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A Simple Culture System for Long-Term Imaging of Individual *C. elegans*

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Abstract

We have miniaturized standard culture techniques to rear arrays of isolated, individual *C. elegans* throughout their lives on solid gel media. The resulting apparatus is compatible with brightfield and fluorescent microscopy, enabling longitudinal studies of morphology and fluorescent transgene expression. Our culture system exploits a novel crosslinking reaction between a polyethylene glycol hydrogel and a silicone elastomer to constrain animals to individual “corrals” on the gel surface. These devices are simple to construct on the benchtop with commercially available reagents, and, unlike microfluidic isolation methods, does not rely on micropatterned materials. We demonstrate that this new culture method has negligible effects on the physiology of *C. elegans* compared to standard culture on agar plates. In addition, RNAi techniques are effective in this system. Finally, the hydrogel–silicone binding chemistry that we developed also allows traditional microfluidic devices to be covalently attached to gel substrates instead of glass.

Introduction

Caenorhabditis elegans, a small and transparent roundworm, is perhaps the simplest multicellular model organism in common use. These animals, approximately 1 mm long and 100 μ m in diameter, are easy to culture in bulk. Since they feed on bacteria, the standard method of culture is to introduce a population of *C. elegans* onto solid agar on which a lawn of food bacteria was previously grown.¹ Protocols for culture in liquid media supplemented with bacteria or in a chemically defined bacteria-free medium are also well developed.² Unfortunately, it is impossible to use bulk culture for longitudinal studies in which specific individuals must be identified and repeatedly analyzed over time. To conduct such experiments, investigators have typically cultured single animals in the same conditions designed for the culture of hundreds or thousands of individuals. The inefficiency, low density, and substantial manual handling that attend such culture arrangements have generally limited longitudinal analysis of *C. elegans* to examinations of relatively few individuals with low time resolution. Despite these limitations, longitudinal studies of *C. elegans* have yielded critical information about development^{3,4} and aging⁵ in these organisms. To enable larger-scale longitudinal analysis, and to make studies requiring such analysis more accessible to the *C. elegans* community, we miniaturized standard solid-media culture techniques using straightforward techniques and readily available reagents. This

work describes in detail our new culture system, which we previously employed to analyze the relationship between health and lifespan among wild-type *C. elegans*.⁶

Various single-animal culture systems have been proposed for *C. elegans*. First, individuals can be grown in liquid culture in microtiter wells (in 96- or 384-well format).⁷ A related technique consists of rearing individuals in an array of micro-droplets of liquid culture media suspended in mineral oil.⁸ Maintenance of appropriate conditions for liquid culture in the static environment of a microtiter well or droplet is challenging, however. Indeed, viability is impaired in micro-droplets when individuals are cultured for longer than approximately four days.⁸ Moreover, it can be challenging to image individuals in liquid culture or droplets, due both to the optical density of the bacteria suspended in the culture medium, and to the rapid thrashing of *C. elegans* in liquid, which requires complex immobilization strategies to counter in microtiter plates.⁹ Microfluidic devices, consisting of arrays of chambers in which animals can be reared from mid-larval stages onward, allow for the constant supply of fresh culture medium and bacterial food in liquid suspension and enable the offspring produced by self-fertile hermaphroditic *C. elegans* to be washed away.^{10–14} In microfluidic designs, immobilization for imaging can be achieved by compression (via e.g. tapering “clamp” channels¹⁰ or deformable culture chambers¹⁴), or by temperature-driven sol–gel transitions.^{12,13}

Liquid culture has significant limitations, however. It increases the rate of death due to matricidal internal egg hatching,¹⁰ has systematic effects on physiology and lifespan, and often requires the use of antibiotics and antifungals.^{15,16} Microfluidic methods require off-chip connections for food and waste, increasing complexity, and often provide relatively low culture density, typically housing only 8–32 individuals. Of these designs, only the original device by Hulme *et al.* has been used for true, lifelong culture (2–4 weeks). Of note, that device was shown to not affect adult lifespan compared to standard liquid culture.¹⁰

To circumvent the difficulty of imaging rapidly thrashing animals and the physiological changes that accompany liquid culture, approaches for culture of individual *C. elegans* on solid gels have been proposed. The chief difficulty in solid culture is preventing animals from escaping by crawling off the imaging surface along the edges of the gel. One approach traps individual larval-stage animals under a coverslip in microchambers molded into agarose, allowing tens to hundreds of individuals to be imaged through the initial stages of development.^{17–19} To study late larval development and early adulthood, the animals must be manually transferred to larger microchambers.¹⁹ Another system consists of an array of agar pads with a geometry that prevents escape,²⁰ with each pad surrounded by a moat of chemo-repellant solution.²¹ In 384-well format, this system permits lifelong brightfield imaging of thousands of individuals, though with very low spatial resolution compared to most other approaches. Last, we previously described a system where gel pads were embedded in, and covalently crosslinked to, an array of holes cut through a glass slide. Individual animals were placed on a bacterial food drop on each gel pad, and sealed from the outside environment with PDMS silicone. This permitted culture of 16 individuals per slide, from hatching to death, with no effect on lifespan.²² All of these approaches require cumbersome setup, most offer relatively low culture density, and few provide the ability to perform lifelong studies.

