Agar-polydimethylsiloxane devices for quantitative investigation of oviposition behaviour of adult *Drosophila melanogaster*

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(Received 6 March 2015; accepted 8 June 2015; published online 23 June 2015)

Drosophila melanogaster (fruit fly) is a model organism and its behaviours including oviposition (egg-laying) on agar substrates have been widely used for assessment of a variety of biological processes in flies. Physical and chemical properties of the substrate are the dominant factors affecting *Drosophila*'s oviposition, but they have not been investigated precisely and parametrically with the existing manual approaches. As a result, many behavioral questions about *Drosophila* oviposition, such as the combined effects of the aforementioned substrate properties (e.g., exposure area, sugar content, and stiffness) on oviposition and viability, and their threshold values, are yet to be answered. In this paper, we have devised a simple, easily implementable, and novel methodology that allows for modification of physical and chemical composition of agar substrates in order to quantitatively study survival and oviposition of adult fruit flies in an accurate and repeatable manner. Agar substrates have been modified by surface patterning using single and hexagonally arrayed through-hole polydimethylsiloxane (PDMS) membranes with various diameters and interspacing, as well as by substrate stiffness and sugar content modification via alteration of chemical components. While pure PDMS substrates showed a significant lethal effect on flies, a 0.5 mm diameter through-hole access to agar was found to abruptly increase the survival of adult flies to more than 93%. Flies avoided ovipositing on pure PDMS and on top of substrates with 0.5 mm diameter agar exposure areas. At a hole diameter of 2 mm (i.e., 0.25% exposure area) or larger, eggs were observed to be laid predominately inside the through-holes and along the edges of the PDMS-agar interface, showing a trending increase in site selection with 4 mm (i.e., 1% exposure area threshold) demonstrating natural oviposition rates similar to pure agar. The surface-modified agar-PDMS hybrid devices and the threshold values reported for the substrate physical and chemical conditions affecting oviposition are novel; therefore, we advocate their use for future in-depth studies of oviposition behaviour in *Drosophila melanogaster* with accuracy and repeatability. The technique is also useful for development of novel assays for learning and decision-making studies as well as miniaturized devices for self-assembly of eggs and embryonic developmental investigations. © 2015 AIP Publishing LLC. [<http://dx.doi.org/10.1063/1.4922737>]

I. INTRODUCTION

Drosophila melanogaster (i.e., fruit fly) is one of the most widely used model organisms of human diseases, which has contributed significantly to various areas of neurobiology, genetics, developmental studies, and toxicological sciences.^{1–6} This is mainly due to its genetic

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homology to human,⁶ rapid reproduction and development, small size, and ease of caring and genetic manipulation.⁷ It is employed in chemical screening and toxicology studies whereby behaviours, such as oviposition,⁸ movement,⁹ foraging,¹⁰ avoidance,¹¹ and mating,¹² are assayed in response to environmental toxicants.^{1,12–14}

Oviposition is the process of egg-laying in *D. melanogaster* that is dependent on responses of the fly to environmental stimuli (such as chemicals, physical conditions of the substrate, temperature, light, population density, and humidity).^{15–19} *D. melanogaster* possesses sensory neurons (such as taste^{20,21} and touch^{22,23} sensing systems) to detect various environmental cues prior to selecting preferred sites for oviposition and egg laying.^{15–19} Therefore, oviposition is used as a metric quantity to provide a clear indication of flies' sensory system health and their overall fitness (the ability to survive and reproduce).^{24,25} It is used predominately in developmental,²⁶ chemical screening,²⁷ and toxicology^{28,29} studies as a simple and effective readout indicator. It is also of interest to biologists for answering fundamental questions about oviposition and reproduction patterns in insects. Thereby, development of a precise and sensitive method for accurate and quantitative analysis of oviposition is necessary. Current ovipositional assays in laboratory settings investigate the effect of environmental cues such as physical surface texture^{30,31} and substrate chemical composition²⁵ on oviposition by using the standard yeast medium and the grape juice agar plates.³²

Current surface texture studies may be categorized into additive and subtractive texturing approaches. In the former method, seeded bisected grapes³¹ or blotters,³³ silk meshes,³⁰ and different kinds of papers³⁰ (such as cardboard strips, filter paper, and Washi paper) on top of standard agar substrates have been used to produce different surface modifications. In the latter approaches, manually scratched agar plates^{33,34} were used to produce texturized surfaces. By using the additive texturing methodologies, more eggs were shown to be laid on certain substrates modified by silk meshes or seeded grapes.^{30,31} However, due to the low mechanical stiffness of the silk meshes, their physical shapes were reported to be deformed upon implantation of the eggs.³⁰ Similarly, subtractive texturing methods have also provided the same conclusion that textured substrates induce more oviposition. It was shown that egg-laying was enhanced and more prominent on rough surfaces and on sharp edges produced by manual incisions of substrates with sharp objects to create slices, slits, and grooves.^{17,33–35} Atkinson showed that texture is important in oviposition when investigating the reason for gregarious ovipositional effects.³⁵ They created 12 egg-laying pots filled with yeast-agar and patterned half of them manually by scratching with a 5 mm deep perpendicular grid. It was observed that 90.6% of the eggs were laid in the conditioned chambers. Aside from the manual techniques that were used to create physical properties on the substrates, chemical properties were also shown to influence *Drosophila*'s oviposition significantly. Bahadorani and Hilliker have shown that ovipositional responses were greatly influenced by heavy metals (such as cadmium, copper, iron, and zinc) and their concentrations.²⁵ It was found that flies avoided high concentrations of zinc and iron, and preferred concentration ranges of 1–2 mM for oviposition. Other studies have likewise shown ovipositional preferences towards chemicals such as acetic acid and limonene but avoidance from harmful ones like geosmin.^{24,36,37} These techniques and studies have greatly enhanced our overall understanding of oviposition behaviour. However, they lack the accuracy, sensitivity and control, as well as the adequate level of repeatability that is required for parametric and quantitative investigation of viability and oviposition of fruit flies on agar substrates, especially in response to precise variations in surface exposure area and physical size of the oviposition site as well as the chemical and mechanical properties of the agar substrate.

