Supplementary Table 1. Comparison of costs for fluorinated oil sealing and adhesive tape sealing

Sealing Method	Assay Component	Cost per assay	Assumptions for calculation
Fluorinated oil sealing	FC40 fluorinated carbon oil	0.07–0.15 USD	38,900 Japanese Yen (JPY) ≈ 389 US Dollar (USD) per 100 mL FC40, 20–40 µL per assay.
	Polyimide double-sided tape	0.03–0.12 USD	45000 JPY ≈ 450 USD per 20 m roll of 210 mm width. 54 pieces of 24 mm × 24 mm from 210 mm × 300 mm sheet. 1–4 assays per piece. Cost for pattern cutting is ignored.
	Glass upper device	0.005–50 USD	2.500 JPY ≈ 2.5 USD per hole drilled manually. With 8 holes, 4 assays can be performed from a single piece. Reusable if washed. Assumed repeated use of 1–1000 times. Cost for the glass itself and cost for washing after each use are ignored.
	CYTOP coating of glass	0.18–0.70 USD	100,000 JPY ≈ 1,000 USD per 100 mL, 70 µL per upper device for 1–4 assays. Required if glass upper device is used.
Adhesive tape sealing	145RN adhesive tape	0.0010–0.0021 USD	347 JPY ≈ 3.5 USD for 20 m roll of 48 mm width. 12–24 mm × 24 mm sheet per assay.

Supplementary Table 2 Reagents used for making different adhesives. Related to Figure 3 and Supplementary Fig. 3.

Product Name	Manufacturer	Reagent	Category
Kraton D1161	Kraton	SIS	Rubber adhesive elastomer
Kraton D1162	Kraton	SIS	Rubber adhesive elastomer
Kraton D1163	Kraton	SIS	Rubber adhesive elastomer
Kraton DX401	Kraton	SIS	Rubber adhesive elastomer
Quintac 3421	Zeon	SIS	Rubber adhesive elastomer
Asaprene T-411	AsahiKasei	SBS	Rubber adhesive elastomer
D1101	Kraton	SBS	Rubber adhesive elastomer
D1155	Kraton	SBS	Rubber adhesive elastomer
Tufprene A	AsahiKasei	SBS	Rubber adhesive elastomer
SEPTON 2002	Kuraray	SEPS	Rubber adhesive elastomer
SEPTON 2063	Kuraray	SEPS	Rubber adhesive elastomer
SEPTON 8004	Kuraray	SEBS	Rubber adhesive elastomer
JSR EP331	JSR	Ethylene-Propylene rubber	Rubber adhesive elastomer
Tetrax 6T	ENEOS	Polyisobutylene	Rubber adhesive elastomer
JSR IR2200	JSR	Polyisoprene	Rubber adhesive elastomer
JSR BR01	JSR	Butyl rubber	Rubber adhesive elastomer
JSR BUTYL065	JSR	Butyl rubber	Rubber adhesive elastomer
T-REZ HA085	ENEOS	Hydogenated hydrocarbon resin	Rubber adhesive tackifier
Quintone DX390N	Zeon	Aliphatic hydrocarbon resin	Rubber adhesive tackifier
PINECRYSTAL KR-85	Arakawa Chemical Industries	Rosin derivatives	Rubber adhesive tackifier
PINECRYSTAL PE-590	Arakawa Chemical Industries	Rosin derivatives	Rubber adhesive tackifier
YS Resin PX1150N	Yasuhara Chemical	Terpene resin	Rubber adhesive tackifier
I-MARV S-100	Idemitsu	Hydrogenated petroleum resin	Rubber adhesive tackifier
YS Resin SX100	Yasuhara Chemical	Styrene resin	Rubber adhesive tackifier
YS Resin TO125	Yasuhara Chemical	Terpene resin	Rubber adhesive tackifier
KR-100	Shin-Etsu Chemical	N/A	Silicone adhesive
KR-130	Shin-Etsu Chemical	N/A	Silicone adhesive
KR-3700	Shin-Etsu Chemical	N/A	Silicone adhesive
KR-3704	Shin-Etsu Chemical	N/A	Silicone adhesive
Dowsil SD 4580	Dow	N/A	Silicone adhesive
Dowsil SH 4280	Dow	N/A	Silicone adhesive
X-40-3237	Shin-Etsu Chemical	N/A	Silicone adhesive
Benzoyl peroxide	Tokyo Chemical Industry	Benzoyl peroxide	Silicone adhesive crosslinker