To overcome these limitations, we developed a simple, high-density solid-media culture system that can be easily constructed from commercially available materials. This system allows 100–200 individual *C. elegans* to be cultured in isolation from one another, from hatching until death, on a solid gel medium supported by a microscope slide. We use a novel binding chemistry between the gel substrate and an elastomeric polydimethylsiloxane (PDMS) silicone cover material to confine each individual to its own miniature bacterial food pad on the surface of the gel. This apparatus is compatible with conventional bright-field and fluorescence microscopy, and requires no additional hardware or automation. We have also demonstrated additional applications for this combination of materials. PEG gels can be molded to form useful features prior to binding with PDMS, and/or can serve a substrate for pre-molded PDMS to form microfluidic devices. This makes possible a new class of microfluidic devices that can directly interface with hydrogel matrices.

Results and Discussion

Device Design

We aimed to produce a *C. elegans* culture device that could house isolated individuals from hatching throughout life (approximately two weeks at 25°C), in conditions as similar as possible to the standard solid-culture medium employed for the vast majority of *C. elegans* studies.²³ In standard culture, 6-cm petri dishes containing nematode growth medium (NGM) and 1.7% agar are “seeded” with a lawn of the slow-growing *E. coli* strain OP50 to provide a food source for up to 500 adults per plate. In miniaturizing these conditions to produce single-animal culture environments, we also aimed to meet the following requirements: (1) It should be possible to rear many individuals in extremely homogenous, high-density conditions. To ensure environmental homogeneity, the culture apparatus should be protected from outside contamination, which is a common problem in long-term *C. elegans* culture. (2) Individuals should remain isolated from one another throughout life. Escape at any stage, or transit of animals between individual enclosures, should be completely prevented. (3) The system should be compatible with brightfield and fluorescence microscopy using 5–20× objective lenses. This requires that individuals be visible at all times, that the surface of the culture medium be extremely flat to ensure that the animals remain on a fixed focal plane, and that the culture apparatus can be easily mounted on a standard microscope stage.

Here, we describe the results of these efforts: a microscope-slide-sized (25×75 mm) culture system in which 100–200 isolated individuals can be reared on a polyethylene glycol (PEG) hydrogel that closely resembles the standard agar plates. Droplets of *E. coli* OP50 are adsorbed to the gel to produce an array of “food pads”, with one animal per pad (Fig. 1a and b). Each individual is confined to its food pad by a tough, gas-permeable, and optically transparent PDMS elastomer membrane that is formed atop the entire apparatus (gel, food pads, and *C. elegans*). As detailed below, the PDMS covalently bonds to the hydrogel except where the food pads separate the PDMS–hydrogel interface. Thus, each individual can move freely along the plane of the gel surface within the extent its food pad, but not beyond; nor can it move out-of-plane in either direction. We refer to each individual’s food pad as its “corral”. The PDMS membrane permits gas exchange but prevents contaminants from the

outside environment from coming into contact with the gel surface, and reduces desiccation. Using this device, high-resolution images of each individual can be acquired throughout their lives (Fig. 1d, Movies S1 and S2). All components of the device are fully compatible with epifluorescence imaging, allowing multi-channel fluorescence images to be obtained throughout life (Fig. 2).

The foundation of the device is a ~1 mm thick PEG hydrogel supported by a standard glass microscope slide. A polyethylene frame is affixed to the surface of a glass slide, producing a reservoir that is filled with PEG reagents that react to produce a covalently crosslinked hydrogel (Fig. 1b and c). Specifically, we employ a large 8-arm thiolated PEG ($M_n \approx 10$ kDa), which acts as a nucleophile in a Michael addition reaction with a smaller linear PEG-diacrylate ($M_n \approx 700$ Da) (Fig. 3a).^{24,25} These compounds are separately dissolved in a variant of the standard nematode growth medium (NGM; see Methods) with the pH adjusted to 6.3 in order to facilitate the Michael chemistry (standard NGM is pH 6.0). The solutions are mixed at a stoichiometric ratio to yield a final concentration of 8.5% w/v PEG, and dispensed into the frame/slide apparatus under a separate glass coverslip. A gel forms in approximately 1.5 hours at room temperature, at which time the coverslip can be gently removed, yielding a very smooth top surface of uniform height.

Once the hydrogel is set, droplets of concentrated *E. coli* OP50 are dispensed onto the surface of the gel in a grid, ensuring that each individual receives the same amount of food. As the droplets adsorb to the surface, a single *C. elegans* egg at the “pretzel stage” (immediately prior to hatching)²⁶ is gently transferred into each droplet. Eggs at this stage are most robust to handling and transfer; this also ensures that the population is roughly synchronized (Fig. 1b and c). Last, we pour pre-mixed but uncured Sylgard 184 on top of the apparatus. Sylgard 184, a PDMS that crosslinks into an elastomeric rubber, is commonly used in the construction of microfluidic devices (Fig. 1b and c). Sufficient PDMS (mixed at the standard 10:1 ratio of base to curing agent) is added to produce a ~0.6 mm thick layer over the surface of the slide. Overnight, as the eggs are hatching, the PDMS cures into a soft rubber membrane. By 24 hours, the larval *C. elegans* are entrapped and unable to penetrate the PDMS; after approximately 48 hours at room temperature the final material properties of the membrane are achieved. (If animals older than the first larval stage are placed under uncured PDMS, we have observed that they are able to crawl upward into the PDMS as it cures.) After fabrication is complete, we store the devices in a humid environment (75–90% relative humidity), which is sufficient to retain the integrity of the gel and viability of its occupants for upwards of six weeks. A detailed, step-by-step protocol for corral assembly is provided as supplementary information (Protocol S1).

