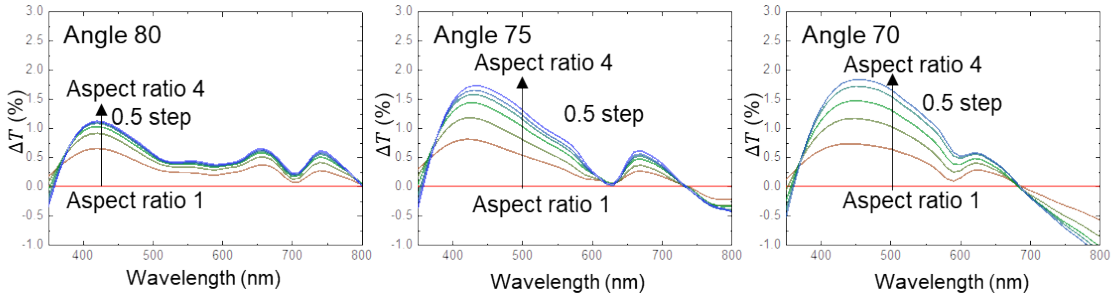

Table S1. Comparison of figure-of-merits of Ca^{2+} influx detection measurements. The detection limit evaluation is shown in Supplementary Note 3.

	Sensitivity	Labeling	Detection limit	Throughput	Temporal resolution	Preparation process	Detectable scale	Viability maintenance	References
Fluorescent Probe	High	O	A few ~ tens of nM	High	High	Complex	~ nm	High (Cell & NV)	1, 2
Patch Clamp	High	X	tens of nM	Low	High	Complex	~ μm	Low (Cell)	3, 4
GECIs	High	O	tens of μM	Low	High	Complex	~ nm	Low (Cell)	5, 6
Conical Au/PDMS	**High**	**X**	**tens of pM**	**High**	**Intermediate**	**Simple**	**~ nm**	**High (NV)**	**Our work**

(O: Labeling is required, X: Labeling is unnecessary)

Supplementary Note 1. Experimental method of sample preparation for analysis

Preparation of nanovesicles for SEM analysis

Nanovesicles were resuspended in 4 % paraformaldehyde for 10 min, then the nanovesicles were dehydrated with ethanol and diluted water (DI water) mixture, gradually increasing ethanol volumetric percentage from 30 to 100 %. Liquids in nanovesicles were dried by nitrogen gas to retain the shape of the nanovesicles in the preparation for SEM analysis. Cell-derived nanovesicles were dropped on a silicon surface and coated with platinum (Pt) by a sputtering method.

Measurement of calcium influx

The Fluo-4 DirectTM Calcium Assay Kits was used to measure TRPA1 activation. Medium containing the cells was replaced by Fluo-4 DirectTM calcium reagent loading solution (that was added directly into wells) with culture media and a probenecid concentration of 5 mM (Invitrogen). Cells were incubated at 37 °C for 30 min under 5 % CO_2. In the cases of nanovesicles, comparison of the produced nanovesicles were performed giving variation of the type of the solvent, in addition, TRPA1 siRNA knockdown processed nanovesicles were also used to confirm the contribution of TRPA1 receptor to the calcium flux with presence of calcium. At first, the Fluo-4 DirectTM calcium reagent-loaded nanovesicles were suspended under two different solvent conditions, PBS solvent with concentration of 1 mM $CaCl_2$, and PBS solvent without $CaCl_2$. The suspended nanovesicles were placed inside the Poly-D-lysine coated 96-well plate at 37 °C for 4 hrs. The basal fluorescence signal was recorded for 10 s followed by an injection of agonists by injector and 180 s continuously recorded.

Quantitative real-time PCR(qRT-PCR) analysis

RNA was extracted from cells and the cells with treatment of TRPA1 knockdown and scramble. 250 ng of RNA was used to synthesize complementary DNA (cDNA) with SuperScript VILO cDNA synthesis kit (Invitrogen). The reverse transcription PCR was processed for 25 min in total (10, 10, 5 min), under temperature conditions of 25, 50, and 85 °C, respectively. PCR amplification was performed with Maxime PCR Premix (i-StarTaq) (Intron Biotechnology). Quantitative real-time polymerase chain reaction (PCR) assay was performed on a real-time PCR system (Applied Biosystems), utilizing a SYBR premix Ex Taq (TaKaRa). The primer sequences were as follows: TRAPA1 forward 5’-

CTGCCACTTTGGTTTCTACG-3', reverse 5'-AGTAAGATCCTTCAGCCGGTA-3'. For TRPA1 and GAPDH, 2 uL of the reverse transcription products were subjected with a thermal program of 20 s at 94 °C for denaturation, 10 seconds at 58 °C for annealing, 30 s at 72 °C elongation, and 5 min at 72 °C final extension step. Each sample was analyzed in triplicate, the measured mRNA levels in each sample were normalized to GAPDH mRNA levels. PCR products were electrophoresed on 2 % agarose gel to confirm the target band size.

siRNA knockdown

A549 cells were plated on 6-well plates (0.25×10^6 cells/well) with 2 mL of RPMI containing FBS (10 %) a day before the transfection. Cells were transfected as follows: TRPA1 siRNA and lipofectamine RNAiMAX were diluted with Opti-MEM separately before being mixed by pipetting. The siRNA-RNAiMAX mixture was added to 6-well cell culture plates. The final concentrations of lipofectamine RNAiMAX and siRNA were 9 mM and 50 nM, respectively. Cells were then incubated for 24 hrs under 5 % CO_2 at 37 °C. Knockdown efficiency was detected at 48 hrs by RT-PCR comparative quantitation analysis.

