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A microreactor sealing method using adhesive tape for digital bioassays†

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Digital assays using microreactors fabricated on solid substrates are useful for carrying out sensitive assays of infectious diseases and other biological tests. However, sealing of the microchambers using fluid oil is difficult for non-experts, and thus hinders the widespread use of digital microreactor assays. Here, we propose the physical isolation of tiny reactors with adhesive tape (PITAT) using simple, commercially available pressure-sensitive adhesive (PSA) tape as a separator of the microreactors. We confirmed that PSA tape can effectively seal the microreactors and prevent molecules from diffusing out. By testing several types of adhesive tape, we found that rubber-based adhesives are the most suitable for this purpose. In addition, we demonstrated that single-molecule enzyme assays can be successfully performed inside microreactors sealed with PSA tape. The results obtained using PITAT are quantitatively comparable to conventional oil sealing, although it is quick and cost-effective. Finally, we demonstrated that single-particle virus counting of the influenza virus can be achieved using PITAT. Collectively, our results suggest that PITAT may be suitable for use in the design of sensitive tests for infectious diseases at the point of care, where no sophisticated equipment or machines are available.

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Introduction

A digital assay is a method of quantifying molecules or microorganisms by partitioning the sample into numerous microreactors.[1–5] In digital assays, each reactor typically contains 0, 1, or a few analyte particles (enzyme molecules, sequences of nucleic acids, virions, or cells, *etc.*). The concentration of the analyte in the sample can be estimated by simply counting the number of positive reactors containing one or more particles. This is possible because the number of analyte particles in each reactor is governed by Poisson statistics when most reactors are negative and a fraction of positive reactors is small. Under such conditions, the concentration of the analyte can be easily calculated from the fraction of positive reactors. This feature of digital assays is particularly advantageous for application in point-of-care testing (POCT). In POCT, quick and affordable testing is desired. To meet such demands, assays are often needed to be performed with crude biological samples and low-cost detection devices. Therefore, the signal-to-noise (S/N) ratio of the assay is usually not very high. Even under such conditions, digital assays can provide precise quantification and can achieve a lower limit of detection (LOD), as long as we could properly distinguish between positive and negative reactors.

Usually, partitioning can be achieved using one of the two major methods, namely droplet microfluidics or physically isolated reactors. More specifically, in the context of droplet microfluidics, droplets of aqueous solution can be generated in non-reactive oils, and so it is possible to observe single-molecule reactions inside them.[6–9] Using specialized microfluidic setups such as T-junctions, the desired number of droplets with a highly uniform size can be generated.[10] Using microfluidics, digital droplet PCR (ddPCR)[11,12] and digital enzyme counting[13,14] techniques have been developed. In particular, the ddPCR technology is now available from many manufacturers and is potentially applicable for the quantification of target nucleic acid sequences in both clinical and research environments.[15,16] One of the drawbacks of these droplet-based microfluidics is that, in many cases, large and expensive instruments equipped with pumps and valves are required, which are not always available in POCT situations. In addition, care must be taken to avoid problems such as clogging of the microfluidic channels, and so these instruments are typically operated by trained experts.

On the other hand, physically isolated reactors are arrays of wells or chambers fabricated on materials such as glass, plastic,

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or silicone.[17–21] Following introduction of the sample reaction mixture into the microreactors of an array chip, each microreactor is separated, or sealed, by pushing away the excess reaction mixture. Such sealing can be performed using solid elastic gaskets,[21–28] immiscible oils,[29–35] or air.[36,37] In contrast to droplet microfluidics, the sealing and imaging of microreactors do not require sophisticated instrumentation, and so sealing is potentially more suitable for POCT.[38] However, sealing is a difficult process to perform for an unexperienced POCT user, and it often requires somewhat complicated structures. For example, sealing with a solid gasket requires additional structures to hold the microreactor array chip and gasket together, while sealing with oil or air usually requires building a flow channel to be created over the microreactor array chip in advance. To mass produce microreactor array chips at a low cost, one of the most common methods is injection molding. However, to build a hollow channel structure by injection molding, the channel part and the microreactor array chip part must be manufactured separately. These parts must be assembled afterwards, leading to increased labor and higher overall manufacturing costs. In addition, oil sealing requires handling of small amounts of liquids, which is not an easy process for standard POCT users to carry out. Thus, to avoid liquid handling by non-experts, the use of a fully automatic liquid handling system is desirable (for example, Simoa by Quanterix).[39] However, such systems are bulky and expensive, and often require maintenance by experts, which is generally unacceptable in small clinics and patients' homes. These difficulties therefore hamper the use of physically-isolated array-type reactors for developing cost-effective, user-friendly POCT kits, and so a simpler method to seal microreactors is desired.