It is important to prevent self-fertile hermaphrodite *C. elegans* from reproducing in the devices, and thus filling their corrals with offspring. We have found it most convenient to use individuals with the temperature-sensitive *spe-9(hc88)* mutation, which at the restrictive temperature of 25°C produce defective sperm that are unable to fertilize eggs. In all other known aspects, *spe-9(hc88)* animals are physiologically wild-type.^{27,28} Below, we also demonstrate that lifelong RNAi against *pos-1* also effectively sterilizes *C. elegans*, relieving the requirement for the use of a specific genetic background at a specific culture temperature.

PEG–PDMS Binding Chemistry

PEG gels produced via Michael addition (Fig. 3a), as described above, interact with Sylgard PDMS in a striking fashion: liquid PDMS that cures atop such a gel will crosslink with the gel. Similarly, liquid PEG constituents will crosslink with pre-cured PDMS as they gel. In either case, the PDMS cannot be removed from the PEG without tearing the gel (Fig. 3d), which demonstrates that the PEG–PDMS binding is at least as strong as the covalent PEG–PEG crosslinks. This PEG–PDMS crosslinking can be blocked by adsorbing a material to the surface of the PEG gel that prevents the physical interaction of the PDMS and PEG layers. In our apparatus, the food bacteria OP50 acts as such a material, and prevents crosslinking between the two polymers in the regions of the food pads (Fig. 3d). *C. elegans* can move freely along the surface of the food pads underneath the PDMS, but are blocked from movement beyond at the edges of the pads by the PEG–PDMS crosslinking.

We observe only limited crosslinking between blocks of PDMS and PEG brought into contact after both have solidified, however. Further, there is no reaction between PDMS and agarose gels, or PEG-diacrylate hydrogels produced via free-radical polymerization.^{22,29,30} PDMS cured atop agarose or free-radical-polymerized PEG gels can be removed cleanly (not shown), and *C. elegans* are not trapped by PDMS on the surface of food pads deposited on such gels.

We hypothesize that the PEG gel crosslinks to PDMS via unreacted PEG-acrylate groups that participate in the PDMS curing chemistry. Sylgard 184 consists of two components (Fig. 3b): a “base” reagent consisting primarily of dimethylvinyl-terminated polydimethylsiloxane, and a “cure” reagent containing poly-(dimethyl methylhydrogen) siloxane and a platinum catalyst. The catalyst promotes a hydrosilylation reaction, in which the Si–H bonds in the methylhydrogen siloxane groups of the cure reagent are added across the unsaturated vinyl groups of the base reagent. Given the chemical similarity of the dimethylvinyl siloxane groups terminating the base reagent and the acrylate groups terminating the PEG-diacrylate chains, we suspected that PEG-diacrylate was also participating in the hydrosilylation that produces the PDMS elastomer (Fig. 3c). We reasoned that if this was true, other hydrogels with free terminal C=C double bonds would also be able to crosslink into a PDMS elastomer. We therefore acquired allyl-functionalized agarose,³¹ which forms a hydrogel via the physical tangling of the agarose polymer rather than covalent linking of separate monomers. We found that PDMS cured atop allyl agarose gels prepared with OP50 food pads are able to trap *C. elegans* as effectively as PEG gels. (We did not observe allyl agarose to tear when the PDMS was removed, however, unlike the PEG gels.) Nevertheless, PEG remains most convenient for this application: unlike allyl agarose, it does not need to be pipetted into place while molten.

Corrals Provide Benign Culture Conditions for *C. elegans*

Our previous work used this culture system to compare the physiology of long- vs. short-lived individuals within a wild-type population of *C. elegans*.⁶ To validate the general utility of these methods for comparisons between different populations, or between different culture regimes, we examined whether this system alters key aspects of *C. elegans* physiology. It is well known that the health and lifespan of *C. elegans* are highly dependent

on culture conditions.^{2,32–34} In particular, suboptimal and/or stressful environments can slow development,³⁵ halt production of oocytes in the germline³⁶ and either extend or shorten lifespan, depending on the degree of stress.^{37,38} Therefore, we set out to compare the biology of *C. elegans* reared in standard culture conditions vs. in our environment along three axes: development, reproduction, and lifespan. We further examined whether various well-characterized genetic mutants and RNAi conditions yielded phenotypes comparable to those obtained using standard culture.

Beyond the switch from agar to PEG as a gelling agent, we made three small changes in the formulation of our growth medium compared to standard NGM. First, in order to promote the Michael addition of the PEG monomers, we increased the pH to 6.3, from the standard 6.0. Next, CaCl_2 is well known to crystallize out of solution in NGM, which interferes with high-resolution microscopy. As its use in NGM has previously been reported to be unnecessary,³⁹ we omitted this salt. Last, we initially noticed symptoms of sterol depletion in animals reared in our apparatus (i.e. poor reproduction and a skinny, clear appearance). As *C. elegans* cannot synthesize sterols themselves, this suggested that cholesterol bioavailability in our conditions was low. We therefore increased the concentration of cholesterol fourfold from standard conditions. We refer to this modified medium (pH 6.3, lacking CaCl_2 , and with supplemental cholesterol) as “corral-NGM”. For the following experiments, we compared phenotypes of animals reared in our corral-NGM/PEG/PDMS culture system to those of animals reared on standard plates with corral-NGM and 1.7% agar (see Methods).