Recently, microfluidic devices and Micro-Electrical-Mechanical Systems (MEMS) have been successfully used in many *Drosophila* assays,^{38–46} mainly because of their capability to provide scalable and controllable environments for quantitative embryonic^{38–43} and larval^{44–46} investigations in flies. These miniaturized devices that are made primarily from the elastomer polydimethylsiloxane (PDMS) have significantly enhanced the throughput, accuracy, and reproducibility of these assays. The techniques and materials used in microfluidic devices can be adopted for whole-organism studies as well, however, biocompatibility (e.g., viability and fitness) of PDMS with *Drosophila* is an important matter that needs to be primarily investigated.

In this paper, we have employed a simple and practical miniaturization technique to develop a novel tool for studying adult fruit flies' viability and oviposition behaviour (collectively and at a single egg level) in a quantitative and convenient manner. In our method, PDMS-based through-hole membranes with various hole sizes and arrangements have been used to pattern agar plate surfaces with various stiffness and sugar contents, in order to provide oviposition sites with controllable physical conditions and chemical compositions (called "*oviposition substrates*"). Flies were allowed to lay eggs on various *oviposition substrates*, and their viability and ovipositional rate (i.e., number of eggs laid inside and outside the holes) were quantitatively studied. Due to the ability to produce highly controllable and precise environments for egg-laying, we have been able to provide additional insights into the oviposition behaviour of fruit flies, from the effect of PDMS on oviposition and viability, to determination of threshold exposure areas and physical sizes of oviposition site for natural egg laying behaviour and survival, to investigation of the effect of other chemical and mechanical properties of the substrate on oviposition with a sensitive single-egg assay device. Our assays showed that pure PDMS substrates were lethal to flies but by introducing access holes larger than 2 mm to agar (0.25% exposure area), adults exhibited full survival. Also, oviposition was significantly inhibited on single-hole agar substrates with hole diameters of 2 mm or smaller or on arrayed seven-hole substrates with holes smaller than 0.5 mm. However, reduction in the agar substrate stiffness re-stimulated egg laying on these substrates significantly. It was also observed that physical patterning of pure agar substrates yielded enhanced oviposition but no preference for site selection. Finally, our experiments provided evidence that in order to achieve natural oviposition with site selection preferences, three factors were required: a PDMS repelling surface, an agar attracting surface, and a mechanical structure in between. This technique is a sensitive and important tool that may be used for fundamental behavioural and genetic studies of oviposition in *Drosophila* and other fruit fly species (e.g., *Ceratitis capitata*) and flying insects (e.g., moths). It also has applications in development of devices for decision-making assays and collection, de-clustering and self-assembly of eggs for imaging and embryonic developmental studies. Moreover, due to the simplicity of this methodology and the use of PDMS, these substrates may be easily integrated with microfluidic devices for toxicological and chemical screening applications.

II. MATERIALS AND METHODS

A. Fly stock

White (w^1) *Drosophilae* were grown on standard yeast media in stock bottles and maintained under standard 12:12 h light:dark lighting regime. For all experimental assays, adult flies were emptied from the bottles on the day of collection. Young flies that emerged from their pupae within half a day were collected into new stock bottles and were aged 5 days. To ensure an adequate amount of food source and constant population density of flies, 0.3 g of flies were anesthetized with ether and put into each of these bottles at 21 °C. When transferring 25 female and 20 male flies from the stock bottles to the assay setup, flies were first anesthetized and counted. Prior to each assay, the flies were given a minimum of 1 h recovery time.

B. Experimental setup and ovipositional assay

Our experimental setup for oviposition assays (Fig. 1) consisted of a stock bottle with air holes on the utmost top, containing $n = 45$ flies (25 females and 20 males), assembled onto a patterned *oviposition substrate* (i.e., agar surfaces patterned with through-hole PDMS membranes, as discussed in Section II C) and a dark cage for conducting the experiments. Using a small paint brush, the counted anesthetized flies were brushed into each stock bottle. Subsequently, the patterned oviposition substrate was capped onto the mouth of the stock bottle via a press fit. To ensure the system was well-assembled, a piece of tape was used to secure the substrate to the bottle prior to the inversion to allow fly substrate contact, as shown in Fig. 1. The whole assembly was then transferred into a dark and enclosed box, and the flies were left to oviposit for 24 h at 21 °C. After the assays, the viability rate of the adult flies (i.e., the percentage of alive animals)

FIG. 1. Schematic of the experimental setup for ovipositional assay is shown in (a) and the corresponding assembly is shown in (b). The setup consisted of 25 female and 20 male flies in an inverted stock bottle capped onto an oviposition substrate. The substrate consisted of a custom-patterned through-hole PDMS membrane that was overlaid on each agar juice plate. Various patterning configurations such as single through-holes (diameter $d = 0.5, 2, 4$, and 8 mm) and seven hexagonally patterned through-holes ($d = 0.5, 2$, and 4 mm) with equal spacing ($s = 0.5, 2$, and 4 mm) were used for the assays.

and the ovipositional rates (i.e., the number of eggs oviposited inside and outside of the holes of the PDMS membrane) were quantified with the aid of a dissection probe and a dissection microscope.