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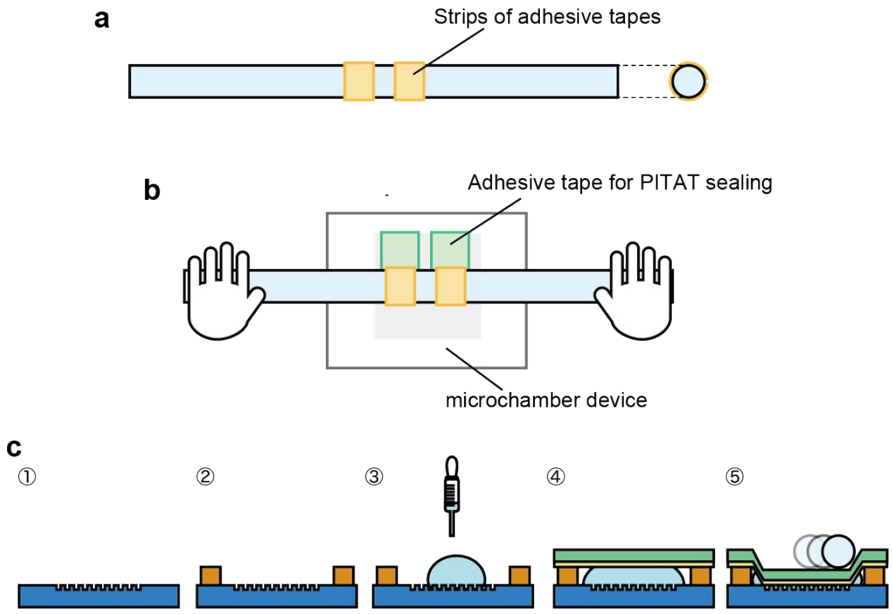
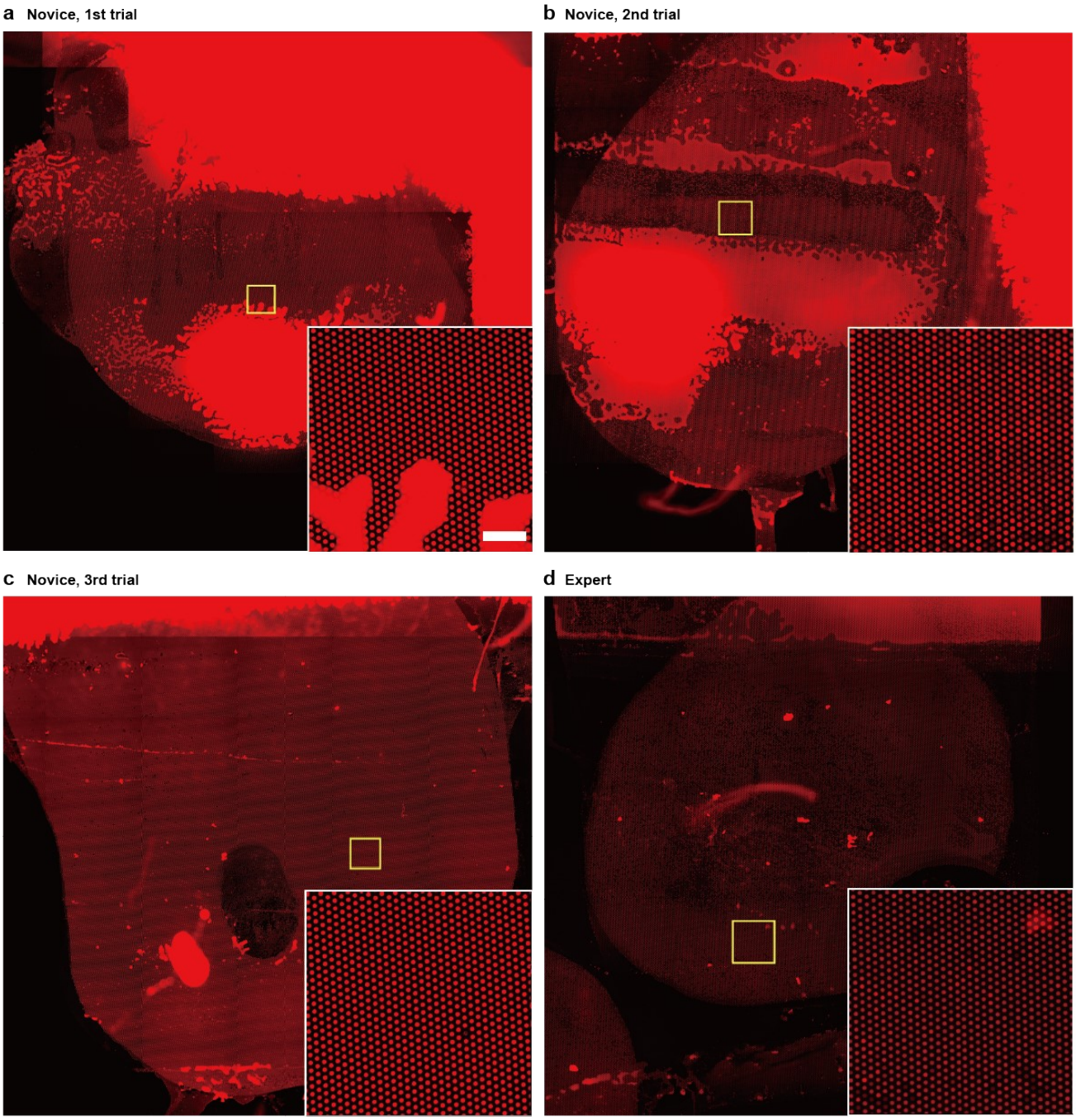
X-92-122C	Shin-Etsu Chemical	N/A	Silicone adhesive crosslinker
PLT-50-PT	Shin-Etsu Chemical	Platinum catalyst	Silicone adhesive crosslinking catalyst
SRX212	Dow	Platinum catalyst	Silicone adhesive crosslinking catalyst
ORIBAIN BPS 5160	Toyochem	N/A	Acrylic adhesive
ORIBAIN BPS 5213K	Toyochem	N/A	Acrylic adhesive
ORIBAIN BPS5296	Toyochem	N/A	Acrylic adhesive
ORIBAIN BPS5375	Toyochem	N/A	Acrylic adhesive
NISSETSU KP-1282	Carbide	N/A	Acrylic adhesive
NISSETSU KP-1405	Carbide	N/A	Acrylic adhesive
NISSETSU KP-2369	Carbide	N/A	Acrylic adhesive
NISSETSU PE-121	Carbide	N/A	Acrylic adhesive
CK-101	Carbide	Isocyanate crosslinker	Acrylic adhesive crosslinker
CK-102	Carbide	Isocyanate crosslinker	Acrylic adhesive crosslinker
CK-131	Carbide	Isocyanate crosslinker	Acrylic adhesive crosslinker
BHS-8515	Toyochem	Isocyanate crosslinker	Acrylic adhesive crosslinker
CYABINE SH-101	Toyochem	N/A	Urethane adhesive
CYABINE SH-205	Toyochem	N/A	Urethane adhesive
T-501B	Toyochem	Isocyanate crosslinker	Urethane adhesive crosslinker
Diana process oil NR-26	Idemitsu	Naphtenic oil	Rubber adhesive plasticizer
M5904	Merck	Mineral oil	Rubber adhesive plasticizer
A0142	Tokyo Chemical Industry	BA	Acrylic adhesive monomer
A0144	Tokyo Chemical Industry	EHA	Acrylic adhesive monomer
A0141	Tokyo Chemical Industry	AA	Acrylic adhesive monomer
M0085	Tokyo Chemical Industry	HEMA	Acrylic adhesive monomer
DMS-V46	Gelest	Vinyl terminated PDMS	Silicone adhesive ingredient
DMS-V41	Gelest	Vinyl terminated PDMS	Silicone adhesive ingredient
DMS-V31	Gelest	Vinyl terminated PDMS	Silicone adhesive ingredient
DMS-V21	Gelest	Vinyl terminated PDMS	Silicone adhesive ingredient
HMS-993	Gelest	Polydimethylhydrosiloxane	Silicone adhesive ingredient
SQO-299	Gelest	MQ resin	Silicone adhesive ingredient

