Computer-Assisted Transgenesis of *Caenorhabditis elegans* for Deep Phenotyping

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ABSTRACT A major goal in the study of human diseases is to assign functions to genes or genetic variants. The model organism *Caenorhabditis elegans* provides a powerful tool because homologs of many human genes are identifiable, and large collections of genetic vectors and mutant strains are available. However, the delivery of such vector libraries into mutant strains remains a long-standing experimental bottleneck for phenotypic analysis. Here, we present a computer-assisted microinjection platform to streamline the production of transgenic *C. elegans* with multiple vectors for deep phenotyping. Briefly, animals are immobilized in a temperature-sensitive hydrogel using a standard multiwell platform. Microinjections are then performed under control of an automated microscope using precision robotics driven by customized computer vision algorithms. We demonstrate utility by phenotyping the morphology of 12 neuronal classes in six mutant backgrounds using combinations of neuron-type-specific fluorescent reporters. This technology can industrialize the assignment of *in vivo* gene function by enabling large-scale transgenic engineering.

KEYWORDS *C. elegans*; high throughput; transgenesis; deep phenotyping; automation

RECENT advances in sequencing technology have made it feasible to genotype hundreds of thousands of patients to identify disease-relevant human gene variants. To take advantage of this new information and rapidly probe gene function there is a need for large-scale high-throughput genetic modification and phenotyping of model organisms. Its small size, ease of culture, rapid development, facile genetics, and large mutant libraries make *Caenorhabditis elegans* a powerful model organism for assigning functions to genes relevant to human disease (Shaye and Greenwald 2011). This organism is also a paramount model for discovering basic biological mechanisms. For example, the neurodevelopmental guidance molecule Netrin (Ishii *et al.* 1992), the programmed cell death caspases and their regulators (Yuan and Horvitz 1990; Yuan *et al.* 1993), the role of small RNAs (Lee *et al.* 1993; Wightman *et al.* 1993), regulators of organismal aging (Kenyon *et al.* 1993; Kimura *et al.* 1997), and many other key pieces of biology have emerged first from studies in this simple model organism.

A major strength of *C. elegans* is the wealth of vector and strain resources available. Vector resources include the Promoterome (Dupuy *et al.* 2004), a collection of 5526 predicted promoter sequences; the ORFeome (Reboul *et al.* 2003; Lamesch *et al.* 2004), a collection of 12,625 cDNAs; and the TransgeneOme (Sarov *et al.* 2012), a collection of 16,102 genomic DNA clones in which protein coding sequences have been tagged with GFP and an affinity epitope. Strain resources include 6841 "knockout" strains generated by the *C. elegans* Deletion Mutant Consortium (*C. elegans* Deletion Mutant Consortium 2012), each of which carries a small genetic deletion disrupting 1 of 6013 genes; 2007 fully sequenced strains generated by the Million Mutation Project (Thompson *et al.* 2013), which together carry 183,327 nonsynonymous mutations in 19,666 protein-coding genes, including 12,594 predicted knockout mutations in 8150 genes; and ~10,000 other strains that have been generated by independent laboratories and made publicly available through the *C. elegans* Genetics Center

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(https://www.cbs.umn.edu/research/resources/cgc). These resources, in addition to tools such as RNAi (Fire *et al.* 1998; Kamath and Ahringer 2003), CRISPR/Cas9 (Friedland *et al.* 2013; Waaijers *et al.* 2013), and cell-specific fluorescent markers—including optogenetic (Nagel *et al.* 2005) and calcium-imaging reagents (Kerr *et al.* 2000; Tian *et al.* 2009)—have made *C. elegans* one of the richest genetic models available.

To fully exploit these resources, it is necessary to systematically introduce collections of genetic vectors into panels of mutant strains (Figure 1A). For example, to assign functions to genes, one could introduce a collection of cell-type-specific fluorescent reporters into a panel of mutants for "deep phenotyping" of multiple cellular features simultaneously as demonstrated in Figure 1B. Or, a collection of epitope-tagged DNA-binding factors could be introduced into a panel of mutants and assayed using chromatin immunoprecipitation and DNA sequencing (ChIP-Seq) to detect altered patterns of DNA binding. Or, a collection of neuron-specific optogenetic reagents could be introduced into a panel of synaptic mutants to identify behavior-relevant deficits. The possibilities for this kind of experiment are limitless.

However, a major bottleneck to these approaches is the rate-limiting step of transgenesis by microinjection (Mello *et al.* 1991), an immensely useful technology that has not changed since it was developed >20 years ago. Although *C. elegans* offers the fastest transgenesis of any animal model, manual injections remain laborious and low throughput, and thus are not amenable to the kind of large-scale screening applications available for bacteria, yeast, or tissue culture. Conventional *C. elegans* transgenesis involves (1) using a dissecting microscope to mount 1–10 animals in a drop of oil on an agar pad on a coverslip, (2) transferring the coverslip to an inverted microscope equipped with a 40× objective and micromanipulator, (3) using a fine glass needle loaded with DNA solution to penetrate and fill the gonad of each animal, and (4) returning to the dissecting microscope and recovering the injected animals to standard growth medium. This procedure must be performed quickly, as animals begin to desiccate within ~10 min under oil. New trainees typically require 2–3 weeks to learn this technique, and even experienced investigators rarely inject more than four to six strains per day due to fatigue from the labor-intensive process. A high-throughput platform for transgenesis could have a major amplifying effect by allowing researchers to fully exploit the massive vector and strain resources available in this powerful model system.