C. Oviposition substrates

Conventional agar-juice plates were used as standard substrates in laboratories for ovipositional assays³² and thereby used in our experiments as positive control and base substrates. We prepared the grape juice-agar solution (10 g agar, 11 g sugar, 333 ml distilled water, and 111 ml Welch's frozen grape juice concentrate) via constant mixing at 700 rpm on a magnetic stirring hotplate at 200 °C. Once a full boiling was obtained, the solution was cooled to 80 °C by natural convection and the bubbles were allowed to escape from the surface. Then 3 ml of the solution was plated onto each petri dish lid (40 mm diameter, 10 mm height) using a syringe and left for solidification. These plates were used as controls and base substrates in the experiments with PDMS through-hole membranes (discussed below). To produce negative control substrates, PDMS was made via mixing Sylgard 184 elastomer and curing agent in a 10:1 ratio (Dow Corning Co., Midland, MI, USA). Similarly, 3 ml of PDMS was plated onto the same petri dishes via a syringe and left to cure for a day at 21 °C.

To vary the concentration of sugar and to study the effect of sucrose content of the agar-juice plates on oviposition, the amount of sugar was altered from the control recipe, which originally had 11 g of sugar. The high- and low-sugar substrates in these assays contained 22 g and 5.5 g of sugar, respectively, with all other components kept unaltered, as discussed above. In the same way, the effect of substrate stiffness was also assayed by modifying the amount of agarose used in the conventional agar-juice plate recipe that contained 10 g agarose powder. The soft and stiff agar substrates contained 5 g and 20 g agarose powder, respectively.

Patterned PDMS through-hole membranes ($250 \pm 50 \mu\text{m}$ thick) were used on top of the abovementioned agar juice base substrates (Fig. 1) to study the effect of surface area exposure on the survival and oviposition behaviour of fruit flies. To fabricate the PDMS membranes, 0.5 ml of PDMS pre-polymer (Sylgard 184 elastomer and curing agent, mixed in a 10 to 1 ratio, respectively) was spun on a $6 \text{ cm} \times 6 \text{ cm}$ transparency film using a spin coater (MODEL P6700

TABLE I. PDMS membranes used to pattern *oviposition substrates*.

Number of through-holes	Through-hole diameter, d (mm)	Interspacing, s (mm)	Exposure area (%)	Schematic
<i>Single through-hole</i>
1	0.5	...	0.016	
1	2	...	0.246	
1	4	...	1	
1	8	...	4	
<i>Seven hexagonally patterned through-holes</i>
7	0.5	0.5, 2, 4	0.112	
7	2	0.5, 2, 4	1.75	
7	4	0.5, 2, 4	7	

Series, Specialty Coating Systems, Inc., Indianapolis, IN, USA) for 20 s at 600 rpm. The pre-polymer was allowed to set and cure for 1 day at room temperature. To pattern the PDMS membranes, a single circular through-hole (diameter $d = 0.5$ mm, 2 mm, 4 mm, and 8 mm) was punched into the fabricated membranes using the corresponding Harris Uni-Core™ hole-puncher (Ted Pella, Inc., Redding, CA, USA). Each of these hole sizes correspond to the percentage exposure areas, as summarized in Table I, defined as $\%(\text{area of through-hole}/\text{area of agar substrate}) \times 100$. On the other hand, multiple-hole membranes were produced similarly with the additional aid of a 3D printed incision guide. For assaying exposure hole diameter and interspacing, nine 3D printed molds, each containing seven hole guides with diameters of 0.5 mm, 2 mm, or 4 mm and equal spacing of 0.5 mm, 2 mm, or 4 mm, were used to pattern corresponding through-holes hexagonally in PDMS membranes. After preparation of the desired PDMS membranes discussed above, they were lifted off their fabrication substrates and placed on the standard agar juice plates for oviposition and survival assays, as shown in Fig. 1 and discussed in Section II B.

The effects of patterning of the pure agar and PDMS substrates on oviposition were also investigated. For this purpose, two additional homogeneous substrates were fabricated: (i) a pure PDMS and (ii) a pure agar substrate. Both substrates contained a 4 mm diameter and 0.25 mm deep circular cavity in the center. They were called *conditioned PDMS* and *conditioned agar* substrates in our assays. To fabricate the *conditioned PDMS* substrate, a PDMS membrane with a punched 4 mm-diameter hole was overlaid on a flat PDMS substrate. To produce the *conditioned agar* substrate, a 4 mm PDMS disc was placed on top of a solidified agar-juice plate. Liquid agar-juice medium was then quickly transferred via a cotton applicator to create a uniform surface by filling up the area around the 4 mm PDMS disc. Upon curing of the agar substrate, the PDMS disc was removed via a set of forceps to expose the agar cavity. These substrates were also tested for oviposition, as discussed in Section II B.