Developmental timing in *C. elegans* is exquisitely sensitive to nutrient availability^{33,35} and other environmental stressors.^{32,40} We therefore examined the total time of larval development, from hatching to the molting event that marks the transition from the fourth larval stage to adulthood. We compared our individual culture system to bulk culture in petri dishes, using 1.7% agar with corral-NGM. We measured time-to-adulthood in side-by-side experiments under these conditions, using a transgenic *C. elegans* strain that begins expressing GFP fused to the adult-specific collagen COL-19 at the larval-to-adult transition.⁴¹ Overall, the timing of development was very similar in our corral system compared to bulk culture on traditional agar plates (Fig. 4a). We did observe a small increase in the median time from hatching to adulthood in our system: 39.5 hours (95% bootstrap CI: 39.1–39.9) in our corral system versus 38.4 hours (38.1–38.8) on plates. This 1.1-hour difference in median developmental time is unlikely to be biologically meaningful, as it is approximately the same as the inter-replicate variability in median time to adulthood on agar plates (1.2 hours).

The rate of progeny production in *C. elegans* is also very sensitive to environmental perturbations.^{32,35} Thus, we assayed the total number of offspring produced within the first 12 hours of adulthood. Again using side-by-side experiments to compare our system to agar plates with corral-NGM, we observed very similar patterns (Fig. 4b). There was a small increase in median progeny production in corrals: 43 (95% bootstrap CI: 40–45) vs. 40.5 (37–48) on agar plates. Again, this difference is unlikely to be biologically meaningful. For comparison, the agar plate replicate differed by one offspring.

Last, we examined lifespan, which is also very sensitive to changes in environment and diet. Stressors can have the expected effect of decreasing lifespan, or of increasing lifespan by a process known as “hormesis” in which induced stress-response programs produce a net benefit to the organism.^{34,37} From side-by-side experiments (Fig. 4c), we observed a consistent but slight increase in median lifespan in our culture conditions (11.7 days, 95% bootstrap CI 11.0–12.5) compared to agar plates with corral-NGM (9.6 days, 8.5–10.4). Again, the magnitude of this difference (2.1 days) was smaller than the inter-replicate difference in median lifespan on agar plates (4.9 days). Minimum and maximum lifespan were essentially identical between agar plates and our corral system.

We quantified the population variability in each assay via the interquartile range (IQR) of our measurements (25th to 75th percentile). For all assays, the corral conditions had a smaller IQR than standard agar culture (Fig. 4d), though this difference was only statistically significant for the lifespan assay. (Using a two-tailed permutation test for difference in interquartile range, $p = 0.0111$ for the lifespan assay; for developmental timing, $p = 0.0834$; and for progeny production, $p = 0.1498$.) In addition, we observed much less inter-replicate variability among our corral samples compared to the agar plates in the developmental timing (Fig. 4a) and lifespan assays (Fig. 4c). Lifespan measures, in particular, are well known to exhibit substantial run-to-run variability in *C. elegans*, as we also observed in our agar plate experiments, but not in the corrals. Furthermore, lifespan data from our previous work,⁶ collected in seven different experimental batches over the course of several months, shows at most one day of batch-to-batch variability in measured median lifespan (Fig. 5a). This further demonstrates the high degree of homogeneity and repeatability offered by this system.

We next tested whether mutations previously known to modify lifespan still produced the expected phenotypes in our system (Fig. 5b). The short-lived mutant *daf-16(mu86)* and the long-lived strains, *ife-2(ok306)*, *clk-1(qm30)*, and *age-1(mu86)* were crossed into the *spe-9(hc88)* background and examined in our corral system. In this environment, these strains modified lifespan to a degree broadly comparable with those reported for standard conditions (see figure legend for details).^{42–45}

Finally, we have found that standard RNAi protocols are effective in this system. We modified our standard system by substituting concentrated RNAi bacteria for the standard OP50 food and adding ampicillin and the IPTG inducing agent to the corral-NGM recipe. Figure 6 shows results using the RNAi-competent OP50-derivative xu363 as feeding bacteria;⁴⁶ we have observed equivalent effects using the standard RNAi strain HT115 (not shown). First, lifelong RNAi against GFP effectively knocks down fluorescence from a *mir-1p::GFP* transgenic strain relative to control RNAi (Fig. 6a,b). Second, RNAi against *pos-1*, a gene essential for germ-cell specification in development,⁴⁷ produces a 100% penetrant embryonic arrest phenotype (Fig. 6c). Thus, RNAi against *pos-1* provides an effective alternate method of preventing reproduction in our culture system. Note, however, that of the two genome-wide RNAi screens for lifespan extension that examined *pos-1*, one but not both reported a moderate increase of lifespan with *pos-1* RNAi.^{48,49} Assays in our system likewise demonstrated a lifespan phenotype with highly variable penetrance (data not shown).

Together, these observations suggest that our corral apparatus does not systematically alter *C. elegans* physiology, is compatible with RNAi protocols, and moreover provides a more homogenous environment than standard bulk culture.