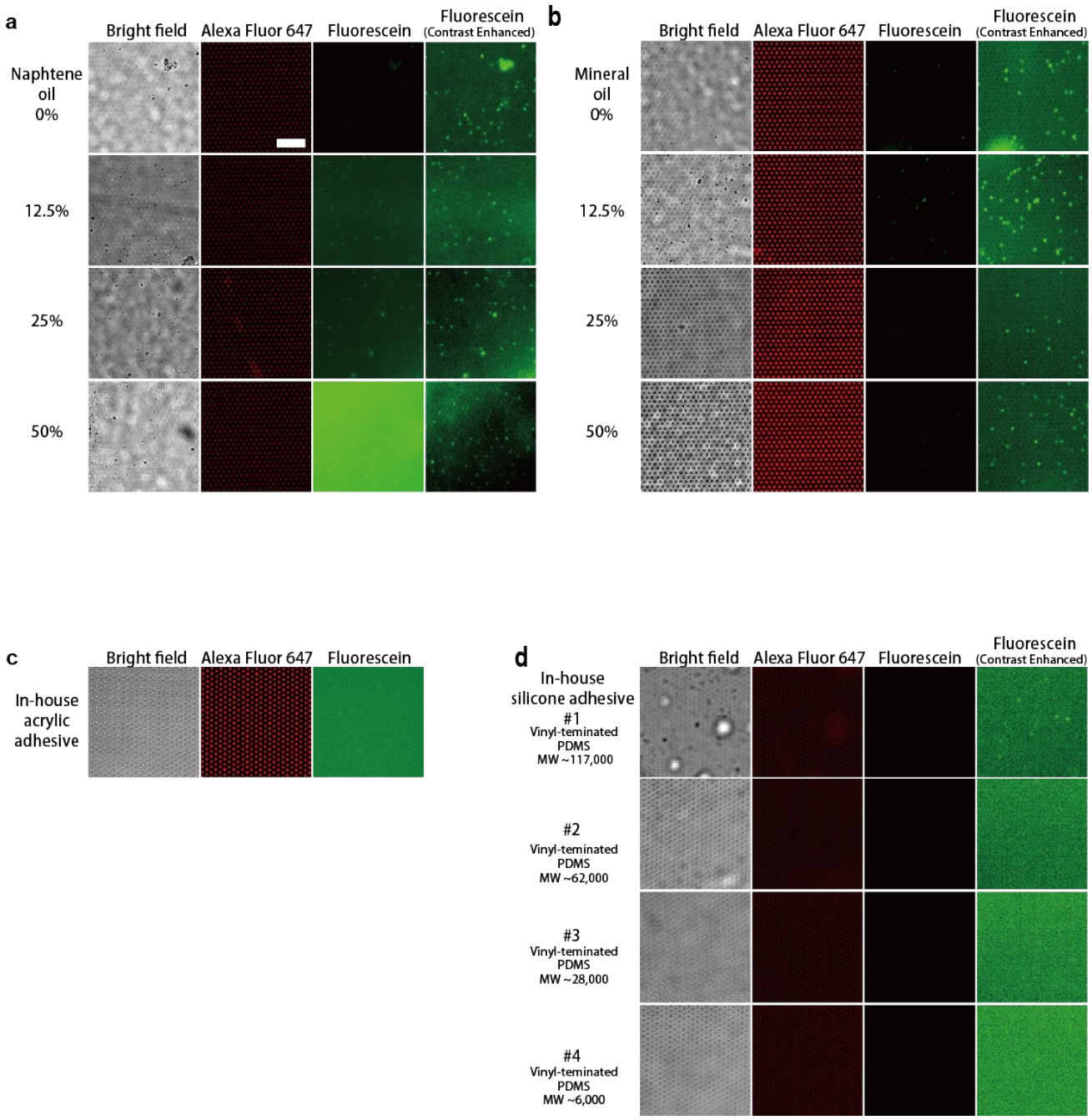
SIS: styrene-isoprene-styrene block copolymer, SBS: styrene-butadiene-styrene block copolymer, SEPS: styrene-ethylene/propylene-styrene block copolymer, SEBS: styrene-ethylene-butylene-styrene block copolymer, BA: butylic acid, EHA: 2-ethylhexyl acrylate, AA: acrylic acid, HEMA: 2-hydroxyethyl methacrylate, PDMS: polydimethylsiloxane, MQ resin: silanol trymethylsilyl modified Q resin, N/A: not applicable.



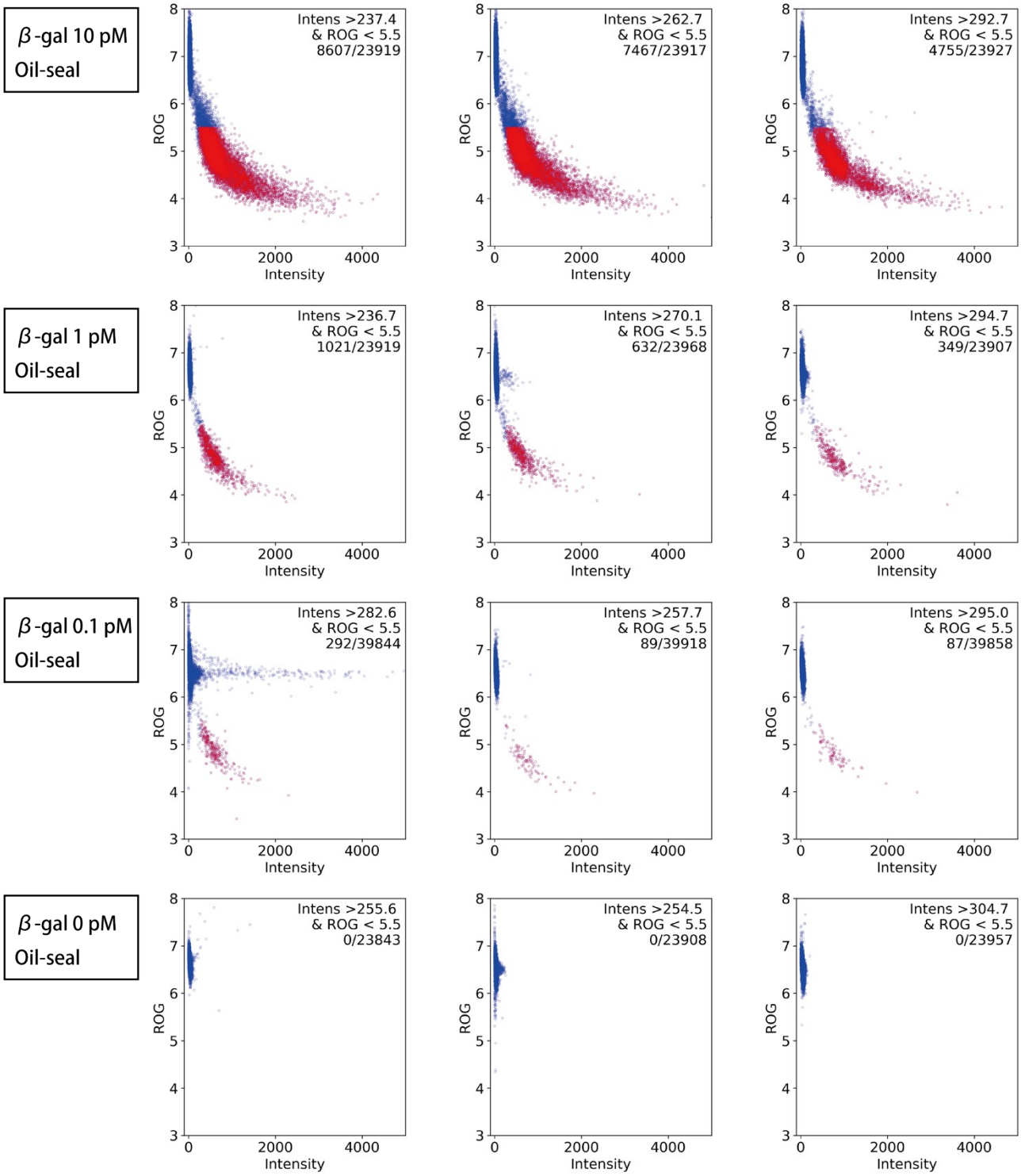
Supplementary Fig. 1 Additional details of sealing procedure. (a) Roller used for sealing of PITAT. To concentrate force, strips of adhesive tape (Kapton double sided tape is used without removing protective film on one side. Width = 5 mm, total thickness ~0.2 mm) were attached to a plastic cylinder (Falcon 10 mL disposable pipette, Corning, NY, USA) (b) Schematic image of manual roller sealing process. Green rectangles are the adhesive tapes. As shown in the scheme, up to two samples can be applied pressure at the same time. The total contact area of cylinder and device was about 1 mm × 5 mm × 2 = 10 mm^2. (c) A modified PITAT setup to prevent assay mix leakage, which is used for the influenza virus assays.The orange reactangles are the Kapton double-sided tapes.