III. RESULTS AND DISCUSSION

D. melanogaster ovipositional assays, studying the effects of physical and chemical substrate factors on egg-laying behavior, were not highly controlled and lack repeatability and precision due to the use of manual texturing approaches.^{30,31,34,35,47} Since they only provided crude results about oviposition behaviour in flies, the PDMS membrane patterning and agar chemical composition manipulation techniques were used to control exposure areas and textures, and to investigate oviposition both in batch format and at a single-egg resolution level. As described in Section II, thin PDMS membranes with patterned through-holes were designed (Table I) and overlaid on conventional agar-juice plates with various physical and chemical properties (Fig. 1), and the oviposition and viability of flies (25 females and 20 males) were quantitatively investigated. When compared to conventional oviposition assays, our technique has provided a

number of new insights about oviposition in a more sensitive and precise manner. This includes the threshold values for agar exposure area as well as physical sizes of the sites and their inter-spacing for full viability and natural oviposition in addition to investigation of the effects of substrate sugar concentration and stiffness on oviposition.

A. Survival and oviposition of *D. melanogaster* on agar and PDMS substrates

Surface quality, texture, and chemistry have been shown to have significant effects on flies' oviposition,^{24,25,30,31,33–37} however, the required area of exposure to agar substrate, in order to keep a population of fruit flies alive and to induce them to lay their eggs naturally (i.e., similarly to pure agar substrates) were not investigated. Accordingly, our aim was to quantitatively investigate the effects and the threshold values of surface exposure area on oviposition of fruit flies using our PDMS membrane patterning technique. To achieve this goal, the investigations started by examining the survival and oviposition of fruit flies on pure PDMS substrates in comparison to that on top of the conventional pure agar substrates. For instance, in a representative experiment, from the 45 fruit flies that were left to oviposit on the pure PDMS substrate for 24 h, 23 were found dead with only 12 eggs deposited on the substrate (Fig. 2(a)). On the contrary, all the flies survived after 24 h on a standard agar plate, and 137 eggs were found to be laid on the substrate (Fig. 2(b)).

A number of $T=44$ trial experiments demonstrated that on the pure agar juice-plates, many eggs (137.23 ± 15.2 standard error (SE)) could be distributed randomly all over the substrate, whereas eggs were found to be oviposited sparingly (11.93 ± 1.11 SE) on pure PDMS substrates. The corresponding viability rates of the adult flies for the pure agar and pure PDMS substrates were found to be $99.56\% \pm 0.17\%$ SE and $50.78\% \pm 5.34\%$ SE, respectively. These results clearly showed that the access to agar is vital for full viability of the animals, and that PDMS alone is not an appropriate site for oviposition or a proper material of choice by itself for fabrication of devices for whole-organism assays. Possible reasons for lack of oviposition on PDMS are the smell of PDMS substrate that was not attractive to *Drosophila*, and its toughness causing obstacles for the ovipositor to be inserted into the media in comparison to pure agar.⁴⁸ Lack of water, food, and nutrients is among the reasons for the fatal effect observed on pure PDMS substrates.^{41,49} With the understanding of flies' viability and oviposition on pure PDMS and agar substrates, additional experiments could be designed to precisely study the effects of exposure area and size of the oviposition site on these behaviors. In addition, understanding the effect of exposure to PDMS is becoming more and more important in design and development of microfluidic devices for *Drosophila* assays.

B. Agar surface exposure area and its effects on survival and oviposition of *D. melanogaster*

To investigate how much access to agar is required to obtain natural responses (*viz.* survival and ovipositional rates similar to those demonstrated by flies on pure agar substrates), an

FIG. 2. Photographs of oviposited eggs on (a) pure PDMS and (b) pure agar substrates, after oviposition of $n = 45$ flies for 24 h. Both substrates showed random oviposition; however, pure agar had a higher ovipositional rate (137 eggs) than the pure PDMS (12 eggs) substrate.

exposure to agar was introduced and controlled via overlaying of PDMS membranes patterned with various through-holes on top of the standard agar substrate. For instance, when a $d = 4$ mm access hole to agar (corresponding to 1% exposure area) was provided through the PDMS membrane (Figs. 3(a) and 3(b)), the survival and ovipositional rates of the flies were observed to increase (as compared to pure PDMS) to 100% and 64 eggs, respectively. It was also interestingly observed that the majority of the oviposited eggs (82%) were deposited inside the PDMS through-hole and aligned at the side edges of the hole.

Fig. 3(c) illustrates the overall survival and ovipositional rate results of $T = 9$ trial experiments for our single-hole oviposition assays with various substrate hole diameters (Table I). When a 0.5 mm diameter (0.016% exposure area) through-hole was introduced, the survival increased from approximately 50% (pure PDMS) to more than 93%, which was significantly different from pure PDMS substrate (P -value of 3.13×10^{-9} using T-test). As the agar exposure