Surface patterning of PEG gels

As the ability to produce raised and/or indented features in soft materials has proven immensely valuable for microfluidic applications, we developed protocols to pattern the surface of PEG hydrogels. Generally, we form the PEG gel under a glass coverslip to produce a smooth top surface. We reasoned that by substituting this coverslip for a surface-patterned material, we could transfer the patterning into the PEG gel. We therefore used replica molding to produce a PDMS cast of an SU-8 master, and used that cast to form a fluorinated ethylene propylene (FEP) replica of the original master via hot embossing.⁵⁰ Curing a PEG gel underneath a molded FEP sheet transferred the pattern to the surface of the gel (Fig. 7a, b). Many materials that can be more readily molded than FEP (such as PDMS or polyurethane) appear to bond to the curing PEG. FEP, however, is sufficiently inert for this application.

Inspired by similar methods using agar gels,¹⁷ we applied these techniques to our *C. elegans* culture system. By using a FEP mold, we were to create an array of pre-formed indentations in the PEG gel (Fig. 7a and b), into which we pipetted bacteria to produce food pads with very uniform size and spacing (Fig. 7c). *C. elegans* can be readily reared in these surface-patterned gels, and appear in all respects phenotypically similar to those reared on smooth-surface gels.

PDMS Microfluidic Devices with PEG-hydrogel Substrates

Though our purpose in developing the PDMS–PEG-gel binding chemistry was to culture individual *C. elegans* on solid media, these methods may have broader utility. One such application is to provide an alternative substrate for PDMS microfluidic devices. Typically, microfluidic devices are constructed by affixing PDMS blocks with pre-patterned micro-channels to glass or another water-impermeable material. There are potential cases where a substrate into which water and dissolved molecules can diffuse would be a benefit, however. For example, in tissue engineering or wound therapy, the delivery of media and trophic factors to cells in or under hydrogels could be assisted by a microfluidic “irrigation system”. The ability to produce temporally and spatially controlled gradients of these factors, or to pinpoint delivery of specific factors or cell types to different gel locations, would also have implications for work on *in vitro* differentiation or tissue modeling.

To demonstrate the feasibility of PDMS-on-PEG-hydrogel microfluidic devices, we first patterned a simple serpentine channel with two inlets and one outlet into a PDMS block using standard soft lithography. Because solid PDMS and pre-cured PEG hydrogels do not spontaneously bond (see above), we transferred a thin layer of uncured PDMS to the bottom of the PDMS block via contact printing.⁵¹ Briefly, we spin-coated a thin layer of uncured Sylgard 184 PDMS onto a slide and pressed the PDMS block gently against the slide. We then placed the PDMS block against a pre-cured PEG hydrogel. The thin layer of chemically active PDMS bonded to both the pre-cured PDMS block and the PEG gel. While the

combined device is more delicate than a PDMS-on-glass assembly, the strength of the PDMS–PEG interaction was sufficient to support the flow of liquid through a 120 mm long channel with a 350 μm deep $\times$ 1.2 mm wide cross-section (Fig. 7c).

Conclusions

We have detailed “worm corrals”, a novel, miniaturized system for long-term longitudinal culture and analysis of individual *C. elegans*. Our approach allows us to comfortably house up to 200 individuals on a glass slide. To image an entire food pad in a single image, 10 $\times$ magnification is most appropriate. With smaller food pads (achieved by surface patterning the PEG gel), or relaxing the constraint that an entire food pad fit in a field of view, higher magnification can easily be used. Overall, this method is simple to set up, requires only commercially available materials, and is relatively inexpensive. Our previously published experiments using this system further demonstrate that it is straightforward to scale this method to the examination of hundreds or thousands of individuals in parallel, or in batches over time.⁶

The chief limitation of this culture system also stems from a significant strength: after the PDMS cures, the apparatus is sealed from the outside environment, save for gas exchange. This complicates retrieving individuals from the system for further characterization. More significantly, it limits our ability to add drugs, RNAi bacteria, or other materials to the culture system at specific time-points. Notably, many useful drugs (such as the sterility-inducing compound 5-fluoro-2'-deoxyuridine, known as FUDR⁵²), lifespan-prolonging compounds, and RNAi conditions must be applied only to adults to avoid altering or inhibiting normal *C. elegans* development. As it is difficult to place animals beyond the first few larval stages in our culture system (larger animals often burrow into the PDMS as it cures), such interventions are currently incompatible with this system. Further, we have anecdotally noted that solubility and/or bioavailability of certain small molecules may be lower in our PEG-based gels than in standard agar gels. As above, supplemental cholesterol is necessary in our system. Perhaps related, we have found that the compound C22, which produces 100% sterility in standard agar plates,⁵³ has more variable effects in our corral system.

The homogeneous nature of each corral within the overall apparatus ensures that the environmental variability inherent in other culture methods is minimized. Moreover, we achieve this without substantial alteration to the basic physiology of *C. elegans* grown on solid media in bulk culture. Specifically, we demonstrated that population medians of three traits that are known to be very sensitive to environmental conditions – developmental timing, reproductive rate, and lifespan – are essentially unaltered by our culture system. Moreover, we showed that the interquartile ranges of these traits across a population are systematically decreased on our system compared to standard conditions, further demonstrating the uniformity of the corral environments.

One significant difference between our corral system and standard solid culture is that animals in the corrals are constrained to stay on their bacterial food source at all times. In standard culture, the lawn of bacteria does not extend to the edges of the petri dish, allowing

animals to leave the food area. While we demonstrated above that this change has no effect on developmental timing, early-life fecundity, or lifespan, there are almost certainly specific phenotypes and/or genetic backgrounds for which the inability to leave food is salient.