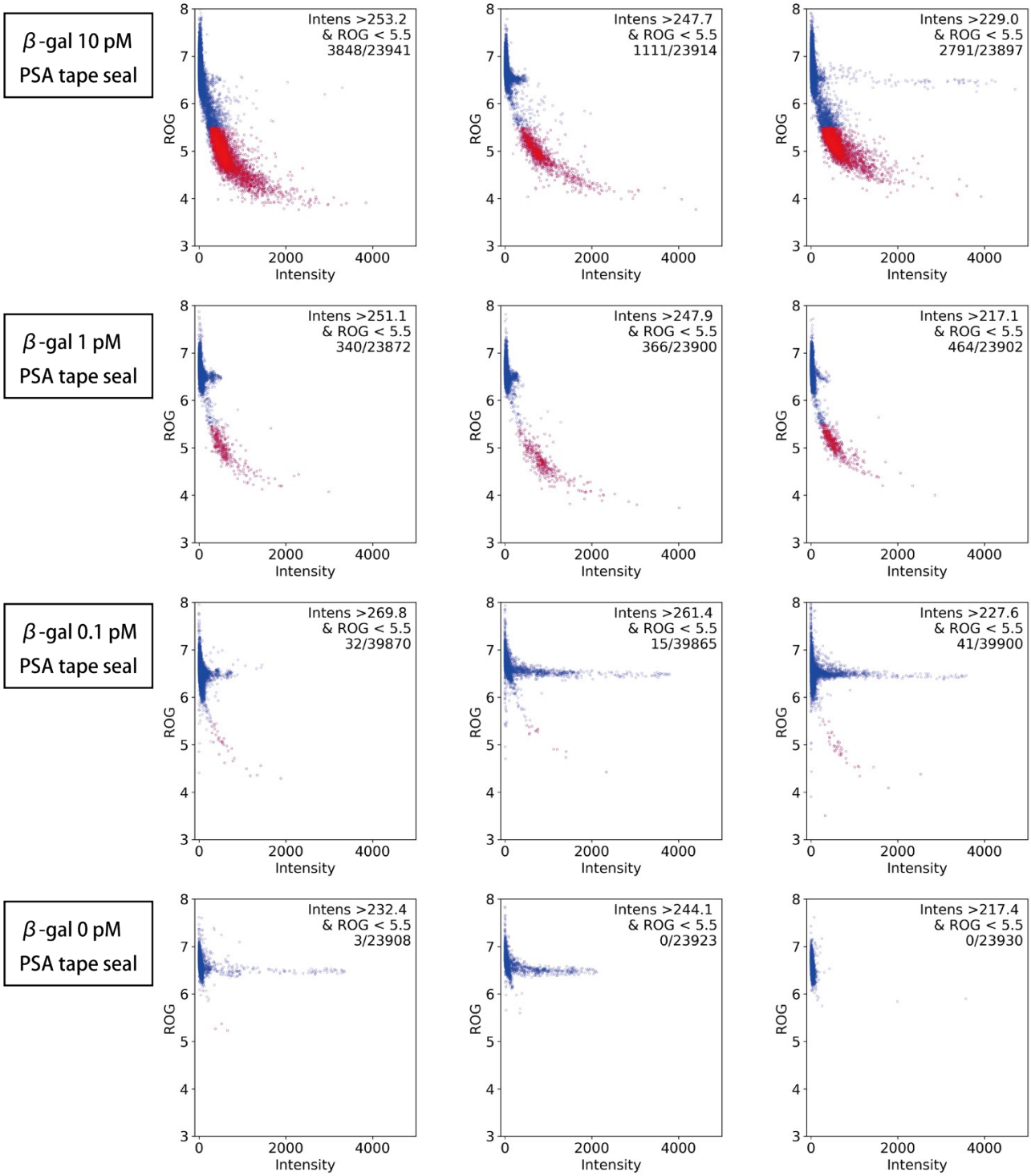
Supplementary Fig. 2 PITAT performed by a novice and an expert. (a–c) A novice of PITAT (volunteer 1st year graduate school student of biochemistry, but had no experience of PITAT) was asked to perform PITAT sealing procedure using 5 μM AF647 solution and 145RN tape. For the first (a) and second (b) trials, the volunteer was provided with an overview of the PITAT method, but no advice about the amount of force was given. Much of the pressure-applied region remained unsealed, and many reactors are interconnected. This is probably because the volunteer was afraid of breaking the device, and the force applied during the rolling process was too low. Then, the volunteer was advised to apply stronger pressure and allowed to watch expert performing the PITAT sealing process. In the third trial (c), the volunteer could seal the chambers as good as the expert. (d) PITAT sealing performed by an expert, who has more than one year of experience. The insets show the magnified views of regions marked by yellow squares. The scale of each image is different, but the scale of inset images are the same. Scale bar = 50 μm.



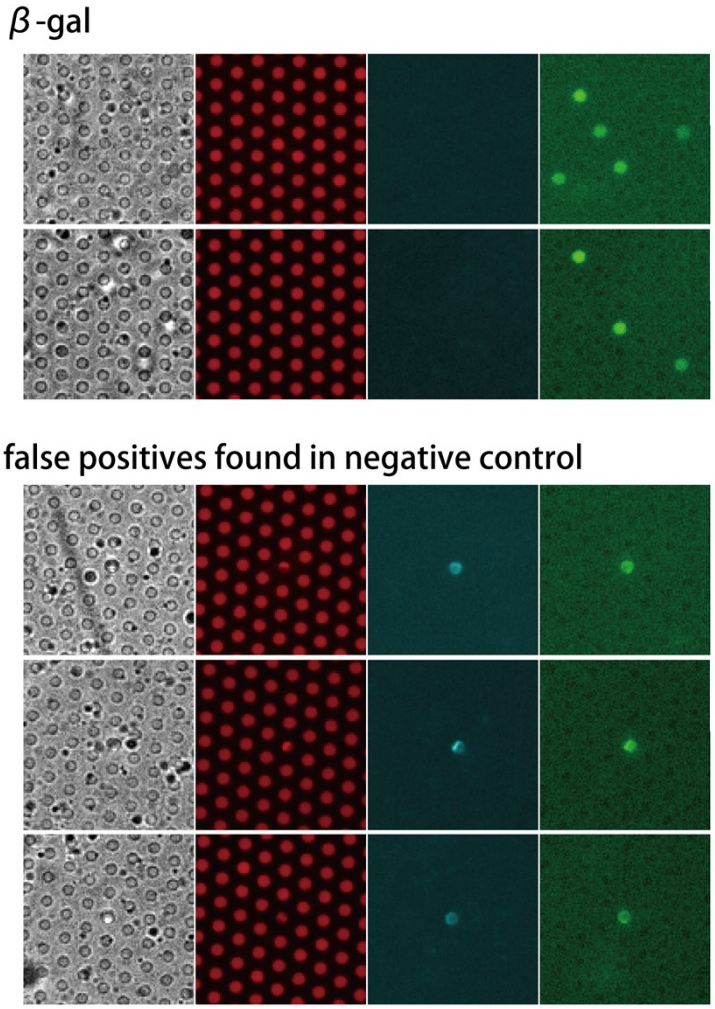
Supplementary Fig. 3 β-gal assay by rubber adhesive tape with plasticizers and in-house prepared acrylic/silicone adhesive tapes. The assay solutions were same as Fig. 3, except that final concentration of β-gal used was 100 pM. The contrasts of AF647 and Fluorescein images (2nd and 3rd columns from the left) are adjusted to the same level as Fig. 4a, and if this gives too bright or too dark images, contrast-enhanced image of fluorescein channel is shown in the 4th column (a, b) Rubber adhesive containing various concentrations of naphtene (a) and mineral (b) oils. (c) Acrylic adhesive prepared in-house from monomers. (d) Silicone adhesive prepared in-house from commercially available functionalized silicone polymers. Vinyl-terminated polydimethylsiloxane (PDMS) of different molecular weights were used as the main elastomer. Scale bar = 50 μm.