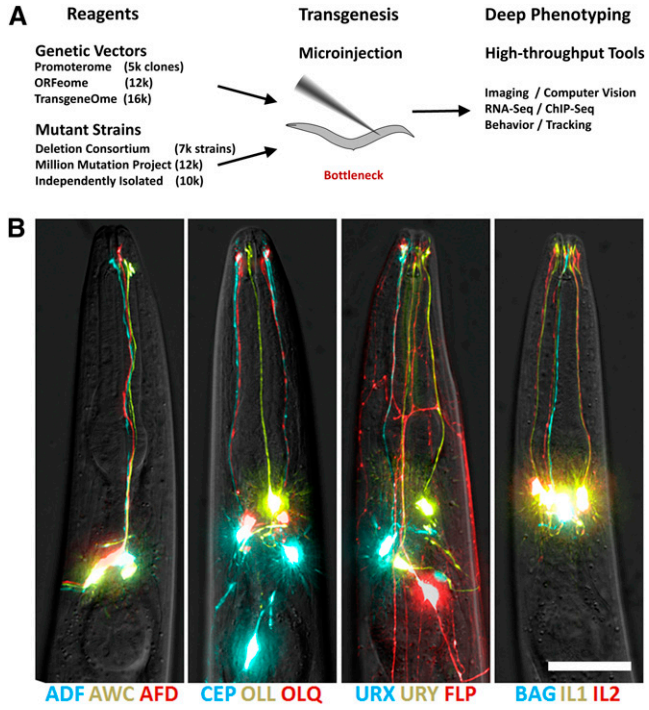

Figure 1 *C. elegans* transgenesis process and deep phenotyping. (A) Large-scale resources of genetic vectors and mutant strains are available and must be brought together via transgenesis (microinjection) to enable high-throughput phenotypic analysis. (B) Deep phenotyping of sensory neuron morphology in the head of wild-type animals generated by our computer-assisted microinjection (CAMI) platform using CFP, YFP, and mCherry cell-type-specific reporters. Bar, 50 μm.

Materials and Methods

Maintenance and imaging of C. elegans

Strains were constructed in the N2 background and cultured under standard conditions (Brenner 1974; Stiernagle 2006). Plasmids, transgenes, and strains are listed in Supporting Information, Table S2, Table S3, and Table S4. Some strains were provided by the *Caenorhabditis* Genetics Center (CGC), which is funded by National Institutes of Health Office of Research Infrastructure Programs (P40 OD010440). Images were collected using a Deltavision Core Imaging System (Applied Precision) using a 40× 1.35 NA objective (Olympus). They were deconvolved and maximum-intensity projections were generated using softWoRx Suite 1.2. The resulting images were pseudocolored and assembled using Photoshop CS5 (Adobe).

Mounting gel preparation

The mounting gel preparation is composed of a mixture of Pluronic F-127 hydrogel (25%) and sodium azide anesthetic (10 mM) in deionized water. The hydrogel (pluronic F-127, poloxamer 407) and anesthetic (sodium azide) reagents are acquired in powder form. A stock solution of 100 mM sodium azide is prepared for later addition to the pluronic solution by adding 650 mg of sodium azide powder (caution: toxic, use gloves and avoid contact with skin) to 100 ml of deionized water. The pluronic F-127 solution is prepared by adding 80 g of powder into 288 ml of chilled deionized water (4°) in a 500-ml flask. The pluronic F-127 powder is dissolved by storing the flask at 4° for 2 days and shaking vigorously by hand twice per day or by storing in a chilled shaking incubator (4°) overnight. Once the pluronic powder is fully dissolved, add 32 ml of the 100 mM sodium azide solution. The flask is gently swirled to mix the

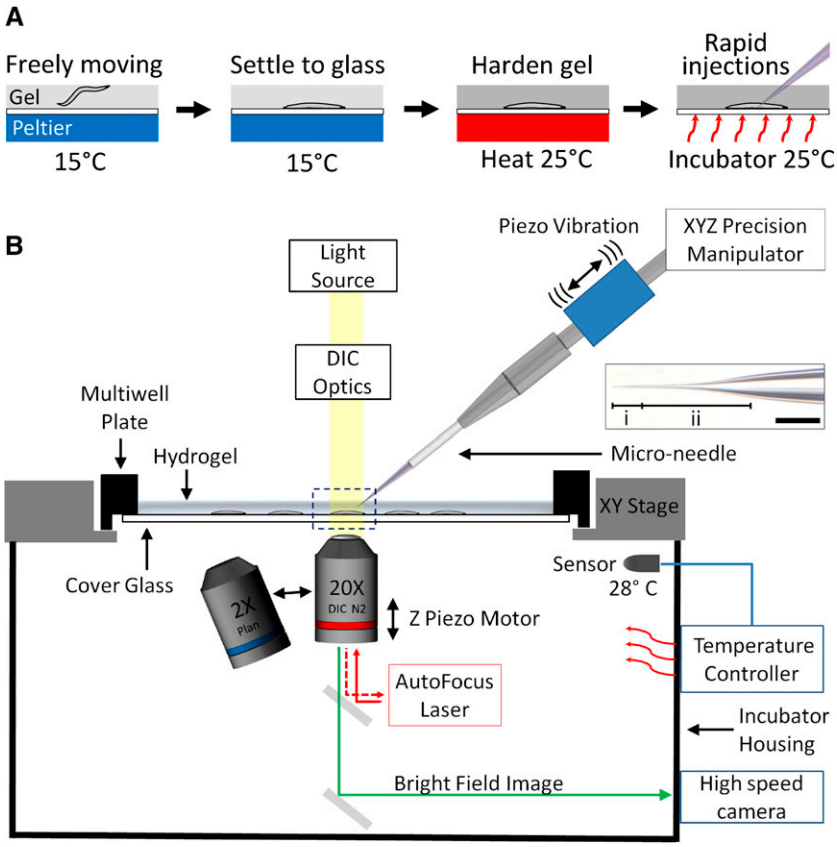

Figure 2 Computer-assisted microinjection (CAMI) platform. (A) Schematic of the gel mounting procedure. All animals in the multiwell plate are simultaneously immobilized flat against the glass within a single image plane enabling rapid XY scanning and mapping of the entire multiwell plate (see Figure 3A). (B) Overview of the instrumentation for the CAMI platform. Dashed box indicates animal undergoing microinjection. Microneedle inset: (i) Indicates penetrating needle tip. (ii) Indicates rapid taper. Bar, 50 μm.

contents yet avoid the creation of bubbles. Store the flask at 4° between each use. This prepared batch can be used for roughly 100 sets of injections.