Finally, the novel interaction between the PEG hydrogel and PDMS may have applicability beyond the specific domain of *C. elegans* culture. This chemistry is easy to achieve, and appears to require only that a material bearing free, terminal C=C double bonds be in immediate contact with Sylgard PDMS. Consistent with our proposed hydrosilylation mechanism (Fig. 3c), experiments with allyl-substituted agarose suggest that there is no requirement for the terminal C=C bond to be part of a larger conjugated double-bond system, as would be the case for a nucleophilic addition mechanism. We believe that the ability to bond hydrophobic PDMS to a hydrated gel represents a generally useful advance, and have developed protocols to both pattern the PEG gel surface and to attach pre-cured PDMS devices to PEG gels. Used with monomeric PEG-acrylates (or other compounds with similar functional groups), this chemistry may also be generally useful for the surface modification of PDMS, beyond the specific application of grafting PDMS to PEG gels.

Methods

Production of culture devices

“Corral nematode growth medium” (corral-NGM) is produced via a slight modification of the standard NGM^{1,23} recipe: 0.3 g of sodium chloride, and 0.25 g of peptone are dissolved in 97.5 mL of distilled water, to which is added 0.1 mL of 1M magnesium sulfate and 2.5 mL of 1M potassium phosphate buffer (0.8M KH₂PO₄, 0.2M K₂HPO₄, titrated to pH 6.3). Filter-sterilized, this solution can be stored indefinitely.

The steps in constructing a corral slide are as follows. (A detailed protocol is provided as supplemental information.) First, frames machined out of adhesive-backed ultra-high-molecular-weight polyethylene (McMaster-Carr; Elmhurst, IL, USA; outer dimensions 25×75 mm; inner dimensions 18×62 mm; thickness 1.2 mm) are affixed to surface of a standard 25×75×1 mm glass microscopy slides (VWR International; Radnor, PA, USA). We have found that machined aluminum frames, adhered to slides via a thin layer of Sylgard 184 (Dow Corning, Midland, MI), perform equivalently well. The assemblies are cleaned with ethanol and DI water, sealed in aluminum foil, and autoclaved.

Next, corral-NGM is used to separately dissolve an 8-armed PEG-thiol (MW=10 kDa; Jenkem Technology; Beijing, P. R. China; Item Number: 8ARM(TP)-SH-10K) and PEG-diacrylate (MW = 700 Da; Sigma-Aldrich; St. Louis, MO, USA; Catalog Number: 455008 Aldrich) at 133 mg/mL and 37 mg/mL, respectively. The two PEG solutions are mixed at a 1:1 ratio, and 4 µL/mL of cholesterol (from a 5 mg/mL stock in 95% ethanol) is added and vortexed vigorously. An autoclaved 25×75 mm glass cover slip is placed atop each polyethylene frame and the interior reservoir is filled with approximately 1.3 mL of the PEG mixture. The completed device is placed in a humid chamber (a 10 cm petri dish lined at the edges with kimwipes soaked with 1.5 mL water) while the PEG hydrogel cures for approximately 1 hour and 45 minutes at room temperature. After gelation is complete, the glass coverslip is carefully removed.

Last, bacterial food and *C. elegans* are transferred to the surface of the gel. To deliver a consistent amount of biomass to each individual well, we spin down and concentrate overnight cultures of *E. coli* OP50. We resuspend bacterial pellets in a volume of M9 equivalent to twice the wet weight of the pellet. (We refer to this as "50% w/v OP50".) An array of 0.4 μ L droplets of the 50% w/v OP50 is deposited onto the gel, and individual pretzel-stage eggs are then placed into each droplet using an eyelash pick. After the *E. coli* droplets have fully adsorbed to the gel surface, 1.2 mL of PDMS (Sylgard 184, Dow Corning, Midland, MI) mixed in the standard 10:1 base:cure ratio, is gently deposited over the PEG gel using a syringe. The liquid PDMS spreads evenly across the surface and cures within 48 hours. (Over a 25×75 mm slide, 1.2 mL of PDMS will yield a membrane ~640 μ m thick.) The assembly is then returned to the humid chamber and maintained there for the remainder of the experiment.

Measurement of *C. elegans* development, reproduction, and survival

Developmental timing was measured using expression of COL-19::GFP as a quantitative marker for the onset of adulthood.⁴¹ For each of two biological replicates, approximately 70 animals of strain TP12 (bearing the COL-19::GFP transgene) were placed on a corral slide (constructed as above) and another 70 were split across two 6 cm petri dishes filled with 5 mL of 1.7% agar in corral-NGM. Each petri dish was seeded with 14 one- μ L droplets concentrated OP50 (0.4 μ L/individual). The corral slide (in its humid chamber) and the petri dishes were incubated side-by-side at 25°C. Every hour, each individual was manually scored for the presence of GFP until 90% of individuals from each condition had reached adulthood.

For the reproductive rate assay, we counted the number of unfertilized oocytes produced by *spe-9(hc88)* individuals at the restrictive temperature. For each of two biological replicates, approximately 40 *spe-9* animals were placed on a corral slide and another 40 were placed in individual wells of 24-well plates, where each well had previously been filled with 1 mL of 1.7% agar in corral-NGM and seeded with 0.4 μ L of concentrated OP50. The total number of oocytes produced by each individual was manually counted 55 hours post-hatching (12 hours after the average onset of adulthood).