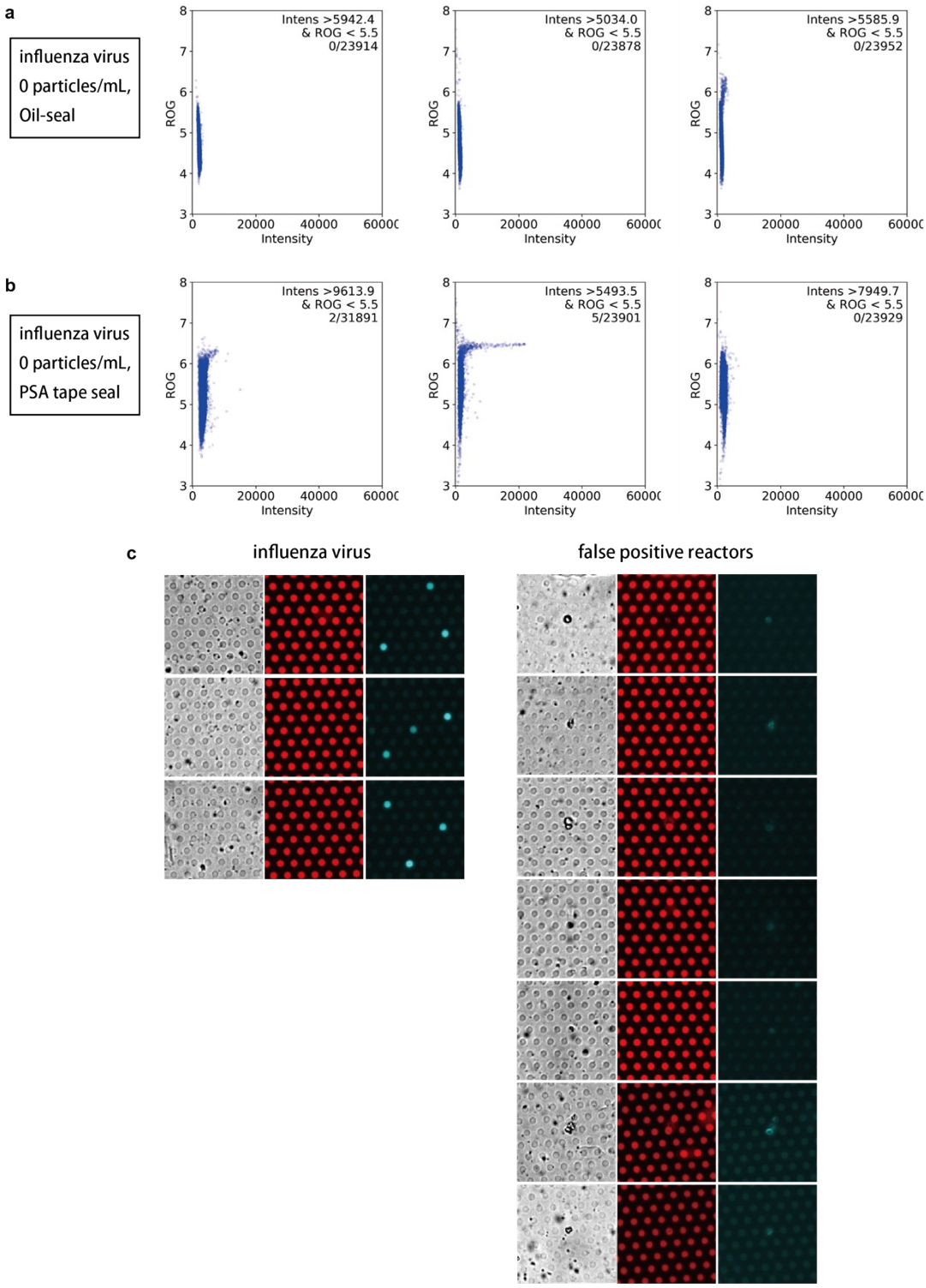
Supplementary Fig. 4 Detailed single cell data for β-gal assay using conventional oil sealing. The vertical axis is the radius of gyration of the cropped 15 px square image of a reactor. The horizontal axis is the intensity of the reactor. Three individual experiments are shown for each condition. Blue and red circles represent negative and positive single reactors, respectively. Threshold values are shown in upper-right corner of each panel. Related to Figure 4.