Large-scale immobilization of C. elegans flat against glass surface

The procedure for mounting animals involves dispensing 3 ml of chilled pluronic gel (4°) onto a small agar dish containing synchronized animals, pouring the resulting mixture into a multiwell plate with a glass bottom, incubating the well plate at 15° (6 min) to allow the animals to settle against the glass, and then applying heat from below via a peltier warmer inside the incubator to quickly harden the pluronic gel and immobilize the animals flat against the glass. The peltier unit is positioned between two copper blocks (1/4 inch thickness) to ensure uniform heat distribution and a plastic plate cover is placed on top of the multiwell plate to retain heat and moisture during warming with a water level on top to ensure uniform distribution of the hydrogel. Alternatively, a programmable thermal cycler commonly used in PCR can be used to harden the gel during the immobilization process (See Supplementary). The multiwell plate is then transferred to the microscope for microinjection without disrupting animal positions. A temperature-controlled incubator maintains the hydrogel at a constant 25° throughout the injection process by feeding warm air to the bottom of the stage surrounded by a plastic sheet ensuring uniform heat distribution. We developed custom multiwell plate configurations with expanded width (three wells: 2 × 12 area of 96-well format, see Figure 3A). This configuration provides large needle-accessible areas and enables simultaneous animal mounting for three independent experiments with large populations. However, commercially available multiwell plates providing sufficient clearance could be substituted.

Clearing plasmid of debris

To allow use of needles with submicrometer inner diameters and avoid interruptions due to clogging, all plasmids were preprocessed with a centrifugation procedure prior to loading needles. Specifically, each plasmid mixture was centrifuged for 20 min (25,000 relative centrifugal force, rcf, 4°), and the top 90% of the supernatant was removed and transferred to a clean tube. This centrifugation/transfer step was repeated a second time. Plasmids were stored at −20° between uses. Each day a plasmid was subsequently used for microinjections, it was thawed and centrifuged for 10 min (25,000 rcf, 4°). Using a thin-tipped pipette, 1 μl of plasmid is extracted from the top 25% of the solution and then loaded from the back of the needle directly into the tip.

Clearing a clogged needle

The constant back pressure (~3 Psi) and submicron tip diameter prevent debris from entering the front of needle while the above plasmid cleaning protocol prevents clogging of the needle by the debris within the plasmid mixture. The

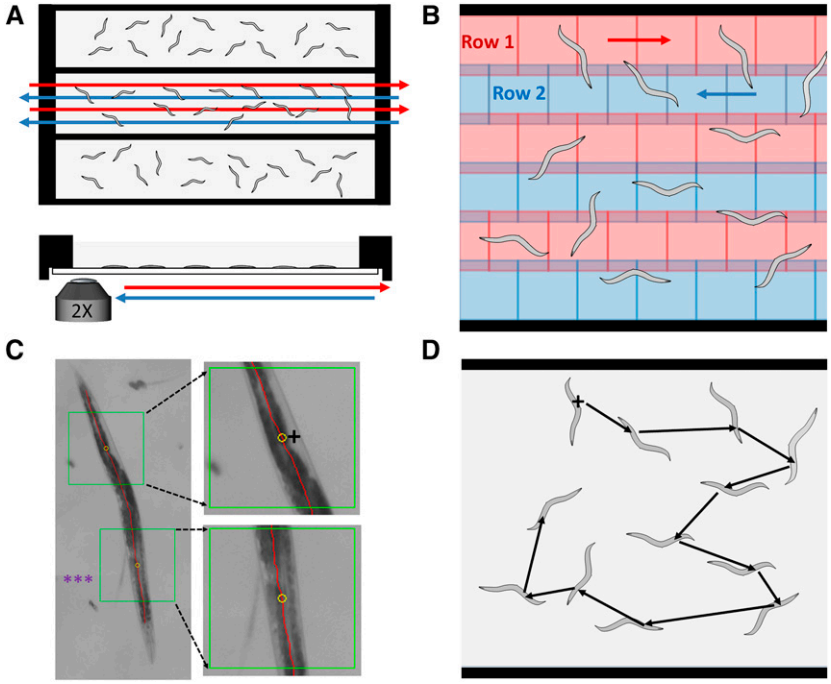

Figure 3 Algorithms for precision mapping and targeting of *C. elegans* gonads at large scale. (A) The entire multiwell plate of immobilized worms is scanned at high speed (blue and red arrows) and stitched to create a high-resolution montage to map worm positions with single-micrometer accuracy under 2× magnification. (B) The montage is generated by rapidly stitching individual rows (red and blue areas) in open-loop via precision timing of XY stage movement and camera frame rate. Each compiled row is overlapped (purple areas) with the lower compiled row by 20% and stitched using computer vision registration algorithms. (C) Automated identification of gonad targets from stitched images. ***, stitch line. The gonads are targeted by first drawing a spline (red) down the center of the animals then creating a region of interest (green box) around a specified distance along the spline (yellow circle). The regions of interest are further processed to detect the gonad targets for microinjection. Top right box indicates preferred orientation for injection. (D) The gonad target locations are then mapped and calibrated to XY stage coordinates and a greedy nearest neighbor algorithm determines the optimal path.

high injection dispense pressure (~60 Psi) and piezo vibration also help to remove any material from the tip during normal operation. An injection needle that is left outside the hydrogel after injections can be clogged with dry hydrogel around the tip. To remove dried hydrogel, soak the needle tip in a droplet of deionized water to rehydrate the dry gel and remove the clog. To prevent clogging from dried hydrogel the system automatically performs a dispense operation before and after the needle enters the gel. If the needle clogs during injections, visualize the clog by adjusting the focus knob, manually adjust the injection dispense pressure to maximum pressure (~90 Psi) and the dispense duration to 10 sec. Dispense the needle repeatedly until the clog is removed, then return to normal operation after adjusting the injection pressure back to normal operating pressure (~60 Psi). If this does not remove the clog, then replace the needle.

Hardware components

See File S1, Table S1 for detailed parts list with sources. A summary of parts include Prior ProScanII stage with controller, Nikon Ti Eclipse microscope with Perfect Focus laser system and DIC optics, Sutter MP-285 manipulator, pressure regulators and gauges (Parker Watts model no. R364-02C), Eppendorf universal capillary holder (cat. no. 920007392), high-speed camera (Allied Vision GX2300), temperature-regulated incubator (In Vivo Scientific), piezoelectric vibrator (RadioShack item no. 273-059), antivibration air table (Newport RS4000), digital output card (National Instruments, USB-9162), DC power supply (30V-5A), computer (PowerSpec G212, 16 GB RAM), cover glass-bottom well plates (In Vitro Scientific item no. P06-1.5H-N), needle puller (Sutter P-97), microneedles (World Precision Instruments, standard glass capillaries, item no. 1B100F-4).