FIG. 3. Single through-hole oviposition assay results for nine trials. (a) A single through-hole substrate ($d = 4$ mm) after the oviposition assay. Eggs were found to be deposited inside and along the edges of the through-hole, (b) a magnified image of the oviposition site in (a), (c) the viability and ovipositional rates with standard errors of mean for oviposition inside the through-hole (white column) and on the entire substrate (dark black column). Increasing the exposure area by a $d = 0.5$ mm through-hole (0.016% exposure) increased the survival rate significantly ($P = 3.126 \times 10^{-9}$, T-test) when compared to pure PDMS substrate. The ovipositional rate over the entire substrate showed a sudden decrease on $d = 0.5$ mm substrates but then increased gradually with an increase in the exposure area. No eggs were deposited inside $d = 0.5$ mm substrates, but the majority (88%) of the eggs were deposited inside the holes on $d = 4$ – 8 mm substrates. Agar exposures exceeding 1% ($d = 4$ mm) yielded to be statistically significant in ovipositional rate when compared to pure PDMS and $d \leq 2$ mm substrates ($P = 8.921 \times 10^{-13}$, T-test).

area was further increased past 0.016% (to 2 mm diameter through-holes and larger), the survival rates reached a plateau showing natural (i.e., pure agar-like) survival. Pure agar showed $99.56\% \pm 0.17\%$ survival in comparison to 1% and 4% exposure areas, which both showed 100% viability. This difference was statistically insignificant and mainly attributed to the occasional death of an animal during our pure agar assays, which may have occurred due to the interruption of excessive egg production and “reproductive overloading.”⁵⁰ Statistical T-test analysis performed on agar and substrates with exposure access areas equal and greater than 0.25% ($d=2$ mm) showed no significant survival differences. This behavior not only validated that agar access is needed for optimal survival but also showed that only a small exposure of 0.016% was adequate in sustaining most ($>90\%$) of the assayed flies.

In Fig. 3(c), the ovipositional rates over the entire substrate (dark black columns) and inside the through-hole (white columns) are also presented. The ovipositional rate over the entire substrate for 0.016% agar exposure ($d=0.5$ mm access hole) unexpectedly decreased from our control PDMS substrate, and yielded a significant difference (T-test, P-value = 3.5×10^{-4}) when both were compared. This decrease in the amount of eggs can be due to possible substrate spatial restriction for oviposition, creating physiological stress on the *Drosophila*. As the agar exposure area was increased further towards 4% ($d=8$ mm), the ovipositional rate on the entire substrate demonstrated an overall increasing trend towards natural (i.e., agar-like) oviposition (P-value = 0.29 for $d=8$ mm compared to pure agar substrate). We also noticed a sudden increase in oviposition between the 0.25% ($d=2$ mm) and 1% ($d=4$ mm) exposure areas, which showed a significant difference (T-test, P-value = 8.92×10^{-13}) in the selection of oviposition sites. The 1% exposure area threshold was adequate for the flies to demonstrate natural oviposition as they would do on a purely agar-based substrate. This can be due to the fact that the adult flies prefer oviposition sites, where food and water are prevalent for the survival of the next generation *Drosophila*.¹⁷ Hence, as a natural metabolic response to situations with limited access to food and water (e.g., $d=0.5$ mm holes), flies potentially conserved their energy by decreasing oviposition to increase their starving resistance in correspondence to the coupled-model of reproduction cost-trade off.^{51,52} This device may be useful for the future design of sensitive assays where the intake of food, water, and even repulsive chemicals has to be controlled precisely for oviposition investigations.

Comparing the number of eggs oviposited inside (white columns in Fig. 3(c)) the through-holes with the overall number of eggs on the entire substrate (black columns in Fig. 3(c)) provided us with some interesting insights for quantifying and interpreting the site selection behaviour in flies. No eggs were found inside the $d=0.5$ mm diameter (0.016% agar exposure) through-holes due to spatial restrictions of the site. As the through-hole diameter was quadrupled; yielding the agar exposure of 0.25%, more than 52% of the total number of eggs were observed to be deposited inside the through-holes, yielding a mediocre site-selection behavior in flies. On the contrary, when the exposures were increased to 1% and beyond ($d>2$ mm diameter), more than 85% of the eggs were laid inside the through-holes, showing that site selection was significantly enhanced. In all of these oviposition assays (Fig. 3(c)), majority of the eggs that were deposited inside the through-holes were also positioned along the edges of the PDMS membrane and embedded inside the agar base (as seen qualitatively under microscope for the 1% substrate in Figs. 3(a) and 3(b)). This trend was true for 72% and 91% of the substrates that had exposure areas greater than 0.016% and 0.25%, respectively. There are a number of advantages associated with our site selection assays that have not been achieved by the conventional oviposition methods. For instance, the 1% and 4% exposure area devices may be used for fundamental investigation of genes that cause defects in oviposition. The 0.016% exposure area device is considered as a sensitive tool that can be used for assessment of other physical and chemical factors that play a role in oviposition, as reported in Section III E.

C. Time-lapse investigation of oviposition on through-hole substrates

To obtain a better understanding of when oviposition occurs in our assays, and to ensure that flies had enough time to come in contact with the substrate before oviposition, we

performed a series of time lapse studies on pure agar as well as the 1% and 4% exposure substrates at the 12th, 16th, 20th, and the 24th hour. For example, in the pure agar experiment, starting from the 12th to the 20th hour, the ovipositional rate increased gradually with an average of 55.3 ± 10.73 SE eggs. At the end of the time lapse, at the 24th hour, a drastic spike in ovipositional rate was observed (T-test, P-value 4.27×10^{-4}), yielding 248 ± 20 SE eggs.