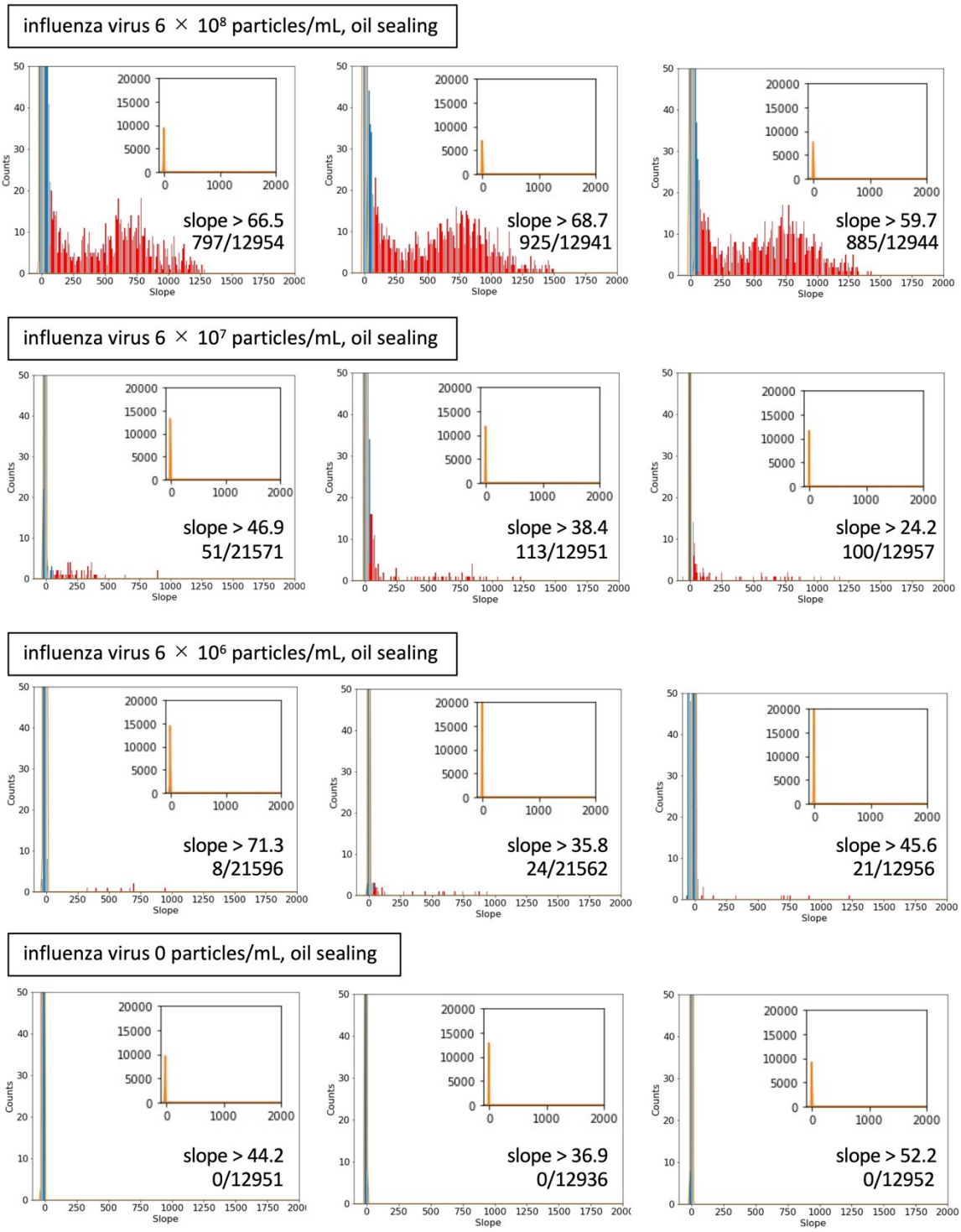
Supplementary Fig. 5 Detailed single cell data for β-gal assay using PSA tape sealing. Related to Figure 4.



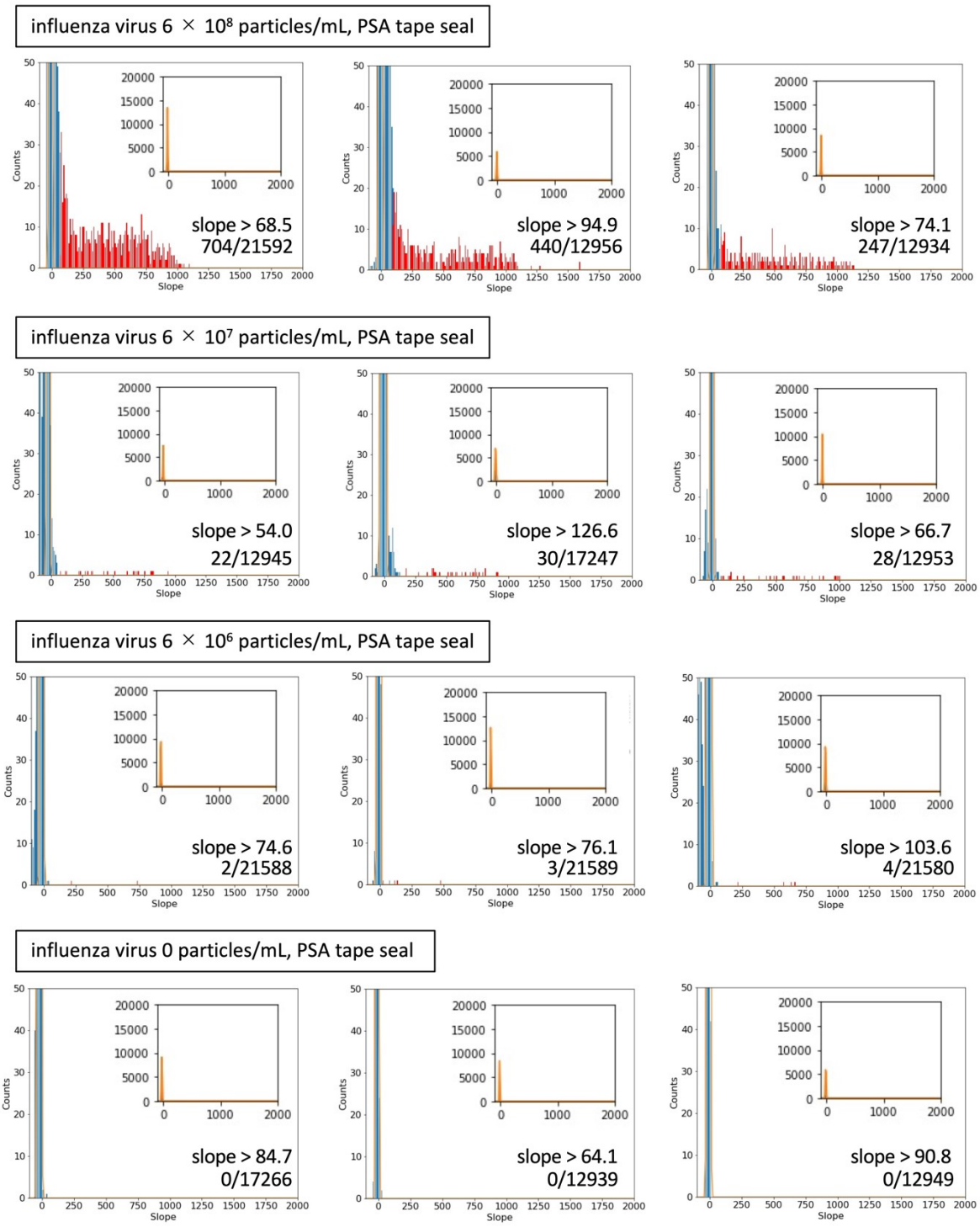
Supplementary Fig. 6 True and false positives in the β-gal assay. Representative true positive reactors in 1 pM β-gal sample (top) and 3 false positive reactors found in the negative control sample (bottom). From left to right: bright field channel (gray), AF647 channel (red), 4MU channel (cyan), fluorescein channel (green). Note that in the 4MU channel, no fluorescence was observed for true positive reactors but autofluorescence is observed for false positive reactors. Related to Figure 4 and Supplementary Figure 5.