Pressure-sensitive adhesive (PSA) tape consists of an adhesive coated on a backing material (*e.g.*, plastic film), and it is widely used to hold things together. For example, in the field of microfluidics, it is not uncommon to use single- or double-sided PSA tape to assemble microfluidic devices.[29,34,40] However, adhesive tape has not been employed for sealing of microreactor arrays. This can be accounted for by considering that adhesives of commercially available PSA tape are a mixture of various materials and additives that exhibit a range of properties. For example, the main elastomer used for the formulation of pressure-sensitive adhesives is usually either natural or synthetic rubber, an acrylic polymer, a silicone polymer, or a urethane polymer. Rubber and silicone elastomers usually do not possess a tack (sticky surface), and so the formulator must add a tackifier to address this issue. In the case of rubber adhesives, the tackifier is usually resin (a mixture of organic compounds) derived from plants or petroleum. In addition, it is normal for adhesive tape to contain various additives that are added during the manufacturing process to achieve different purposes. These additives include plasticizers, cross-linkers, and antioxidants, among others.[41] The detailed composition of the adhesive of commercially available PSA tape is often not disclosed. Biochemists might want to avoid direct contact of such adhesives with their reaction systems. On the other hand, it takes too much work for a researcher in this field to make PSA tape by themselves. These problems of the PSA tape may therefore account for the fact that it has been generally considered unsuitable for direct sealing of microreactors.

Thus, we herein report the development of physical isolation of tiny reactors with adhesive tape (PITAT), a novel, easy, and cost-effective method to seal microreactors for high-sensitivity detection and accurate quantification of proteins and viruses. In PITAT, the microreactors are sealed to prevent leakage using commercially available pressure-sensitive adhesive tape. The quantification accuracy of digital assays using PITAT, as well as the assay cost and time, is compared with that of a previous microreactor sealing method using an immiscible oil. Furthermore, we propose that this novel method will be useful in developing inexpensive and easy-to-use POCT kits for the diagnosis of infectious and other diseases.

Experimental

Cells and viruses

The influenza virus (H1N1/Puerto Rico/8/1934) was cultured using MDCK (ATCC, CCL-34) cells as previously described.[42] The influenza virus and MDCK cells were distributed from the Kawaoka Laboratory, Institute of Medical Science, University of Tokyo. The cell culture supernatant containing the virus was collected, aliquoted, and stored at −80 °C.

Preparation of the femtoliter reactor array device

The femtoliter reactor array devices were prepared as previously described.[40] Micropatterns were fabricated on a 24 × 32 coverslip (no. 1, Matsunami Glass, Japan). First, coverslips were cleaned by sonication for 30 min in 8N KOH solution, washed with MilliQ water and dried. This glass was soaked in 0.05 vol% (3-aminopropyl)triethoxysilane in ethanol (Sigma-Aldrich) for 1 hour, rinsed with ultrapure water and dried. Perfluoropolymer CYTOP (CTL-816AP, AGC) was spin-coated on the coverslip at 3400 rpm for 30 s, followed by heating at 80 °C for 30 min and 200 °C for 1 hour. Positive photoresist AZ P4903 (Merck) was spin-coated on top of the CYTOP layer at 7500 rpm for 60 s, followed by heating at 110 °C for 5 min. After 30 minutes of spontaneous rehydration, the photoresist was exposed to UV through a chrome-coated photomask patterned with 3 μm diameter pores and a pitch of 9 μm, using a mask aligner (BA100it, Nanometric Technology). The photoresist was developed in an AZ 300 MIF developer (Merck). The patterned photoresist layer functions as a mask for the subsequent etching process. The CYTOP layer with no photoresist covering was etched with O_2 plasma using a reactive ion etching system (RIE-10NR, Samco). Then the device was rinsed sequentially in acetone, 2-propanol and deionized water. The final size of each individual reactor, as determined using a scanning microscope (VK-X200, Keyence, Japan), was 4 μm in diameter

and 3 μm in depth. The volume of a single reactor was therefore ~40 fL.

Droplet array formation by sealing with oil

The flow cell for oil sealing was created as previously described,[40] with some minor changes. Instead of a piece of polydimethylsiloxane (PDMS), we used a piece of thick glass (the "upper device") to create the flow cell. This upper device consists of a piece of 5 mm-thick planar glass with drilled holes as the inlets and outlets of the flow cell. Although the initial cost for the upper device is high compared to the PDMS block, the glass upper device can be used virtually infinite times by cleaning it after each experiment (Table S1†). To increase the hydrophobicity of the surface, M-type CYTOP (CTL-809M, AGC, Japan) was spin-coated on one side of the upper device at 1000 rpm for 30 s and then baked at 180 °C for 1 h.

For each experiment, a femtoliter reactor array device and an upper device were assembled using double-sided tape (7602 #25, Teraoka Seisakusho, Japan, total thickness 85 μm), which was cut into the shape of a frame in advance. The reaction mixture (15 μL for β-galactosidase (β-gal) or influenza; see below) was then injected into the flow cell. To remove air from the femtoliter reactors, the device was chilled by placing it on an aluminum block on ice for ~1 min and then returned to 25 °C.[40] After introducing the reaction mixture into the reactor, perfluorocarbon oil (Fluorinert FC40, 3M, MI, USA) was injected into the flow cell to push away the excess reaction mixture and separate the reactors. FC40 was subsequently exchanged with non-volatile perfluoroether oil (Fomblin Y-25, Solvay, Belgium) prior to imaging.[40] Since this last oil exchange step is optional, the time for this step was not included in the sample preparation time described in the text. After the experiment, the upper device was cleaned by washing sequentially in acetone, fluorinated solvent Asahiklin AC2000 (AGC), and 8N KOH solution.