Similar to pure agar substrates, from the 12th to the 20th hour, the mean ovipositional rates over the 1% ($d = 4$ mm) and 4% ($d = 8$ mm) exposure substrates remained fairly constant at 17.54 ± 3.37 SE and 25.29 ± 4.84 SE eggs, respectively (Fig. 4). As the 24th hour was reached, a significant increase was observed in the number of oviposited eggs (T-tests, P-values = 2.50×10^{-4}). From these observations, the 24 h assay time period was sufficient enough to allow for our investigations of survival and oviposition of fruit flies in response to surface modifications.

D. Effect of oviposition site size and spacing versus the exposure area on survival and oviposition of *D. melanogaster*

Knowing that the physical size of the oviposition site and the exposure area to agar were important factors for full viability of all 45 flies and their natural oviposition behaviour, we sought to investigate for the first time if there were any preferences in oviposition towards these two parameters. Thus, we patterned the agar substrates with 7 holes of $d = 0.5$ mm, 2 mm, and 4 mm diameter (Table I) and conducted the oviposition and survival assays, as discussed before. Each array of through-holes (i.e., seven 0.5 mm, 2 mm, and 4 mm diameter) in the substrate corresponded to the exposure areas of 0.112%, 1.75%, and 7%, respectively. This enabled increasing the exposure areas of the substrates significantly as compared to equivalent single-hole assays, while maintaining the physical sizes of the oviposition sites constant. We were also interested in investigating whether site interspacing ($s = 0.5$ mm, 2 mm, and 4 mm) had a significant effect on oviposition. In an experiment, when seven $d = 2$ mm through-holes (1.75% exposure area) with equal inter-spacing of $s = 2$ mm were provided through the PDMS membrane (Figs. 5(a) and 5(b)), the survival and ovipositional rates of the flies were observed to increase to 100% and 111 eggs, respectively. As shown, the majority of these oviposited eggs (90%) were deposited inside the PDMS through-holes and along the side edges of the holes. To observe the effects of spacing, the results from the 1.75% exposure substrate ($d = 2$ mm; $s = 2$ mm) were compared with the same arrayed through-holes but with 0.5 mm and 4 mm spacing. Similarly, the survival rates for both were 100% and the ovipositional rates were 132.5 and 122.5 eggs, respectively.

Fig. 5(c) shows the overall viability and the ovipositional rate of *Drosophila* on the aforementioned patterned seven-hole substrates ($T = 7$ trials). For all of the assays, the survival rate

FIG. 4. Time lapse investigation of ovipositional rate for the last 12 h of the assays with pure agar, and single hole ($d = 4$ mm and $d = 8$ mm) substrates. In all the substrates, the number of eggs was low and stable until the 20th hour. There was an acute increase between the 20th and the 24th hour in the number of eggs, which was significantly different from the times before.

brusquely increased to 100% as expected. The inter-spacing between the through-holes did not affect the survival rate of animals.

Similar to the agar surface exposure area assay (i.e., single-hole experiments in Fig. 3), which showed that single $d=0.5$ mm hole substrates exhibited few to no eggs, the arrayed $d=0.5$ mm holes (0.112% agar exposure) with spacing of 0.5, 2, or 4 mm also showed no significant differences (T-test, P -value < 0.78) in ovipositional rates on the entire substrate (dark black columns in Fig. 5(c)). Despite a drastic increase in the exposure area to agar, oviposition rate still did not increase significantly, demonstrating that the physical size of the site played the more important role in oviposition. Furthermore, on the arrayed 2 mm through-hole substrates, there was a significant and acute jump (T-test, P -value $= 4.97 \times 10^{-16}$) in ovipositional rate (Fig. 5(c)) due to the increase in energy and the resources available to be spent on reproduction. This substrate showed similarity in oviposition rate to pure agar substrates as opposed to the single-hole 2 mm-diameter assays (Fig. 3(c)). This observation demonstrated the importance of exposure area provided that an adequate oviposition site size was available for flies to lay their eggs. Additionally, the varying interspaces showed no effect on the quantity of eggs oviposited over the entire substrates or inside the sites for all the through-hole conditions tested. This suggested that oviposition behaviour is highly dependent on the size of the sites and the

FIG. 5. Effect of site spacing on survival and oviposition of *D. melanogaster* ($T = 7$ trials). (a) The spacing assay substrate ($d = 2$ mm, $s = 2$ mm) under a microscope with one hole magnified in (b). Eggs were found to be laid inside and along the edges of the holes when $d \geq 2$ mm. (c) The overall mean ovipositional rate (white columns for eggs laid inside through-hole; black column for eggs on entire substrate) and the viability (diamonds) of flies over various oviposition substrates. Spacing between the sites had no effect on ovipositional rate and survival of flies. Agar exposures higher than 1.75% (seven $d = 2$ mm holes) yielded to be statistically significant ($P = 6.96097 \times 10^{-16}$, T-test) in oviposition as compared to exposure areas of 0.111% (seven $d = 0.5$ mm holes).

overall exposure area to agar rather than their spatial proximity and the resultant physical constraints to approach the sites.

Fig. 5(c) also shows the effect of exposure area and through-hole inter-spacing on the ovipositional site selection behaviour (white columns compared to the black ones). With a closer examination, $d = 0.5$ mm (0.112% agar exposure) substrates had some random occurrences (47.6%) of very few eggs ($n < 3$) being deposited inside the through-holes. Although site selection was still not seen as 95.2% of the eggs were scattered randomly across the substrate, this demonstrated that, by increasing the exposure percentage via six more through-holes, the substrate was deemed slightly more attractive than the single 0.5 mm hole. Also, on substrates with seven holes greater than $d = 0.5$ mm (e.g., substrate in Figs. 5(a) and 5(b)), most of the eggs (96.68%) were found on the edges inside the arrayed through-holes between the PDMS and the exposed agar interface, leading to a significant increase in site selection in fruit flies (Fig. 5(c)).