Data availability

Plasmids and strains are available upon request. File S1 contains instructions for assembling and using the system. Table S1 contains a purchasing list for all hardware components. Software is available through GitHub (https://github.com/CodyLGilleland/CAMI_Gilleland_2015_GENETICS.git). Plasmids, transgenes, and strains used in this study are listed in Tables S2, S3, and S4 respectively.

Results

To address the need for rapid transgenics, first we developed the computer-assisted microinjection (CAMI) system. Next, we demonstrated the utility of the CAMI method by creating a collection of cell-type-specific fluorescent transgenes, labeling 12 classes of sensory neurons (Figure 1B) and using them to screen a small panel of mutants for defects in neuronal morphogenesis. CAMI is scalable and thus allows transgenesis to be industrialized for large-scale applications. The overall workflow of CAMI resembles the conventional method—animals are mounted on a slide and transferred to an inverted microscope, a glass needle filled with DNA solution is used to microinject the gonad, and then animals are recovered—but CAMI introduces a scalable method of mounting and recovering animals, and a computer-assisted gonad targeting and microinjection system.

In contrast to the conventional oil-mounting protocol, CAMI makes use of a temperature-sensitive hydrogel (Ko and Van Gundy 1988) (poloxamer 407, pluronic F-127) to pipette animals from standard culture on agar plates to a multiwell plate with a thin cover glass bottom. Because standard liquid-handling approaches and multiwell plates

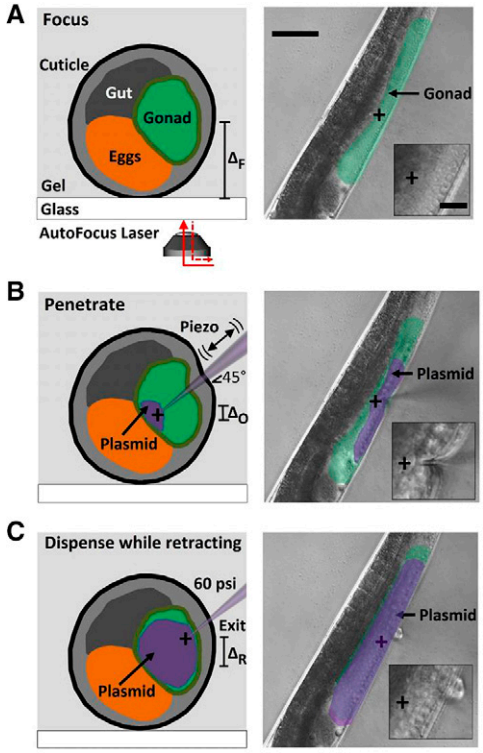

Figure 4 Computer-assisted delivery of genetic vectors via gonadal microinjection. The schematic (left) illustrates a cross-sectional view of an animal during the injection process while the images (right) demonstrate microinjection under 20× DIC objective using the CAMI platform. (A) The autofocus laser brings the gonad (green overlay) into focus, Δ_F indicated set distance above glass surface. The cross indicates target for injection and the inset is magnified to show honeycomb pattern of the gonad nuclei. Bar, 50 μm; inset, 10 μm. (B) The microneedle penetrates the cuticle and gonad sheath (green overlay) from the right side via precision robotic control and piezoelectric vibration with a 5-μm overshoot (Δ_O) to ensure full penetration. A constant back pressure (3 Psi) dispenses a small amount of plasmid (purple overlay) into the gonad, which indicates correct positioning and prevents biological debris from clogging the needle. The magnified inset shows needle penetration. (C) The plasmid (purple overlay) is then dispensed at high pressure (60 Psi) over a short duration (200 ms) as the needle is retracted 5 μm (Δ_R) to free the needle tip from obstruction. The needle is then fully retracted above the gel along its 45° axis. The magnified inset shows the minor damage at the entry point of the needle.

are used, this gel-mounting procedure can be automated by commercially available robotics. Recently, capturing *C. elegans* in hydrogels by optical heating has been used for subsequent manual microinjection but the transgenesis method remained low throughput and labor intensive (Hwang *et al.* 2014). In the CAMI platform, first all animals are simultaneously and easily confined to a uniform image plane near the cover glass: To achieve this, the multiwell plate is placed on a peltier heating unit at 15°, then animals are allowed to settle flat by gravity, and finally the hydrogel is quickly heated to 25° causing it to harden, immobilizing the animals, and trapping them against the surface of the slide (Figure 2A). The hardened gel, combined with 10 mM sodium azide as anesthetic, causes the animals to adopt

Table 1 Microinjection timing

Step	Mean time ± SD (sec)[a]
1. Stage XY travel to target	2 ± 0.5
2. Z-focus/fine alignment	5 ± 1
3. Needle insertion	10 ± 1
4. Plasmid dispense	5 ± 1
5. Needle exit	3 ± 0.5
Total	**25 ± 4**

[a] Timing rounded to nearest half second.

a straightened conformation, simplifying gonad targeting and needle penetration. The gel concentration is optimized to be stiff enough to restrict animal movement while still permitting the fine glass injection needle to travel through the gel without bending. Because the gel provides moisture to prevent desiccation, the animals can remain immobilized greater than 45 min with no loss of viability, compared to conventional mounting in which all animals die in <30 min (Figure S4E). We designed needles (Figure 2B, inset) with sufficiently long and thin penetrating shaft (i = 25 μm) to minimize tissue damage and rapid taper (ii = 100 μm) to prevent bending and enable rapid penetration. The needles are pulled with an open-end tip with submicrometer diameter to eliminate the need for manual needle breaking and thus reduce variability. We also used procedures to prevent clogging of needles (see *Materials and Methods*).

Next, the multiwell plate is transferred to a temperature-controlled microscope stage for microinjection. The CAMI hardware system consists of a high-speed camera mounted on an automated inverted microscope with objective turret, DIC optics, robotic XY stage, temperature controlled incubator, micromanipulator for needle positioning, piezoelectric unit for needle vibration pulsing, and pneumatic valves for pressurized dispensing of reagents (Figure 2B). The multiwell plate is scanned at high speed under a 2× objective to collect an image of the entire plate at ~1-μm resolution, allowing fine anatomical features including gonad positions to be discerned automatically. Computer vision algorithms detect animal locations, define the target gonad locations, and plan an efficient travel path between targets using a nearest neighbor optimization (Arya *et al.* 1998) (Figure 3). Once targets are mapped at 2×, the system can then remain at 20× magnification throughout the entire set of injections while traveling from one predetermined target to another, which avoids time delays due to objective changing and refocusing (see File S2, Supplementary Video).