Droplet array formation by sealing with PSA tape

To perform the β-gal single molecule assay using the PSA tape sealing method, an aliquot (~3 μL) of the reaction mixture was initially dropped on the femtoliter reactor array device. A piece of commercially available PSA packaging tape (145RN, 3M) was gently placed on top of the droplet. The device was then chilled on an aluminum block placed on ice for 1 min. After returning the device to 25 °C, pressure was applied using a plastic cylinder roller to seal each reactor (Fig. S1a and b†). The cylinder was rolled over the tape at a force of ~3–5 kgf. To concentrate force, strips of adhesive tape were attached to the cylinder, so the total area of contact between the cylinder and the device was approximately 10 mm^2, and the pressure at the contact point was about ~4 MPa. Sliding of the cylinder without rolling should be avoided for uniform sealing. This rolling process was repeated 2–5 times to push away any excess liquid to give the reactors separated by the adhesive of the PSA tape (Fig. 1b).

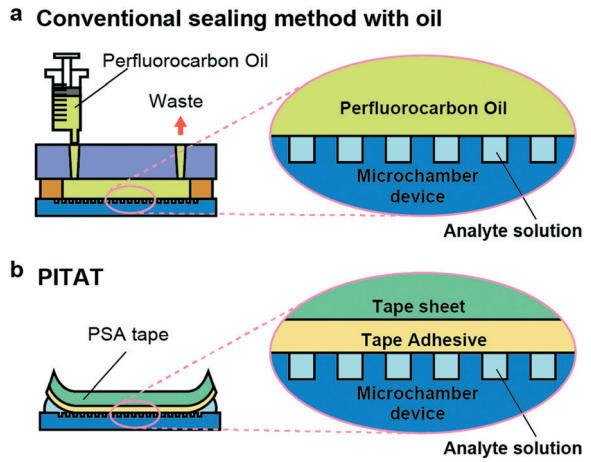

Fig. 1 Schematic representations of the conventional oil sealing (a) and PITAT (b) procedures.

For the influenza virus counting assay using the PSA tape sealing method, it was necessary to completely confine the reaction mixture inside the device to prevent leakage since live infectious virus particles were used for our assay. Therefore, the sealing process was slightly modified (Fig. S1c†), wherein a square frame of double-sided Kapton adhesive tape was placed on the femtoliter reactor array device. Subsequently, an aliquot (~3 μL) of the reaction mixture was dropped inside this frame, and PSA tape was placed gently on top of the droplet. At this point, the PSA tape and double-sided Kapton tape frame were allowed to stick together, but the reactors were not sealed. The device was then chilled on an aluminum block placed on ice for 1 min, pressure was applied to the center of the PSA tape to seal the reactors, and excess liquid was pushed away from the center of the device. As a result of this modified procedure, even after sealing, the excess infectious reaction mixture was contained inside the sealed space of the device.

Leak-check of the microreactors by photobleaching

To check for leakage from the microreactors sealed with PSA tape, Cy3 (Q13108, GE Healthcare, IL, USA) was dissolved in buffer 1 (1 M 3-morpholinopropanesulfonic acid, 4 mM $MgCl_2$, pH 7.0) to prepare a 1.8 μM Cy3 solution. This solution was sealed in the microreactors using 145RN packaging tape, and confocal microscopy (A1R, Nikon, Japan) was used for imaging and photobleaching of the reactors. The imaging interval after photobleaching was 10 s.

Testing of different PSA tape formulations

Different formulations of pressure-sensitive adhesive tape were prepared in-house. The reagents used for preparing the adhesive tape are summarized in Table S1.† For adhesives of the rubber category, the elastomer and tackifier were dissolved in toluene to obtain an adhesive solution. For the silicone, acrylic, and urethane adhesives, the main adhesive reagent was mixed with a crosslinking reagent according to

the manufacturer's instructions or recommendations. The solution was then dropped onto a piece of polyimide film (100H-A4, Toray-DuPont, Japan) and allowed to spread spontaneously. The film was then heated for 10–60 min, and subsequently cooled. The obtained tape was incubated at 25 °C for 1–7 d prior to testing. For details of the different tape preparations, please see Text S1.†

The PSA tape was tested in the same way as the single-molecule β-gal assay described below. The concentration of β-gal was fixed at 1 pM unless otherwise stated, and images were taken 10 min after sealing. For each acquired image, approximately 3000 reactors were observed by the naked eye, and the number of positive reactors was recorded.

Single molecule enzymatic assay of β-galactosidase

The β-gal assay was performed as previously described[33] with some modifications. β-Gal (10745731001) and fluorescein-di-β-galactopyranoside (FDG, ab273643) were purchased from Merck and Abcam (UK), respectively. The concentration of β-gal was determined by measuring the absorbance at 280 nm (ε = 241 590 M^{-1} cm^{-1}).[43] Alexa Fluor 647 (AF647) dye stock solution was prepared by dissolving Alexa Fluor 647 C2 maleimide (Thermo Fisher Scientific, MA, USA) in water at a concentration of 2 mM. The AF647 stock solution was then incubated at 25 °C for 3 h. The incubation step was to reduce the reactivity of the maleimide group, which is unnecessary for our experiments. To prepare the β-gal reaction mixture, 1 mM FDG, 5 μM AF647, and various concentrations of β-gal were dissolved in buffer 1. After sealing the reaction mixture using either the oil sealing method or the PSA tape sealing method, the device was maintained at 25 °C for 20 min. Bright field and epifluorescence images were then obtained using an IX83 microscope (Olympus, Japan) equipped with a 20× objective lens and a sCMOS camera (Neo, Andor, UK). An Xcite XYLIS (Excelitas Technologies, MA, USA) was used as the light source for epifluorescence. The filter units for AF647 imaging were composed of an FF02-628/40 excitation filter (Semrock, NY, USA), an FF660-Di02 dichroic mirror (Semrock), and an FF01-692/40 emission filter (Semrock). The filter units for the imaging of fluorescein (the product of the β-gal reaction) were composed of a 460-480GFP excitation filter (Olympus), a 490GFP dichroic mirror (Olympus), and a 495-540GFP emission filter (Olympus). The acquired images were analyzed using ImageJ/Fiji macro (see Text S2† for details).