E. Single-egg level site selection assay for studying the effect of agar properties on oviposition

Despite providing both texture and exposure to agar, few eggs were laid inside the single (Fig. 3(c)) and arrayed (Fig. 5(c)) oviposition substrates with $d = 0.5$ mm through-holes. Because of this binary observation (i.e., egg or no egg) and the convenience in scoring results from this substrate, it was used as the most sensitive tool developed to date to study the effect of other substrate factors on oviposition site selection at a single-egg resolution level (i.e., $n < 5$ eggs per hole). A set of experiments with 1 to 7 through-holes ($d = 0.5$ mm) provided confirmatory evidence that increasing the exposure area had no effect on egg deposition inside the 0.5 mm diameter holes and site-selection was not achieved (data not shown). To further investigate site selection and to determine whether the oviposition avoidance phenomenon on the aforementioned substrates were due to geometrical constraints of the hole, physical conditions of the substrate base, or chemical composition of the oviposition site, a series of experiments on the sugar concentration and the stiffness of the substrate were conducted, as discussed below.

1. Effect of substrate sugar content on oviposition site selection

To understand the effect of agar chemical composition on oviposition site selection and to investigate if sugar composition is more predominant in oviposition, 5.5 g and 22 g of sugar were used in preparation of the agar plates as the low and high sugar concentration substrates in reference to 11 g in the control assay (used in all other experiments reported in this article). PDMS membranes with seven hexagonally patterned through holes ($d = 0.5$ mm, $s = 2$ mm) were overlaid on top of these base substrates to produce the sugar modified substrates and the oviposition assays were conducted. The ovipositional rates were similar for all substrates with most of the eggs ($> 78.3\%$) deposited randomly outside the through-holes with no preference for site selection (data not shown here). Therefore, altering the sucrose content had no effect on re-stimulating oviposition and site selection.

2. Effect of substrate stiffness on oviposition site selection

Variations in the exposure area and sugar content had no effects on oviposition of flies on $d = 0.5$ mm through-hole substrates. Here, the effect of agar substrate stiffness on the oviposition of flies on the same substrates was studied. As in the previous assays, PDMS through-hole membranes ($d = 0.5$ mm) with seven holes were fabricated and overlaid on top of three different agar substrates containing 5 g (softer substrate), 10 g (standard substrate used in our other assays), and 20 g (stiffer substrate) of agar powder to vary their stiffness. After oviposition, the substrates were imaged under a microscope and the mean ovipositional rate inside the through-hole (white columns) and on the whole (dark black column) substrate were quantified for six trials (Fig. 6).

As shown in Fig. 6, in comparison to the control, the ovipositional rates on the entire substrate for the soft and stiff agar platforms were statistically similar (T-test, $P\text{-value} \geq 0.43$). However, when considering the ovipositional rate inside the through-holes (white columns in Fig. 6), there was an increasing ovipositional trend with the softening of the agar substrate. The stiff substrate exhibited no eggs inside the through-holes while reducing the agar quantity to 10 g and 5 g yielded 0.83 ± 0.40 SE and 12.26 ± 2.91 SE ovipositional rates, respectively. Moreover, the corresponding percentages for the number of eggs deposited inside the stiff, regular, and soft substrates were 0%, $10.20\% \pm 5.03\%$, and $61.86\% \pm 24.44\%$, respectively. This could potentially be due the fact that altering the stiffness of the substrate influenced the ability of the flies to insert their eggs deeper into the substrate using their ovipositors. Softer substrates permit more ovipositional activity with lesser effort since *Drosophila* have a tendency to puncture substrates to implant their eggs.^{53–55} In addition, all of the through-holes ($d = 0.5$ mm diameter) in the soft substrate had less than four eggs per hole due to the small spatial constraints.

These substrates may be used in the future as sensitive tools to study oviposition in details and at a single-egg resolution level. Additionally, they would allow for sorting and collection of eggs in embryonic developmental imaging studies and DNA extraction applications. Moreover, since PDMS is the material of choice in most microfluidic devices, these substrates would provide the flexibility and the possibility to design miniaturized devices for decision-making applications⁴⁸ and self-assembly of eggs at desired locations on a platform for high throughput chemical screening and toxicological studies.

3. Effect of mechanical structuring on oviposition site selection

We became interested in learning if the oviposition site selection behaviour observed in our agar-PDMS substrates was affected more significantly by the mechanical structure of the hole or by its chemical composition. This investigation was done via patterning a 4 mm diameter hole (selected based on the agar-like viability and ovipositional rates as reported in Fig. 3(c)) in pure PDMS (called “conditioned PDMS”) and pure agar (called “conditioned agar”) substrates and the conductance of standard ovipositional assays, as discussed in Section II.