The computer then rotates the objective turret to 20× for microinjection, and begins to visit each gonad target. The autofocus laser system brings the gonad into view and, if necessary, the user can make a small XY alignment using only a single mouse click, which also initiates a digital zoom feature (Figure 4A). The needle is brought into the image plane enabling axial penetration of the cuticle and gonad sheath with the assistance of piezoelectric vibration, simulating the manual tapping used in a standard microinjection procedure (Figure 4B). After penetrating the gonad, a single

Table 2 Transformation efficiency

Trial	Single gonad injections	F1 Rollers			Heritable expression F_2 lines/total F_1 (%)
		Total	Average per injected gonad	Maximum per injected gonad	
1	14	266	19	46	15/266 (5.6%)
2	15	272	18	79	10/272 (3.7%)
3	10	208	21	46	18/208 (8.7%)
Total	39	746	19 ± 1.4[a]	79	43/746 (6 ± 2.5%)[a]

A standard assay of rol-6 plasmid expression efficiency was conducted to determine the efficiency of the injection process. In each trial 100 μg/ml of rol-6 was injected into single gonads. The resulting F_1 transformants were placed on individual agar plates and heritable expression was scored in the F_2 generation.

[a] Mean ± SD.

mouse click is used to dispense the DNA mixture followed by axial removal of the needle (Figure 4C). The stage then travels to the next target where the injection procedure is repeated. After all injections are complete, the hydrogel is diluted with M9 medium and placed in a shaking incubator at 15° for 10 min to return the gel to liquid state, following which animals can be pipetted or poured onto standard growth medium for recovery.

CAMI operates at a rate of ~25 sec per gonad target with minimal user fatigue, in contrast to conventional manual microinjection at ~2–3 min per animal where, in addition, most users cannot sustain injecting for more than 1–2 hr (Table 1). Mounting and recovery of animals brings the total time per set of injections to ~20–30 min; however, recovery can be performed in parallel with mounting for the successive set of injections (see Supporting Methods). Typically, one needs to inject ~15 animals to ensure obtaining a transgenic line. Importantly, however, recent genome-editing techniques such as CRISPR/Cas9-mediated genome editing require larger cohorts of ~50 animals, which are challenging using manual methods but are easily accommodated using CAMI.

To quantify the transgenesis efficiency using CAMI, we replicated a classic experiment that was used to establish the conventional transgenesis protocol (Mello *et al.* 1991). In this experiment, a 100 μg/ml solution of the plasmid pRF4 bearing a semidominant allele of the *rol-6* gene was injected into the gonads of young adult animals. The F_1 progeny that bear the transgene exhibit an easily scored "roller" (Rol) phenotype, in which animals crawl in circles. Most of these transgenic F_1 progeny are transient, and only a small fraction transmit the transgene to the F_2 generation. In three independent trials, we injected 10–15 gonads each and obtained 19.0 ± 1.4 transgenic F1 animals per gonad, comparable to a published rate of 12.5 F1 animals per gonad by the conventional method (Table 2). We found that 6.0 ± 2.5% of these F1 animals transmitted the transgene to the F_2 generation, comparable to a published rate of 9.4% by the conventional method (Table 2). Thus, transgenesis by CAMI occurs at an efficiency well in line with conventional techniques.

To test if this method can be used effectively to combine a vector and strain library as illustrated in Figure 1A, we performed a small screen focused on sensory neuron development. This screen was motivated by the following rationale: first, in a previous genetic screen we found that the zona pellucida (ZP) domain protein DYF-7 is required for sensory neurons to extend their dendrites to the nose tip (Heiman and Shaham 2009); second, ZP domain proteins are secreted factors that often heteromultimerize into a matrix; third, the *C. elegans* genome encodes dozens of predicted ZP domain proteins, raising the possibility that other ZP domain proteins may cooperate with DYF-7 to promote sensory dendrite extension. Therefore, we wished to determine if mutants in other ZP domain proteins might exhibit defects in sensory dendrite extension similar to those seen in the absence of DYF-7.

As a vector library, we generated a collection of plasmids bearing cell-specific markers to individually label most of the head sensory neurons. These include glial-ensheathed neurons—namely, 3 of the 12 amphid neurons (ADF, AWC, and AFD) and all of the outer and inner labial and cephalic neurons (OLL, OLQ, IL1, IL2, and CEP)—as well as non-glial-ensheathed neurons that also extend dendrites to the nose tip (BAG, FLP, URX, and URY). Together, these markers allow us to assay morphology of 38 individual neurons in 12 classes. We divided these markers into four plasmid mixes, each containing CFP, YFP, and mCherry-based plasmids (Figure 1B and Table S2). As a mutant library, we selected a small panel of mutants that represent the three major *C. elegans* strain collections: publicly available mutants generated by individual laboratories (*ram-5*) (Yu *et al.* 2000), deletion mutants generated by the North American and Japanese knockout consortium (*cut-1*, *cut-5*, and *cut-6*), and predicted null mutants identified through the Million Mutation Project (*cutl-14* and *T23F1.5*) (see Table S4).

We used CAMI to introduce each of the four plasmid mixes into each of the six mutant strains, generating 24 lines, allowing us to look for phenotypes in a total of 78 mutant neuron classes (4 transgenic lines × 6 mutants × 3 neuron classes per line) (Figure 5 and Table S4). Although we observed very low penetrance defects in CEP neurons of the *cut-5* mutant strain (not shown), in most cases these mutants had no effect on neuronal morphology, suggesting that ZP domain proteins do not broadly contribute to sensory neuron dendrite extension and thus that the phenotypes we observe with DYF-7 are likely to be specific.