TRPA1 siRNA: AUGACAUCCAUCGGUUGUG=tt(1-AA)

TRPA1 siRNA: CACAACCGAUGGAUGUCAU=tt(1-AS)

Supplementary Note 2. Evaluation of effective agonists per nanovesicle.

By estimating the number of effective agonists per single nanovesicle, the number of nanovesicles undergo Ca influx in the conical Au-PDMS biosensor can be approximately estimated. The effective agonists per single nanovesicle can be estimated using equation below.

$$Effective\ agonists\ per\ nanovesicle\ (in\ unit\ volume) = \frac{EC50\ * NA}{Number\ of\ nanovesicle\ per\ unit\ volume}$$

where, EC50 is half maximal effective concentration and NA is Avogadro's number. From the fluorescence probe method, the EC50 values are 2.027 and 1.793 μM for AITC and cinnamaldehyde, respectively. The number of nanovesicle per unit volume is estimated to be 1.86 x 10^{13} by using nanoparticle tracking analysis, as shown in Fig. S12. The effective agonists per single nanovesicle is calculated as 6.6 and 5.8 x 10^4 for AITC and cinnamaldehyde, respectively. When 100 pM of AITC is introduced, it is estimated that effective Ca influx events can be triggered in approximately 9.8 x 10^5 nanovesicles on the Au-PDMS biosensor. Likewise, the Ca influx occurs in 1.0 x 10^4 nanovesicle by 100 pM of cinnamaldehyde introduction.

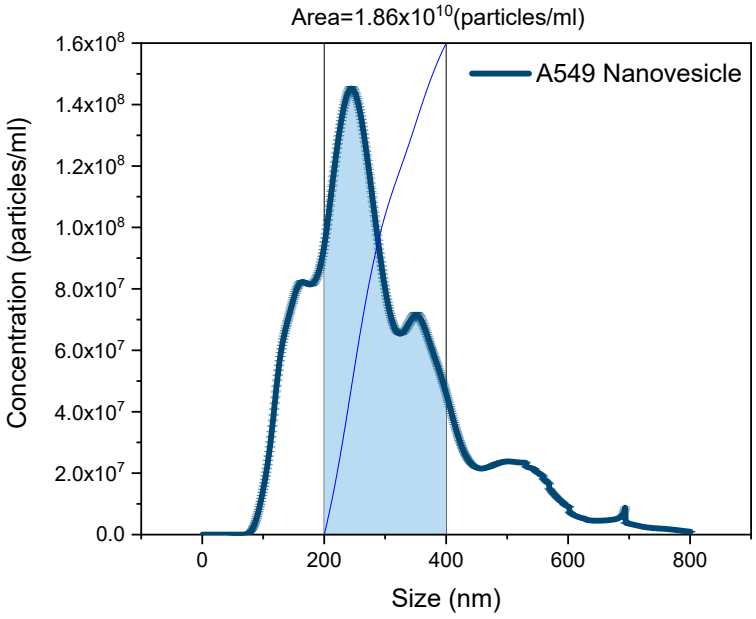

Figure S12. Nanoparticle tracking analysis of A549 nanovesicle between 200 and 400 nm size distribution.

Supplementary Note 3. Determination of detection limit of the conical Au-PDMS biosensor.

To determine the detection limit, we calculated the instrument detection limit using the equation below.

$$S(DL) = mean\ (blank) + 3\ \sigma\ (blank)$$

where, S(DL) is analyte signal at detection limit, mean (blank) is the mean value of the signal for a blank measured multiple times, and σ (blank) is the standard deviation for the signal of blank. The S(DL) of conical Au-PDMS biosensor was calculated as 0.066 at blank signal (transmittance variation of nanovesicle solution in the conical Au-PDMS biosensor without any agonist injection). For determine the detection limit of conical Au-PDMS biosensor, we decreased concentration of each agonist to 10 pM and ΔT value exhibited as 0.036 and 0.045 for AITC and cinnamaldehyde, respectively, which were lower than the S(DL). The value of ΔT with respect to the agonist concentration has the following linear relationship in the low concentration range.

$$y = 0.12\log x + 1.37\ (for\ AITC)$$
$$y = 0.26\log x + 2.54\ (for\ cinnamaldehyde)$$

Based on the linear equation and lower level of ΔT at 10 pM, we estimated the detection limit of conical Au-PDMS biosensor as about tens of pM.

References

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