Digital influenza virus counting

A digital influenza assay was performed as previously described[44] with some modifications. More specifically, the reaction mixture [1 mM 4-methylumbelliferyl-*N*-acetylneuraminic acid (4MU-NANA, Merck), 5 μM AF647, and influenza virus in buffer 2 (1 M diethanolamine–HCl, 4 mM $CaCl_2$, pH 9.0)] was sealed inside the femtoliter reaction array device by either the oil sealing method or PSA tape sealing method. After sealing, the device was placed on a microscope system at 25 °C. Epifluorescence images were then recorded at 10, 20, and 30 min after sealing, wherein the microscope setup was the same as described for the β-gal experiment. The filter units for the imaging of 4-methylumbelliferon (4MU) were composed of an FF01-377/50 excitation filter (Semrock), an FF409-Di03 dichroic mirror (Semrock), and an FF02-477/60 emission filter (Semrock). Data analysis for the increase in fluorescence inside the reactors was performed using a combination of custom-made ImageJ macro and the Python 3 program (see Text S3† for details).

Volume measurement of sealed reactors

500 μM fluorescein solution was sealed in the femtoliter reactor array device, using either oil-based or PSA tape-based sealing. The reactors were imaged using a confocal microscope (Leica Microsystems, Wetzlar, Germany) equipped with a white laser.

Results and discussion

Sealing of the microreactors using commercially available PSA tape

In many digital assays using a physical microchamber, fluorinated oil (perfluorocarbon, hydrofluoroether, *etc.*) is commonly used as a sealing material to compartmentalize the assay mixture in microreactors. One of the simplest ways to achieve fluorinated oil sealing is by using a piece of thick cover glass with inlet and outlet holes (*i.e.*, the "upper device"), together with double-sided adhesive tape[38,44,45] (Fig. 1a). A variation of this method uses a piece of PDMS with channels and holes instead of glass.[40] In either case, creating a flow cell structure over the microreactors is mandatory for the efficient exchange of water and fluorinated oil.

Here, we developed PITAT, a simple digital assay that does not require flow cells to be built over the microreactors. In PITAT, fluorinated oil was not used, and the femtoliter reactors were isolated using commercially available PSA tape (Fig. 1b). More specifically, the reaction mixture was dropped onto the femtoliter reactor array device and a piece of rubber-based PSA packaging tape (Cat#: 145RN, 3M) was gently placed on top of the droplet, causing the droplet to deform and spread over the reactor array. Pressure was then applied to the PSA tape using a roller so that it was pushed against the upper surface of the femtoliter reactor array device. Pressing with a roller several times pushed the excess liquid away, and the reaction mixtures in the different femtoliter reactors were isolated from one another by the adhesive layer of the PSA tape (Fig. 2a). We confirmed successful sealing by using a fluorescent dye solution, which was successfully isolated in separate reactors (Fig. 2b). In addition, we confirmed separation of the reactors by fluorescence recovery after photobleaching (FRAP). More specifically, the fluorescence in the photobleached reactors did not return for at least 30 min, suggesting that each reactor was well separated by the PSA tape (Fig. 2c).