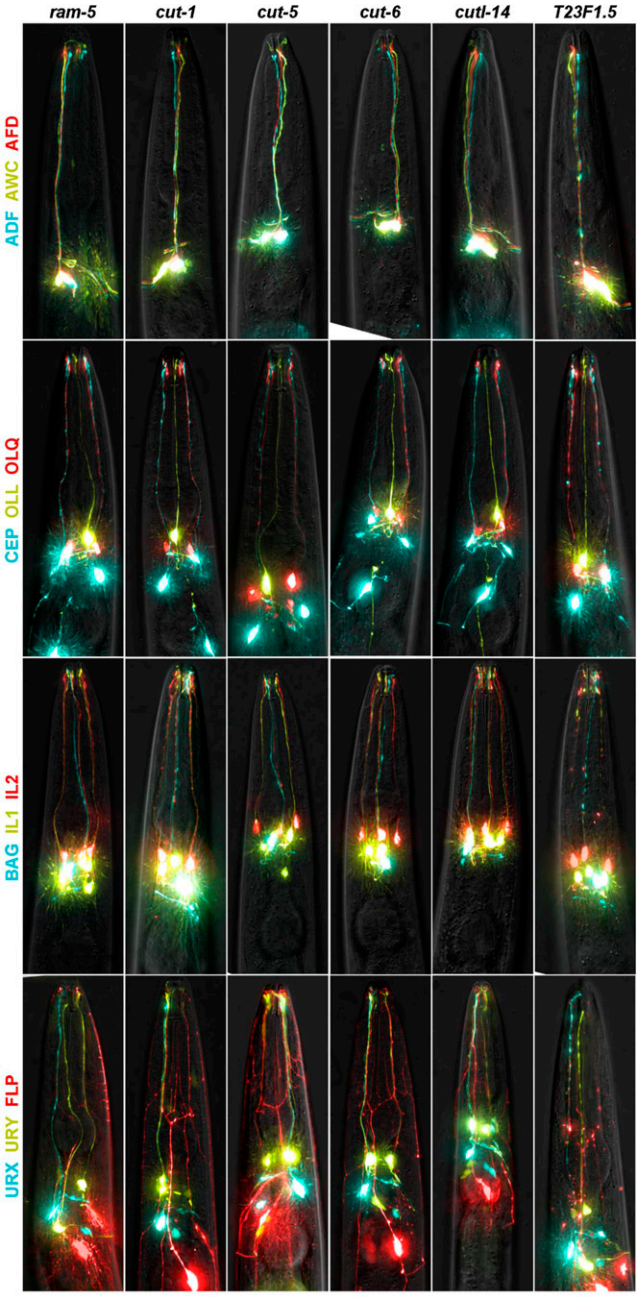

Figure 5 Deep phenotyping of sensory neuron morphology. A total of 24 transgenic strains were generated, each consisting of a plasmid mix bearing fluorescent markers of three sensory neuron classes, indicated at left, introduced by microinjection into a different mutant strain, as indicated at top (see Table S4). Dendrite length, morphology, and position were analyzed in at least 25 individuals per strain. Representative images are shown.

Discussion

Microinjection is a powerful method for manipulating model organisms. For example, several groups including ours have developed automated microinjection of zebrafish embryos (Wang *et al.* 2007; Hogg *et al.* 2008; Spaink *et al.* 2013; Chang *et al.* 2014). However, the small size of *C. elegans* and its special handling requirements have rendered it previously inaccessible to such higher-throughput microinjection. Here, we have demonstrated how CAMI can be used to simplify and accelerate microinjection, and we used it to screen for novel phenotypes in a panel of *C. elegans* mutants and thus assign new functions to genes. This method can similarly be used to deliver small molecules in a screen for novel therapeutics, to deliver RNAi constructs in cases where traditional feeding RNAi is not potent enough, to perform large-scale genome editing using CRISPR/Cas9 or TALENs, or to assay the pathogenicity of human gene variants.

The hardware set-up cost of the system as presented (~$100,000, File S1, Table S1) is suitable for a core facility, and roughly comparable to employing an injection technician for 1–2 years, but will be prohibitive for smaller labs. Future improvements can certainly allow for less expensive microscope systems and robotics and can also be integrated with more streamlined software. Additional modifications can increase the speed of the system; for example, the ability to flush the needle would allow plasmid mixes to be changed automatically as we demonstrated in another previous study (Steinmeyer and Yanik 2012). Combining this system with recently reported antibiotic selection vectors for *C. elegans* can speed the isolation of transgenic lines following microinjection (Cornes *et al.* 2014). Portions of the CAMI protocol can be adapted to existing microinjection systems; for example, the hydrogel mounting methods can, in principle, be used with manual injection systems, provided that the micromanipulator offers "on axis" movement in addition to more conventional XYZ movement. It could also be combined with recently reported manual optical immobilization of *C. elegans* in hydrogel (Hwang *et al.* 2014). Bombardment is another technique that can potentially be used for large-scale transgenesis (Semple and Lehner 2014); however, bombardment requires the transgenes and selection markers to be cloned into a single plasmid, and thus is not compatible with existing vector libraries and lacks the versatility of plasmid mixtures (Wilm *et al.* 1999). Bombardment also produces low-copy number transgenes, which can provide more physiological expression but are not compatible with visualizing weaker fluorescent markers. The facile transgenesis offered by CAMI brings the field one step closer to Brenner's pioneering goal of "microbiologizing" an animal system to be as manipulable as bacteria (Wood 1988).

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Author contributions: C.L.G. designed the microinjection platform, developed the hydrogel immobilization technique and wrote the supplementary information. C.L.G., A.T.F., and J.N. developed algorithms for precision gonad targeting of injections. C.L.G. and A.T.F. performed all microinjection screening experiments and designed microneedle parameters. M.G.H. designed the biological assay, prepared all plasmids, and imaged resulting transgenic strains. C.L.G., M.G.H., and M.F.Y. wrote the manuscript. M.G.H. and M.F.Y. supervised the research at all times.