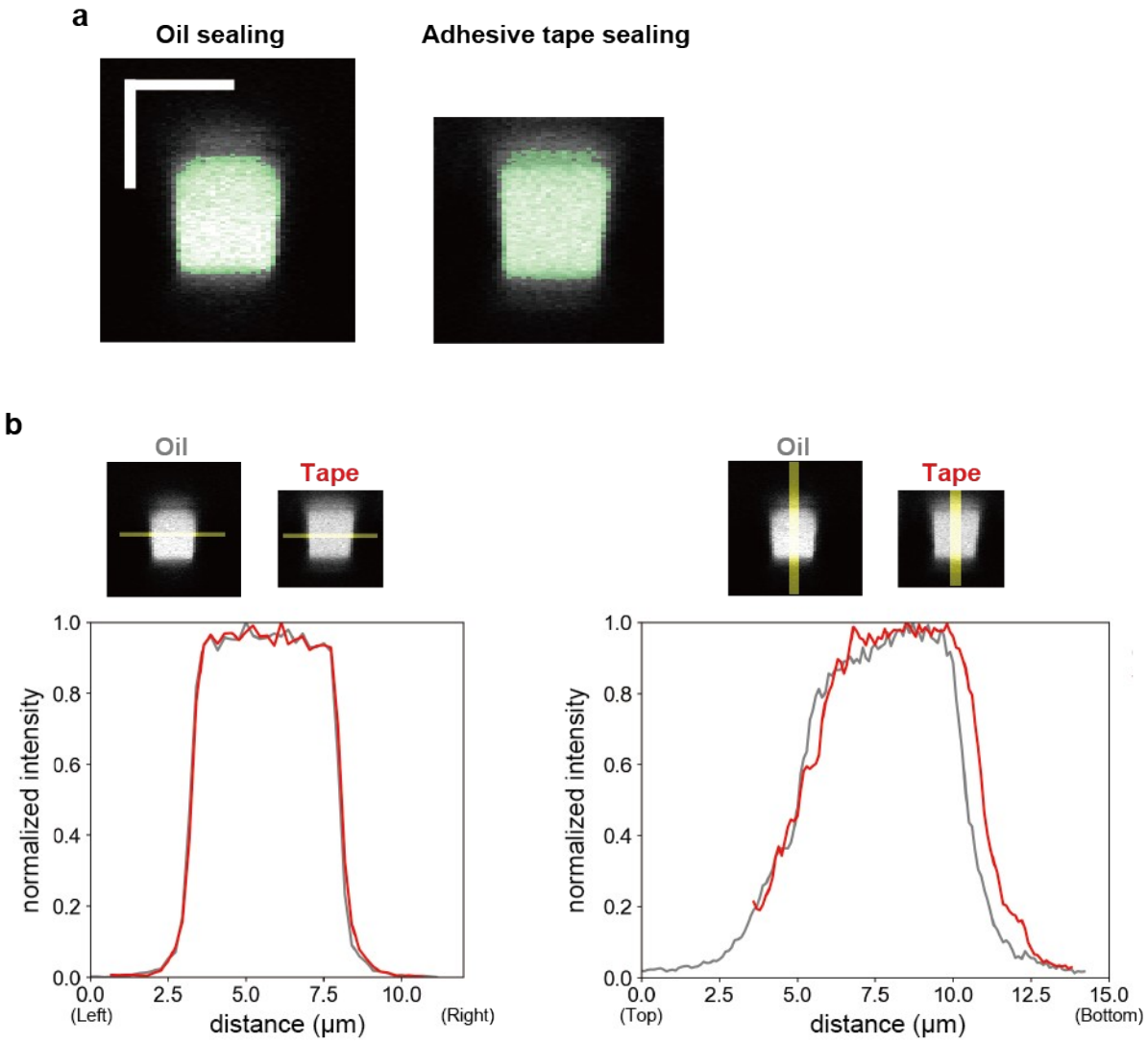
Supplementary Fig. 7 Analysis of influenza digital counting by reactor intensity. (a, b) Results of negative control samples prepared by oil-sealing (a) and PITAT (b). Analysis was done in the same way as Supplementary Fig. 4 and 5. (c) Representative positive and false-positive images of PSA tape-sealed reactors found in influenza digital counting assay. (c, left) Representative virus-positive reactors in 6.0×10^8 particles/mL influenza virus sample. (c, right) All 7 reactors with autofluorescence found in negative control samples of (b). From left to right columns: bright field channel (gray), AF647 channel (red), 4MU channel (cyan).



Supplementary Fig. 8 Detailed data for digital influenza virus counting assay using conventional oil sealing method. Histogram of fluorescence increase rate is plotted for each set of experiment. Three individual experiments are shown for each condition. Blue and red columns indicate negative and positive data points, respectively. Threshold slope value and numbers of positive/total reactors are shown for each experiment. Orange line is the Gaussian fitting result of the histogram. (Inset) Zoomed-out histogram and Gaussian fitting result of the same data. Related to Figure 5.



Supplementary Fig. 9 Detailed data for digital influenza virus counting assay using PSA tape sealing. Related to Figure 5.



Supplementary Fig. 10 Confocal scanning microscope image of reactors sealed by PITAT and conventional oil sealing. Fluorescein solution was sealed in the femtoliter reactors, and imaged by confocal microscopy. (a) x–z plane cross section of a single reactor. Region of signal intensity larger than half maximum is indicated by green. (b) Quantification of signal intensity along horizontal (left) and vertical (right) line.

Supplementary Text 1. Details of in-house preparation of various PSA tapes.

All rubber adhesive solutions, except for "IR2200 & HA085" adhesive and those with plasticizing oils, were prepared by mixing main elastomer, tackifier and toluene by 1 : 1 : 13 (by weight). "IR2200 & HA085" adhesive solution was prepared by mixing IR2200, HA085 and toluene by 1 : 0.33 : 13. Rubber adhesive solutions were then dropped on polyimide film, and heated at 140ºC for 60 minutes.

KR-100 adhesive solution was prepared by mixing KR-100, benzoyl peroxide (BPO) and toluene by 100 : 2 : 400. KR-130 adhesive solution was prepared by mixing KR-130, BPO and toluene by 100 : 2 : 400. SH4280PSA adhesive solution was prepared by mixing SH4280PSA, BPO and toluene by 100 : 2 : 400. SD4580PSA adhesive solution was prepared by mixing SD4580PSA, SRX212 and toluene by 100 : 0.9 : 300. These adhesive solutions were then dropped on polyimide film, heated at 90ºC for 5 minutes and then at 200ºC for 5 minutes.

KR-3700 adhesive solution was prepared by mixing KR-3700, CAT-PL-50T and toluene by 100 : 0.3 : 200. KR-3704 adhesive solution was prepared by mixing KR-3704, CAT-PL-50T and toluene by 100 : 0.5 : 300. X-40-3237 adhesive solution was prepared by mixing X-40-3237, X-92-122, CAT-PL-50T and toluene by 100 : 0.5 : 1 : 200. These adhesive solutions were then dropped on polyimide film, and heated at 140ºC for 10 minutes.