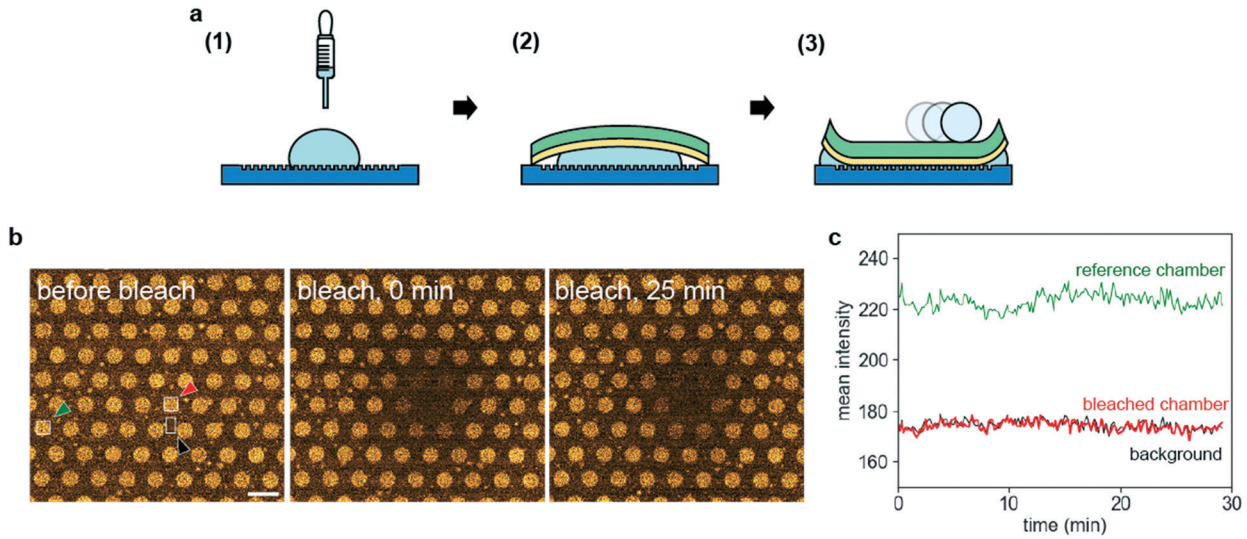

Fig. 2 Microreactor sealing by the PITAT method. (a) Schematic representations of the PITAT procedures. (1) A droplet of the assay mixture (light blue) is created on a microreactor array chip (blue). (2) A piece of PSA tape (green and yellow) is placed on top of the droplet. (3) Pressure is applied using a roller and the microreactors are sealed. (b and c) Leak check of the PSA tape-sealed reactors by FRAP. (b) Confocal microscopy images of the Cy3 dye solution sealed inside the femtoliter reactors using PSA tape. From left to right, the images were acquired before photobleaching, immediately after the 5 min photobleaching step, and 25 min after photobleaching. The arrowheads and white rectangles indicate the regions measured in (c). Scale bar = 10 μm. (c) Time-lapse change of the fluorescence intensity of the reactors immediately after photobleaching.

The cost and time required for PITAT should be noted. The cost of the PITAT sealing technique is <0.01 US dollar (USD) per assay. This is in contrast with the fluorinated oil sealing method, where the total cost of oil itself and materials for construction of the flow cell is significantly higher (*i.e.*, at least ~0.3 USD per assay) (Table S1†). Therefore, the final cost of the assay is expected to be significantly lower in PITAT. The sealing procedure is simple, and a novice of PITAT could seal almost as good as experts after 3 trials (Fig. S2†). When performed by trained experts, the total time to seal was ~1–2 min for PITAT. In the case of conventional fluorinated oil sealing, the time to seal was ~4 min, including the time required for flow cell construction. Thus, PITAT is not only inexpensive but also a relatively quick method to achieve the sealing of femtoliter reactors. In addition, since PITAT is very simple, it can be performed without any special equipment, except for the femtoliter reactor array itself.

Performances of different adhesives for PSA tape sealing of the microreactors

Next, we explored which type of PSA tape was the most suitable for PITAT. For this purpose, the single molecule β-galactosidase (β-gal) assay was selected as a model experiment. In this assay, if the β-gal molecule is present in the reactor, substrate FDG is hydrolysed, fluorescent dye fluorescein is produced and the reactor becomes fluorescent. Here, several types of PSA tape were prepared in-house and were used to seal the β-gal single-molecule assay mixtures inside the femtoliter reactors. A fixed concentration of β-gal (1 pM) was used. By counting the number of positive reactors, we investigated the PSA tape that yielded the most reliable results. Initially, we tested several kinds of rubber adhesives because the adhesive of 145RN packaging tape used in the FRAP experiment was based on synthetic rubber (Table S2†). Typically, rubber adhesives consist of a main elastomer and a tackifier, and so different formulations of elastomer and tackifier mixtures were used and tested. As shown in Fig. 3a, the performances of the various rubber adhesive formulas were generally consistent, giving a positive reactor count of ~30–70% of the number of the fluorinated oil (FC40) sealing technique. This result suggests that differently formulated rubber adhesives perform similarly in sealing the femtoliter reactors for the single-molecule β-gal assay. We further tested if adding plasticizing oil (naphthenic or mineral oil) to rubber adhesives has an effect on the β-gal reaction. The effect of such oils was small even at high concentrations (Fig. S3a and b†), suggesting that these plasticizing oils can be added without any notable negative effects on single molecule assays.

Furthermore, we also tested other types of adhesives, namely silicone, acrylic, and urethane adhesives, in the same way (Fig. 3b). In this case, the results varied among the different adhesive types and formulations. Some silicone PSA tape materials performed moderately well for sealing the β-gal assay mixture, while others showed no fluorescence increase after sealing. The number of positive reactors was 0–50% of that obtained using the fluorinated oil sealing method, and so it was apparent that the silicone PSA tape materials did not outperform most rubber PSA tape materials. In contrast, when the β-gal assay mixture was sealed into a femtoliter reactor array using acrylic and urethane PSA tape materials, little to no reactors showed an