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Computer-Assisted Transgenesis of *Caenorhabditis elegans* for Deep Phenotyping

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File S1

Supplementary Methods

Gilleland *et al*., Computer-assisted transgenesis of *C. elegans* for deep-phenotyping, **GENETICS** 2015

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1. CAMI hardware components and assembly (Timing: 2 days)

This section describes the hardware components that comprise the CAMI system. The CAMI software package is designed to work with the specific hardware components listed in Table S1 and shown in Figure S1 (diagram) and Figure S2 (assembled). The local microscope sales and support representative will assemble your automated Nikon microscope with DIC optics, Perfect Focus System, Prior XY motorized stage, and Sutter XYZ micromanipulator. (Nikon Ti eclipse brochure Link) Tip: The Sutter XYZ micromanipulator should be centered over the objective in XY and placed at a 45° angle of approach for axial penetration (Figure S2b). The Z height of the manipulator should be mounted to allow the micro-needle tip to touch the bottom of the glass well plate at 23 mm in travel leaving 2mm in Z height tolerance. This also allows for maximum needle clearance for taller well plates. Assemble the high pressure regulators and gauges with the tubing, fittings and pneumatic valves as shown (Figure S3a,b). Assemble the peltier unit hardware and connect the electrical components to the peltier heating unit and piezoelectric vibrator as shown (Figure S3c). The peltier unit has a 1/4" copper block above and below to ensure uniform temperature distribution. Thermal paste is used to ensure thermal conductivity from the peltier to the copper blocks. The copper blocks are cut using a water jet machine to match the size of the glass bottom of the well plate. To calibrate the peltier unit temperature and electrical parameters, cut a small hole in the top of the well plate cover and insert a small temperature sensor into the hydrogel and track the temperature over time. Adjust the amount of current as necessary to achieve 25°C at the end of a 3 min cycle (as an example, 4 Amps were needed to raise the hydrogel temperature from 15°C to 25°C in 3 min using our equipment). Alternatively, a thermal cycler unit commonly used for PCR could be used to provide the temperature changes necessary for hydrogel immobilization in place of the custom peltier system (See Troubleshooting).

1a. Hardware diagram and component list with price and source

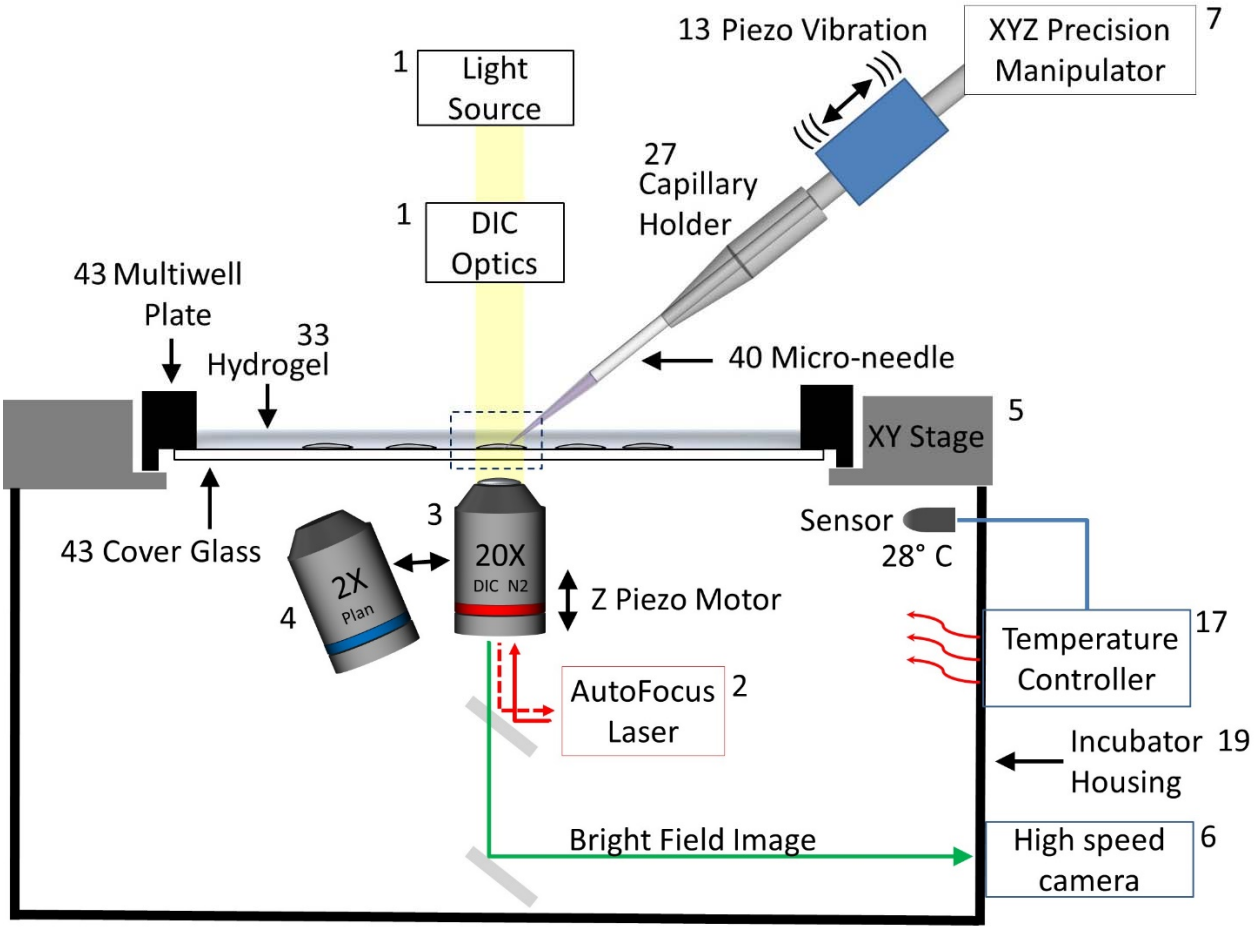


Figure S1. CAMI system hardware diagram and parts list. See list of parts (Table S1) and corresponding images of the assembled components in Figures S2 and S3. The parts are labeled with a reference number that corresponds to the part list information in Table S1.