BPS5160 adhesive solution was prepared by mixing BPS5160 and toluene by 100 : 100. BPS5213K adhesive solution was prepared by mixing BPS5213K, BHS8515 and toluene by 100 : 2 : 100. BPS5296 adhesive solution was prepared by mixing BPS5296, BHS8515 and toluene by 100 : 2 : 100. BPS5375 adhesive solution was prepared by mixing BPS5375, BHS8515 and toluene by 100 : 1.7 : 200. KP-1282 adhesive solution was prepared by mixing KP-1282, CK-101 and toluene by 100 : 2 : 200. KP-1405 adhesive solution was prepared by mixing KP-1405, CK-102 and toluene by 100 : 1 : 200. KP-2369 adhesive solution was prepared by mixing KP-2369, CK-131 and toluene by 100 : 2 : 100. PE-121 adhesive solution was prepared by mixing PE-121, CK-101 and toluene by 100 : 3 : 100. SH-101 adhesive solution was prepared by mixing SH-101 and T-501B by 100 : 4. SH-205 adhesive solution was prepared by mixing SH-205 and T-501B by 100 : 3. These acrylic or urethane adhesive solutions were then dropped on polyimide film, and heated at 140ºC for 10 minutes.

Rubber adhesive with plasticizing oils in Supplementary Fig. 3 was prepared by mixing elastomer QTC3421, tackifier HA085, toluene and either naphtene oil (NR-26) or mineral oil (M5904). Adhesive with 12.5%, 25% and 50% oil was prepared by mixing QTC3421, HA085, oil (either NR-26 or M5904) and toluene by 0.438 : 0.438 : 0.125 : 13, 0.375 : 0.375 : 0.25 : 13 and 0.25 : 0.25 : 0.5 : 13. This mixture was dropped on polypropylene film [autoclave bag, nerbe plus, Winsen (Luhe), Germany], and heated at 140ºC for 1 h.

In-house prepared acrylic adhesive was polymerized from monomers by a modified method of Ref 47. BA 4.5 g, EHA 5.1 g, AA 0.3 g, HEMA 0.02 g and ethyl acetate 8.17 g was mixed well. This mixture was heated at 80 ºC in nitrogen gas. BPO 13.3 mg was dissolved in 1 g ethyl acetate and added to the mixture dropwise for ~1 h. The mixture was kept at 80 ºC for 7 h, and then cooled to room temperature. 1 part of crosslinker CK-101 was added to 100 part (total weight) of this solution, and this was dropped on polyimide film, and heated at 140ºC for 10 minutes.

In-house prepared silicone adhesive was polymerized from commercially availabe functionalized silicone polymer reagents by a modified method of Ref. 48. Vinyl-terminated polydimethylsiloxane (PDMS) 4.3 g, MQ resin SQO-299 5.7 g and toluene 16.7 g was mixed. The product numbers of vinyl-terminated PDMS used here was DMS-V46 (MW = 117,000) for in-house silicone adhesive (IHSA) #1, DMS-V41 (MW = 62,700) for IHSA #2, DMS-V31 (MW = 28,000) for IHSA #3 and DMS-V21 (MW = 6000) for IHSA #4. The mixture was heated at 110ºC for 3h, and cooled to room temperature. 100 part (dry weight) of this mixture was mixed with 1.2 part of polydimethylhydrosiloxane (HMS-993) and 1 part (total weight) of platinum catalyst (CAT-PL-50T). Then, the mixture was dropped on polyimide film, and heated at 130ºC for 1 minute.

Supplementary Text 2. Details of image analysis of single-molecule β-gal assay

For automated image analysis of single-molecule β-gal assay, ideally we wanted to count the positive reactors while excluding false positives due to (auto)fluorescence from interconnected reactors, impurities or air bubbles. In order to do so, we wrote an ImageJ/Fiji macro. First, local maxima positions were detected from AF647 channel image. These positions are the candidates for positive femtoliter reactors. Next, median filter (radius 1 px), mean filter (radius 1 px) and white top hat filter (square, radius 50 px) was applied to fluorescein channel image, and we obtained white-top-hat image. For each local maxima position, a circular region of interest (ROI, radius 1 px) was created. Mean intensity inside the ROI of the white-top-hat image was considered as the intensity (f_i) of the ith femtoliter reactor. Next, 15 px × 15 px region around each candidate position was cropped out from the white-top-hat image. Radius of gyration value of ith candidate reactor (ROG_i) was calculated for each of this cropped image by the following definition.

$$ROG_i = \sqrt{\frac{m_{20} + m_{02}}{m_{00}}}$$

where

$$m_{20} = \sum_{y=0}^{M-1} \sum_{x=0}^{N-1} \{x - (N-1)/2\}^2 g(x,y)$$

$$m_{02} = \sum_{y=0}^{M-1} \sum_{x=0}^{N-1} \{y - (M-1)/2\}^2 g(x,y)$$

$$m_{00} = \sum_{y=0}^{M-1} \sum_{x=0}^{N-1} g(x,y)$$

M and N is the height and width of the image in pixels (i.e. M=N=15 in this case), and $g(x, y)$ is the signal intensity at pixel position (x, y). A smaller value of ROG_i means that pixels with high intensity is concentrated close to the center of 15×15 cropped region. Larger impurities and dark negative chambers usually have larger ROG_i, because brighter pixels are more uniformly distributed in the cropped 15×15 image. In our automated analysis, reactors with intensity larger than a certain threshold value ($f_i > T_1$) and ROG_i smaller than 5.5 was classified as positive chambers. Intensity threshold T_1 was determined for each experiment as following,

$$T_1 = \mu_1 + 15\sigma_1,$$

where μ_1 and σ_1 are the mean and standard deviation values of gaussian distribution fitted to reactor intensity histogram. Since most reactors are negative, μ_1 and σ_1 are good estimates of mean and standard deviation of negative reactor signal levels. The concentration of β-gal in the reaction mix (C_g) was calculated as:

$$C_g = n_P / n_T / (V * N_A)$$

where n_P / n_T is the fraction of positive reactors from all observed reactors, V is the volume of single femtoliter reactor, and N_A is the Avogadro number. V was assumed as 2 × 2 × 3.14 × 3 ≈ 40 fL.

Supplementary Text 3. Details of image analysis of influenza virus counting assay

Data analysis of influenza virus counting assay was done by combination of custom-made ImageJ macro and home-made Python 3 program. To count the reactors, we used timelapse data for influenza virus assay to count reactors with increasing signal intensity. After correcting for stage drift, the positions of femtoliter reactors was detected in the same way as β-gal experiments using ImageJ macro. For each reactor, circular ROI (radius 5 px) was created, and the mean intensity in 4MU channel was measured. The results were exported in text file and opened in Python 3. Slopes of intensity change were calculated by linear-fitting of intensity change in each reactor from 10 to 30 min. Histogram of slope values were created. The high peak around zero was fitted to Gaussian distribution, and mean (μ_2) and standard deviation (σ_2) were obtained from fitting. Threshold slope value for positive reactor was determined as,

$$T_2 = \mu_2 + 15\sigma_2$$

The concentration of virus particles in the assay mix (C_v) was caluclated as,

$$C_v = n_P / n_T / V$$

The expected theoretical concentration of virus sample was determined by a separate result of particle counting using fluorinated oil sealing method, which was measured immediately after preparation of influenza virus solution.

Supplementary References

47. J. Watanabe, Jpn. Pat., JP3219461, 2001

48. T. Kobori, I. Moriizumi, M. Takarada and K. Isobe, US Pat., 5254644, 1993