We examined lifespans by measuring survival in bulk culture in petri dishes and in "worm corral" devices incubated side by side at 25°C. Our bulk-culture conditions consisted of 40 *spe-9(hc88)* individuals per 6 cm petri dish, filled with 5 mL of 1.7% agar in corral-NGM and seeded with 16 one- μ L droplets of concentrated OP50 (0.4 μ L/individual). For each of two biological replicates, we compared 80 individuals in bulk culture (on two petri dishes) to 80 in a single corral device. On each day of adulthood we assayed survival via response to touch stimulus. Survival curves were constructed using the Kaplan-Meier procedure to account for individuals that escaped the bulk-culture petri dishes.

RNAi

Single colonies of RNAi bacteria (strain xu363)⁴⁶ were selected from a plate and inoculated overnight at 37°C with shaking, in LB supplemented with 100 mg/mL ampicillin. The overnight cultures were spun down and resuspended in M9 to 50% w/v and stored in aliquots

at 4°C until use. Standard corral media was prepared as above, supplemented with 100 mg/mL ampicillin and 1 mM IPTG. Bacteria and animals are transferred into the system as described above.

Surface patterning PEG gels

To produce a surface-patterned FEP sheet, we employed a two-step replica-molding process. First, we patterned features onto a silicon wafer with SU-8 photoresist using standard photolithography, and cured a 1mm thick PDMS (5:1 base to cure ratio) on top of the wafer at 70°C for 30 minutes. The PDMS was removed, inverted, placed atop a standard glass slide, and baked on a hotplate at 250°C for an additional hour. This protocol, originally described in reference,⁵⁰ produces PDMS stable at the high temperatures required for FEP molding.

A 0.5 mm thick sheet of FEP (2000A Teflon FEP Film, American Durafilm, Holliston, MA) was then clamped between the PDMS mold and a glass slide and placed inside an aluminum foil enclosure on a 275°C hot plate for 35 minutes. After removal from the heat, the clamped apparatus was allowed to cool to room temperature before the FEP was removed. Patterned PEG gels were formed by curing PEG underneath the embossed FEP for 12 hours (a shorter duration of curing leads to destruction of the PEG gel when the FEP is removed, which we did not observe with glass coverslips).

Bonding of PEG gels to microfluidic devices

We attached a pre-formed PDMS block to a pre-formed PEG gel using micro-contact printing with PDMS as an adhesive.⁵⁴ We fabricated the PDMS portion of the microfluidics device with standard soft lithography and a 5:1 PDMS base:cure ratio, using 350 µm tall features 3D printed onto an ABS surface as a master. Channel inlets were made with a biopsy punch. We then polymerized a flat PEG hydrogel surface as described above. To bond the PDMS block to the PEG hydrogel surface, 1 mL of uncured PDMS (mixed at a 5:1 base:cure ratio) was dispensed onto a glass slide and spun at 1200 rpm for 5 minutes in a spin coater, creating a layer approximately 20 µm thick. We then gently brought the PDMS block into contact with the surface of the glass slide to transfer uncured PDMS to the base of the block, and then placed the block on the surface of the hydrogel. We put the assembly into a humid chamber (a petri dish with moistened kimwipes), and heated it at 60°C in an oven for 30 minutes to cure the PDMS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Culture system and fabrication

(a) Schematic of device, in which individual *C. elegans* are reared in a dense array. Each animal occupies a single pad of food bacteria adsorbed to a PEG hydrogel, which is supported by a polyethylene frame attached to a glass slide. PDMS silicone cured atop the device binds covalently to the PEG gel wherever those materials are in direct contact (i.e. all locations except the positions of the food pads); thus each animal is trapped on the two-dimensional surface of its food pad by covalent bonds in all directions. (b) Cartoon "exploded view" showing components of assembled device. (c) Fabrication process. 1: A pre-cut polyethylene frame is affixed to the surface of a standard glass slide. 2: Reactive multifunctional PEG monomers, suspended in nematode growth media, are pipetted into the reservoir produced by the frame and glass slide. After approximately 90 minutes, the PEG monomers crosslink into a hydrogel. 3: Small droplets of a food bacterial are pipetted onto the gel surface. As the droplets adsorb to the surface, a single *C. elegans* egg is deposited into each droplet. 4: Liquid PDMS is poured over the surface of the device and allowed to cure at room temperature for 24 hours. (d) Micrographs of one individual *C. elegans* imaged throughout its life within the device (time is shown as days and hours post-hatching). The inset shows the anatomical detail visible in a live, unanesthetized, and freely moving animal: the nuclei of intestinal cells (visible as clearings in the dark granules of those cells' cytoplasm), structures of the developing vulva (the egg-laying apparatus), and the anterior and posterior pharyngeal bulbs.

Figure 2. Fluorescence imaging

(a) Individuals bearing a *mir-63p::GFP* transgene⁵⁵ were imaged throughout life, capturing brightfield, GFP, and red-wavelength autofluorescence.⁵⁶ Exposure time remained constant throughout the experiment. These images show that the GFP signal peaks in early adulthood (around three days post-hatch), while intestinal autofluorescence builds up slowly over time.