As seen in Fig. 7, both homogenous substrates, whether conditioned or unconditioned, exhibited similar viability rates, demonstrating that mechanical structuring did not contribute to altering the viability of 45 adult flies in the assays. The ovipositional rates for the

FIG. 6. Ovipositional rate inside the through-hole (white column) and on the entire substrate (black column) for seven hexagonally patterned $d = 0.5$ mm holes (with $s = 2$ mm) on stiff, standard, and soft agar substrate, which corresponds to the modified agar quantities of 20 g, 10 g, and 5 g ($T = 6$ trials). The total mean ovipositional rates on the entire substrate were similar; however, as the stiffness decreased, there was a trending increase in oviposition inside the through-holes. For the soft agar, most of the eggs were deposited inside the through-holes in reference to the entire substrate, showing an enhancement in site selection.

unconditioned pure PDMS and the pure agar substrates were 9.40 ± 1.51 SE and 232.3 ± 18.39 SE, respectively. Oviposition on pure PDMS was found to be statistically similar to the *conditioned PDMS* substrate (T-Test, P-value = 0.41), and no oviposition inside the PDMS holes was observed. The *conditioned agar* demonstrated some minor significance in overall oviposition compared to the pure agar (T-Test, P-value = 0.04). This demonstrated that the agar exposure along with surface texture made the substrates slightly more desirable. However, looking at the ovipositional rate inside the through-holes, *conditioned agar* only had 19.70 ± 17.07 SE eggs ($\sim 6\%$ of the overall oviposited eggs) laid inside the hole. This showed that mechanical structuring alone was not sufficient to induce site selection in fruit flies. Overall, our experiments show that in order for site selection to occur, the substrate surface ought to have a component that repels the flies (i.e., the patterned PDMS membrane), a component that attracts the flies (i.e., agar-juice base surface), and a mechanical texture (i.e., PDMS-agar step interface).

IV. CONCLUSIONS

Parametric and precise investigation of viability and oviposition of fruit flies on laboratory-based agar substrates, and in response to surface exposure area and physical size of the oviposition site, as well as the chemical and mechanical properties of the agar have not been achieved using the conventional oviposition assays. In this paper, we have developed a novel miniaturization-based technique for investigating oviposition and successfully conducted a series of parametric assays (at collective and single egg levels) without manual manipulation of the substrates as practiced in previous oviposition studies, which were lacking accuracy and precision. By using PDMS membrane-based through-hole micro-patterning, we have studied fruit fly oviposition quantitatively in response to accurate and repeatable exposures to various agar surfaces.

Pure PDMS platforms alone were found to be lethal to flies, yielding around 50% survival; however, a small 0.5 mm diameter hole (0.016% agar exposure) was capable of increasing the adult fly viability by approximately twofold. The viability in the assay was primarily affected by the amount of agar exposure and other parameters such as spacing between the access holes had no effect on sustaining 45 flies during the assay. With regards to oviposition, strong avoidance was exhibited on pure PDMS substrates. At 0.016% agar exposure area (0.5 mm through-holes), flies avoided laying eggs inside the through-holes due to spatial and mechanical constraints. Aside from this, any other substrate having agar exposure holes greater than 0.5 mm diameter had most of their eggs oviposited inside the through-hole along the edges of the PDMS-

FIG. 7. Viability and ovipositional rate inside the through-hole (white column) and on top of the entire substrate (black column) for pure PDMS, $d = 4$ mm conditioned PDMS, $d = 4$ mm conditioned agar, and pure agar substrates ($T = 10$ trials). Both pure and conditioned substrates had similar survival rates. The number of eggs deposited in total was similar in PDMS substrates; however, conditioned agar showed more eggs than pure agar yielding a difference (P-value = 0.04). Despite patterning the substrate in the conditioned agar assays, site selection was not observed for flies.

agar interface of the membrane. In our experiments, acute jumps in the ovipositional rate was observed at single $d=4$ mm (1% agar exposure) or seven $d=2$ mm (1.75% agar exposure) through-holes. The presented device also provided us with a unique advantage to study oviposition site selection simply by comparing the number of eggs deposited inside the through-holes and the overall number of eggs laid on the entire substrate. We observed that by softening the agar, ovipositional rate inside the through-hole increased even if geometric constraints from the $d=0.5$ mm through-holes were in place, increasing attractiveness in site selection. Finally, we discovered that in order for site selection to occur, the substrate surface ought to have a repelling surface, an attracting surface, and a mechanical structure in place.

To date, this is the first study to use micro-technology to devise a sensitive tool for assaying ovipositional behaviors in adult *Drosophila* at a collective or single egg resolution. It can be used for investigating the genetic and neuronal basis of oviposition not only in *Drosophila* but also in other fruit fly species (such as the *Ceratitis capitata*) and flying insects (such as moths). As PDMS is a widely used material in miniaturized devices, this methodology has the flexibility of being integrated into miniaturized and microfluidic devices to develop higher throughput, controllable, and automated experimentation platforms for chemical screening and decision-making assays. Integration of the presented oviposition substrate with microfluidic devices may be achieved by development of novel fabrication methods for assembly of agar layers onto PDMS. The porous structure of the agar will allow for infusion of chemicals via the PDMS microchannels into the oviposition sites. Therefore, on-chip dosing and chemical screening may be performed dynamically and controllably for various assays. Finally, automated egg collection, self-assembly, and arraying devices can also be designed and developed for embryonic developmental studies based on the presented technique.

ACKNOWLEDGMENTS

The authors would like to acknowledge the financial support from the Natural Sciences and Engineering Research Council of Canada. They also thank Professor Alidad Amirfazli for providing access to fabrication and characterization equipment in his laboratory.

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