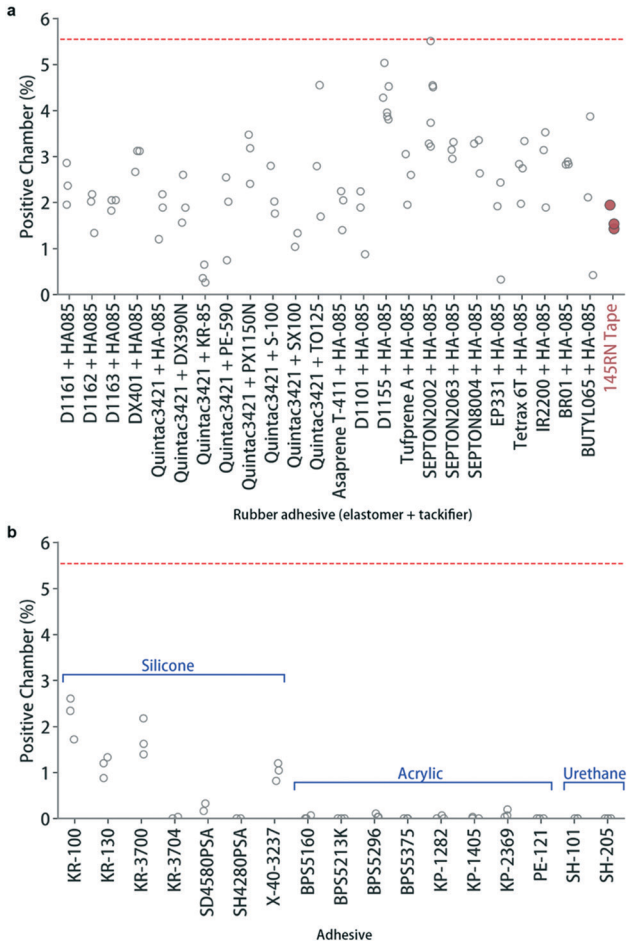

Fig. 3 The different types of adhesives tested for PITAT. (a) Formulations of the rubber-type adhesives prepared in-house and tested for the 1 pM β-gal assay, wherein the number of positive reactors was counted. The results obtained using the commercially available 145RN tape are also shown for reference (same data as presented in Fig. 4). The red horizontal dashed line indicates the average signal level in the oil-sealing experiments for the 1 pM β-gal assay. (b) Various silicone-, acrylic- and urethane-type adhesives were tested as in part (a). See Table S1 and Text S1† for details of the adhesive formulations.

increase in fluorescence. This suggests that acrylic and urethane PSA tape materials either inhibit the enzymatic reaction, inhibit the fluorescence of the fluorescent product, or allow the fluorogenic substrate to leak out of the reactor through the adhesive layer of the tape.

We could not find any previous work addressing the effects of adhesive composition on enzymatic reactions. In addition, since the silicone, acrylic, or urethane adhesives used herein are products of private companies and their detailed compositions are not disclosed, it is difficult to determine which component has the most effect on the signal level. However, it should be noted that we found adhesives of the same category to show similar results. In addition, we also tried to make silicone and acrylic PSA tape materials from acrylic monomers or silicone oligomers, so as to obtain these tape materials with completely known components. Unfortunately, the β-gal reaction was either not observed or only weakly observed in reactors sealed by our in-house prepared silicone and acrylic PSA tape materials (Fig. S3c and d†). For silicone adhesives, we tried several types of silicone oligomers with different molecular weights for preparation, but the difference was small (Fig. S3d†). Therefore, the basic properties of the elastomer itself may be responsible for the observed differences rather than any particular additive or difference of formula. Another possible explanation may be that, since we added a crosslinking reagent for the majority of silicone, acrylic, and urethane adhesives but not for the rubber adhesives, some trace amounts of the reactive crosslinking reagent present in the adhesive tape may impart a negative effect on the enzymatic reaction or the product fluorescence.

Overall, our results suggest that rubber adhesives are the most suitable adhesives for the single-molecule PITAT assay of β-gal. Note that in the following experiments, we used the commercially available 145RN tape for PITAT, instead of self-prepared rubber adhesives. Although some self-prepared rubber adhesives show better performance in Fig. 3a, we did not use them because we chose to avoid the labor-intensive PSA tape preparation step.

Single molecule digital enzyme assay by PITAT

We then performed single-molecule β-gal assays[33] using both the conventional oil-sealing assay and the PITAT assay to demonstrate the performance of our PITAT for an analytical chemistry application. Assay mixtures containing various concentrations of the β-gal enzyme and the fluorogenic substrate were sealed in a femtoliter reactor. If β-gal enzyme molecule(s) exist in the femtoliter reactor, substrate hydrolysis will be catalyzed to generate a fluorescent product, namely fluorescein. Here, a small fraction of the reactors showed fluorescence (Fig. 4a and Fig. S4 and S5†). The reason for the slightly higher background levels of oil-based sealing (Fig. 4a) is unknown, but this might be caused by fluorescein leakage from the reactors into the oil. According to Poisson statistics, only one β-gal enzyme molecule is expected to be present in the majority of these reactors. In our analysis, to automatically count the positive reactors using ImageJ macro, we used two criteria, namely the fluorescence intensity and the radius of gyration of each chamber (see Text S2† for details). The number of positive reactors per image was then converted to an enzyme concentration. Thus, different concentrations of β-gal solutions were measured using this single-molecule assay, and the theoretically and experimentally measured concentrations of β-gal were plotted, as shown in Fig. 4b. The results obtained by both methods agreed well with the theoretical concentrations of β-gal. On closer examination, PITAT yielded slightly lower β-gal enzyme concentrations compared to when the microreactors were sealed using fluorinated oil. The reason for this difference is unknown, but it is discussed in the next section.