(b) Brightfield and GFP images of individuals from the microRNA reporter strain *let-7p::GFP*. The fluorescent signal is dramatically upregulated in 4th-larval stage animals (middle panels), as initially reported.⁵⁷

Figure 3. Crosslinking chemistry

(a) Mechanism of PEG gelation. An 8-arm PEG-thiol and a PEG-diacrylate suspended in aqueous buffer form a gel via Michael addition at room temperature and neutral pH. In this reaction, the thiol groups perform a nucleophilic attack on the terminal C=C bond of the acrylate groups, resulting in a covalently crosslinked PEG network. (b) Mechanism of PDMS curing. A platinum catalyst mediates the addition of a methylhydrogen siloxane group in the "cure" reagent across the terminal vinylic C=C bond of the "base" reagent. (c) Proposed mechanism of PEG-PDMS crosslinking. The similarity between the terminal C=C functional groups in the PDMS base reagent and in the PEG-diacrylate suggests that a

methylhydrogen siloxane in the PDMS cure reagent can be added across the terminal double bond of the PEG-diacrylate. **(d)** The strength of the PDMS–PEG interaction is such that PDMS cured atop a PEG gel will rip the surface of the gel when it is removed, except at locations where bacterial food pads separate the two materials.

Figure 4. Comparison of “worm corral” system to standard bulk culture

(a) Overall developmental timing in our system versus standard conditions was assayed by measuring the time from hatching to adulthood. The fraction of animals expressing a fluorescent reporter for an adult-specific collagen gene is plotted against time. Two replicates were conducted for each condition; individual replicates are in lighter lines while the pooled data are plotted with heavy lines. (b) To measure effects of our system on reproduction, we measured the total number of offspring produced in the first 12 hours of adulthood for individuals in our corral system or in bulk culture. Kernel density estimates of the distribution of offspring count are shown (x-axis: number of offspring; y-axis: probability density). (c) Survival curves show the modest effects of our system on overall lifespan. (d) For each assay, the 25th, 50th, and 75th percentile of the pooled “corral” and “standard agar plates” populations are plotted on the same axes as above. The 25th and 75th percentiles are the left and right ends of the bars, respectively, and the median is marked by a vertical line. For both developmental timing and lifespan, the difference between medians is smaller than the inter-replicate variability in standard conditions. In all three assays, the interquartile range (75th – 25th percentile) is narrower in our corral conditions.

Figure 5. Corral system produces consistent replicates and recapitulates known lifespan phenotypes

(a) Lifespan curves are shown for batches of *spe-9(hc88)* individuals reared in corral assemblies produced on different weeks. The curves are largely superimposable, showing little variation in median lifespan. (b) Lifespan curves for: the *spe-9(hc88)* strain (all batches from panel a, pooled) which is “wild type” for the purpose of longevity comparisons; the short-lived mutant *daf-16(mu86)*; and the long-lived mutants *ife-2(ok306)*, *clk-1(qm30)*, and *age-1(hx546)*. Each mutant allele is in the genetic background of *spe-9(hc88)*. These mutations alter lifespan to a similar degree to that reported in standard culture conditions. In standard conditions, *daf-16(mu86)* was reported to shorten lifespan by 20% compared to wild-type controls at 25°C;⁴² in coralls this figure is 25%. Different reports provide varying degrees of lifespan extension for *ife-2(ok306)* in standard conditions; one well-powered study at 25°C measured a 23% extension.⁴³ We observed a smaller lifespan extension of 5% in coralls. In standard conditions at 25°C, *clk-1(qm30)* yields 21% lifespan extension;⁴⁴ we observed 38% extension in coralls. Finally, in standard conditions at 25°C, *age-1(hx546)* extends lifespan by 105%;⁴⁵ we also observed 105% extension in coralls.

Figure 6. RNAi in corrales

(a) Individuals bearing the *mir-1p::GFP* fluorescent transgene (in the sterile *spe-9(hc88)* background) show strong GFP signal in the pharynx when fed bacteria producing RNAi against *pos-1* (which does not target GFP). (b) RNAi against GFP drastically reduces the fluorescent signal compared to panel a, which shows images acquired with the same exposure time. The average fluorescence intensity of GFP RNAi images is 66.0% of that of the control RNAi in panel a. (Each image's intensity was summarized by the 90th percentile of pixel intensities across the pharyngeal region. The mean intensities differ between control and GFP RNAi conditions with $p=0.001$ per a two-sample *t*-test.) (c) RNAi against *pos-1*, essential for germ-cell specification in development,⁴⁷ completely prevents reproduction of wild-type (N2) individuals. *pos-1*(RNAi) arrests all of the approximately 300 embryos produced throughout life, for all individuals so treated. Without RNAi, roughly 100% of N2 embryos hatch, leading to rapid depletion of the bacterial food (not shown).

Figure 7. Pre-patterning of PEG and PDMS

(a) The surface of PEG gels can be physically patterned. PEG hydrogels that are crosslinked under an embossed FEP surface will retain those features after the FEP is removed. (b) Micrograph of PEG gel with 1.6 mm diameter, 60 μ m deep circular indentations molded into its top surface. (c) Image of portion of a “worm corral” apparatus produced using these divots. (d) The bottom of a pre-cured PDMS block can be coated with a thin layer of uncured PDMS via contact printing and bonded to a pre-cured PEG gel. This allows PDMS microfluidic devices to be attached to a PEG gel.