The LOD of the assay was then calculated from the number of false-positive reactors in the negative control

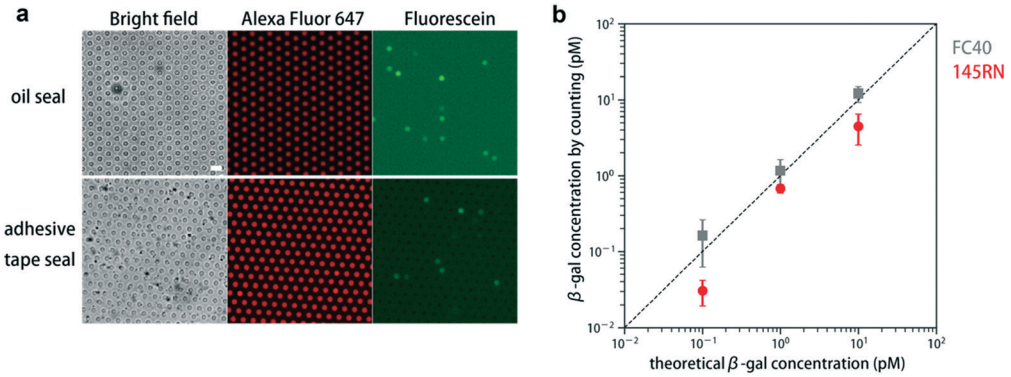

Fig. 4 β-Gal assays carried out using the PITAT and conventional oil sealing methods. (a) Images of the femtoliter arrays prepared using the different sealing methods. A 1 pM β-gal assay mixture was sealed in the reactors. Scale bar = 10 μm. (b) Measured *vs.* theoretical concentrations of β-gal molecules determined by assays using PITAT (red) and fluorinated oil sealing (gray). Error bar = s.d.

sample. In the negative control assay with no enzyme, 0 false-positive reactors were found for fluorinated oil sealing (Fig. S4†). This corresponds to an estimated upper limit of 1.7 fM for the LOD, assuming that the volume of a single reactor is 40 fL. On the other hand, three false positive chambers were found for the negative control of the PITAT β-gal assay (Fig. S5†). This corresponds to an LOD of 5.1 fM. According to the criteria used in our automatic counting macro, some reactors in the negative control experiment were unexpectedly classified as positive. However, careful observation of these false positive reactors revealed that they contained debris that emitted autofluorescence (Fig. S6†). Such false-positive reactors can probably be excluded by improving the sample preparation process or by image analysis. These results suggest that the LOD for detection is comparable between the two assay setups.

Single influenza virus particle counting assay by PITAT

To demonstrate another digital assay using PITAT, we performed a single influenza virus counting assay.[44] In principle, substrate 4MU-NANA is hydrolysed by neuraminidase of the influenza virus, giving rise to fluorescent molecule 4-methylumbelliferone. Therefore, if influenza virus(es) are present in a reactor, the reactor becomes fluorescent. The reaction mixtures containing 4MU-NANA and various concentrations of influenza A virus (H1N1/Puerto Rico/8) were sealed inside the reactors using the two methods described above. In this assay, the PSA tape sealing method was slightly modified; a frame of double-sided Kapton tape was used to ensure that any excess liquid remained inside the device even after pressing with a roller. This modification was necessary to avoid leakage of the solution containing the infectious virus particles. As expected, both PITAT and fluorinated oil sealing separated the chambers and produced uniform droplets (Fig. 5a). The background level was slightly higher for PITAT compared to that for oil-sealing, which is most probably because of the autofluorescence of the adhesive layer of the PSA tape.

In this assay, autofluorescence originating from dust and air bubbles was occasionally observed, and as a result, these false-positive reactors interfered with the reactor counting stage. In PITAT sealing of the negative control sample, ~1 false positive per 10 000 chambers was found by intensity-

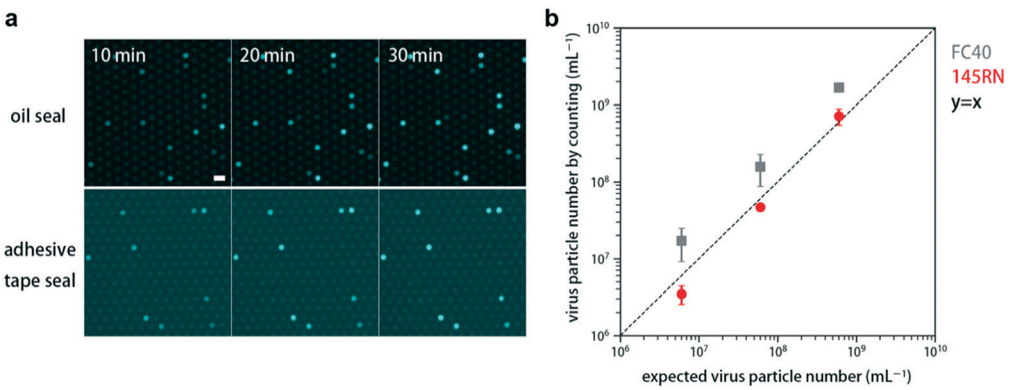

Fig. 5 Digital influenza virus counting assay by PITAT and conventional oil sealing. (a) Time lapse images of femtoliter arrays with different sealing methods. The expected concentration of virus particles in the assay mix was 6.0×10^8 particles per mL. Scale bar = 10 μm. (b) Measured *vs.* expected concentrations of influenza virus particles determined by assays performed by PITAT (red) and oil sealing (gray). Error bar = s.d.

based counting (Fig. S7a–c†). Thus, to avoid miscounting such reactors, we used the rate of the fluorescence increase instead of simple fluorescence intensity to count the number of positive reactors. Counting based on rate has the advantage of robustly omitting false signals caused by autofluorescence. As expected, we were able to eliminate false-positive reactors by rate-based counting, and the number of positive chambers was determined for both sealing methods (Fig. S8 and S9†). It was found that the estimated numbers of virus particles per sample volume were generally comparable for the oil sealing and PSA sealing methods (Fig. 5b). Again, PITAT tended to give a slightly smaller number of virus particles compared to the fluorinated oil sealing method. In our assays, a total of ~13 000 reactors were observed per assay, which corresponded to a total volume of ~0.5 nL. No reactors were identified as false positives by rate-based counting in our negative control sample (Fig. S8 and S9†), and so the LOD for this assay was $\leq 2 \times 10^6$ virus particles per mL for both sealing methods. These results suggest that the result from a single influenza virus counting PITAT assay is comparable to that of the conventional oil-sealing assay.

It should be noted that counting based on rate used here requires somewhat longer observation time, and if the user wishes to do multipoint time lapse imaging, it requires a sophisticated stage controller. The latter might be a substantial drawback, especially if the user intends to do low cost imaging with PITAT. In practice, requirements for a stage controller for timelapse imaging can be circumvented by using lower magnification, as long as the user can tolerate lower image resolution. Thus, we consider that PITAT sealing combined with rate-based counting is one of the viable options for low-cost single molecule detection/quantification systems.

In our results, PITAT yielded slightly smaller concentration estimates than when oil sealing was employed for both the β-gal and influenza assays. The volumes of PITAT-sealed and oil-sealed chambers were very similar (Fig. S10†), so the difference in concentration estimates cannot be attributed to the volume difference. The reason for this is unclear, but we speculate that this may be due to the deactivation or loss of the enzymatic activity upon non-specific adsorption. In the microspace used in digital assays, the ratio of the surface area to the volume is high. For example, a cylindrical reactor with a diameter of 4 μm and a depth of 3 μm has a volume of 3.8×10^{-17} m^3 (38 fL), and its surface area is 6.3×10^{-11} m^2, which gives a specific surface area of 1.7×10^6 m^3. In contrast, for a cylindrical reactor with a diameter of 4 cm and a depth of 3 cm, the specific surface area is only 1.7×10^2 m^3. Thus, as the reactor becomes smaller, the ratio of the surface area increases dramatically, and in microreactors with high specific surface areas, the frequency of a single molecule of interest colliding with the wall by Brownian motion increases, and the probability of non-specific adsorption also increases. The non-specific adsorption of enzyme molecules at hydrophobic interfaces has been suggested to dramatically decrease enzyme activities.[46] In our microreactors, the key difference between the oil sealing and PITAT methods is the aqueous/hydrophobic interface between the water and the oil or adhesive, which is 1.3×10^{-11} m^2, or 17% of the total surface area per microreactor. It is therefore possible that a substantial fraction of enzyme molecules or virus particles may have lost their activity due to their non-specific adsorption at the water/adhesive interface, leading to slightly smaller measurement values. However, in a practical context, such slight differences would be negligible for the majority of applications. Digital assays often have a wide quantification range spanning more than 5 orders,[44] so in most cases, a difference of less than 1 order does not affect the interpretation of results. However, users need to be aware of this difference, especially when comparing the results obtained by PITAT with those of conventional oil sealing.

Conclusions

We herein proposed the physical isolation of tiny reactors with adhesive tape (PITAT) using simple, commercially available pressure-sensitive adhesive (PSA) tape as a separator of the microreactors, and demonstrated that our PITAT assay can be used for digital assays using a femtoliter reactor array device. A single-molecule β-galactosidase (β-gal) assay and a digital influenza virus counting experiment were then performed successfully using PITAT, and the obtained results were comparable to those of a conventional oil sealing technique. We expect that other reactions are also possible in these PITAT microreactors, which constitute a simple, easy, quick, and cost-effective assay method. In the future, this system could find application in point-of-care testing kits, where it is highly important to provide a low-cost testing method to the end user. We tested several types of adhesives and found that rubber adhesives provided optimal results for sealing of the femtoliter reactors in a single-molecule β-gal assay. Although silicone adhesives can also be used, giving slightly poorer results, acrylic and urethane adhesives are not suitable for this purpose. However, further studies are needed to determine the key factor for this difference. In this study, we tested only two types of reactions, but it is possible that for different kinds of reactions, the optimum type of adhesive may be different. Finally, we believe that PITAT is a promising platform for delivering reliable and cost-effective single-molecule testing to locations where sophisticated machines are not available, such as individual patients' homes and geographical areas with limited access to medical care. There are ongoing projects in our group, and we expect to address some of these problems in the future.

Author contributions

H. Y. and K. V. T. designed the study and wrote the paper. H. Y., K. O., and T. A. performed the experiments and analyzed the data. H. N. provided critical discussion and comments.

Conflicts of interest

H. Y. and K. V. T. filed a patent application regarding the technique described in this paper. K. V. T. is a cofounder of Sothis Technologies and has equity in this company. Sothis Technologies developed detection and quantification tools based on the microchamber technology. The remaining authors declare no conflicts of interest.

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