Table S1. CAMI system parts list with price and source

Ref.	Item	Brand	Catalog No.	Quantity	Price	Link
CAMI System Hardware						
1	Fully automated Nikon microscope, DIC prisms and polarizers	Nikon (installed by local sales rep)	Ti eclipse inverted microscope, T-C DIC module LWD N2 dry, D-C DIC slider 20X, T-P2 DIC polarizer, Ti-A analyzer block (installs in epi fluor turret)	1	$44,000	Link
2	Perfect focus system with motorized nosepiece (objective turret)	Nikon (installed by local sales rep)	TI-ND6-PFS-S	1	$18,000	Link
3	Objective 20X DIC	Nikon (installed by local sales rep)	CFI plan apochromat λ 20X	1	$2,400	Link
4	Objective 2X	Nikon (installed by local sales rep)	CFI plan apochromat λ 2X	1	$1,200	Link
5	XY motorized stage, controller & joystick with plate holder	Prior Scientific (installed by local sales rep)	Proscan II H117 (stage, controller, joystick), H223XR (insert plate holder)	1	$17,500	Link Link2
6	High speed, high resolution camera (C-mount adapter), Network Cable (Cat5e, 25')	Allied Vision (camera), Microcenter (cable)	Camera GX2300 (4.1 MP @ 32 FPS), (Cable) CC711-25GR)	1 camera & 1 cable	$5,000	Link Link2
7	XYZ Micromanipulator & Controller with mount to Nikon Ti microscope (user could also use metal posts/plate from Thor Labs for manipulator mounting)	Sutter Instruments (installed by local sales rep)	MPC-385 (manipulator), ROE-200 (controller), MD-54-1/MUP (mount to Nikon Ti with motorized stage & stage up kit), Dovetail extension(285204)	1	$9,500	Link Link2
8	Desktop computer (64bit, 16GB RAM, Windows 7 pro) keyboard, mouse	PowerSpec (MicroCenter)	G212(discontinued) or G423 (newer model)	1	$800 or $1,800	Link or Link2

9	Computer monitor 23.8” & DVI cable	Dell	S2415H 23.8" IPS LED HD	2	$200	Link
10	Electrical DC power supply	Circuit Specialists	CSI3005XIII	1	$219	Link
11	USB module carrier (paired with NI USB-9472)	National Instruments	NI USB-9162	1	$360	Link
12	Portable USB high voltage digital output Card	National Instruments	NI USB-9472	1	$520	Link
13	Piezoelectric vibration buzzer (12V, 2.8kHz, 76dB)	RadioShack	273-059	1	$1	Link
14	Air compressor for vibration isolation table (if no wall air source)	Newport (installed by local sales rep)	AWCS	1	$650	Link
15	Height adjustable desk (isolate vibration from user, allow user to sit or stand)	Mayline	Varitas XR	1	$1,315	Link
16	Vibration isolation table (36” x 72”)	Newport	VIS3672-SG4-325A	1	$5,677	Link
17	Temperature Incubator (used controller only, not enclosure)	In Vivo Scientific	CH.HC5.SAT full enclosure	1	RFQ	Link
18	Flexible Duct Hose (3” x 6’)	Hakko	999-189	1	$13.36	Link
19	Plastic Sheet (cut to wrap around XY stage)	Bed, Bath & Beyond	13876258 (clear shower curtain)	1	$6	Link
Peltier Heating System						
20	Thermoelectric peltier module with thermal paste	Custom Thermoelectric (peltier), newegg (thermal paste)	19911-5M31-28CZ (peltier), arctic MX-4 (4g), (Carbon-Based Thermal Compound)	1 + 1 backup for each	$56.25 $10	Link Link2
21	Copper Sheet (user cuts sheet by water jet to fit under well plate)	McMaster Carr	8995K11, (1/4 Hard Sheet, 0.250" Thick, 12" x 12")	1	$142.95	Link
22	Electrical wire red & electrical tape	DigiKey	6710 RD005-ND (red wire),	1	$31.21 $4.14	Link Link2

			3M15506-ND (electrical tape)			
23	Electrical Wire Black	DigiKey	6710 BK005-ND	1	$31.21	Link
24	Air Bubble Level	DealExtreme	SKU 164069	1	$5	Link
High Pressure Injection Components						
25	High Pressure Pneumatic Actuator (0-100 PSI, 24VDC, 1/4” OD tube)	Norgren	Q212315-1351B-D	1	$60.60	Link
26	High Pressure Gauge (0-160 PSI), Regulator (0-100 PSI)	Parker Watts	K4510N18160 & R364-02C	2 & 2	$13.94 $26.64	Link Link2
27	Universal capillary holder	Eppendorf	920007392	1	$370	Link
28	Rigid tubing PTFE 1/4”, 25ft	Cole Parmer	EW-06605-32	1	$80	Link
29	Rigid tubing PTFE 1/8”, 25ft	Cole Parmer	EW-06605-29	1	$63	Link
30	Female luer lock 1/8” NPT	Cole Parmer	C-31200-60	6	$8	Link
31A	Male luer lock ring 1/8” to barb (pack of 25)	Cole Parmer	W-45505-04	1	$8.75	Link
31B	Male luer lock ring 1/4” to barb (pack of 25)	Cole Parmer	SC-45505-19	1	$8.75	Link
31C	Female luer Lock ring 1/8” to barb (pack of 25)	Cole Parmer	EW-30800-08	1	$9.75	Link
32	Pneumatic Y-connector fitting for 1/4" tubing	WIC Valve	PYU-T1/4	5	$2.09	Link
Pluronic Hydrogel Immobilization Mixture						
33	Plutonic F-127 (1KG, powder)	Sigma-Aldrich	P2443-1KG	1	$151.50	Link
34	Sodium azide (NaN_3, 100g, powder) !CAUTION: Toxic, use gloves and avoid contact with skin	Sigma-Aldrich	S2002-100G	1	$133.50	Link
35	Deionized water (1 gallon bottle)	Science Company	NC-3064	5	$16.50	Link

	or use lab source					
36	Glass bottle & cap (1000 mL , case of 12)	VWR USA	89042-748	1	$118.65	Link
37	Ice bucket for hydrogel (4 liter)	VWR USA	89202-272	1	$105.93	Link
Consumables						
38	Equipment and reagents for culturing *C. elegans*		agar plates,M9 media, incubators, stereo microscope, worm picks, pipet with tips, ethanol			
39	Pipet tips (1000 µL, sterile, 96 hinged rack, 7.6 cm, pack of 6)	VWR USA	83007-380	1	$74.54	Link
40	Standard glass capillaries with filament for micro-needles (4 in., 1 / 0.58 OD/ID, pack of 500)	World Precision Instruments	1B100F-4	1	$54	Link
41	Serological pipets (10 mL, case of 200)	VWR USA	89130-888	1	$116.87	Link
42	Pipette tips (long & thin for loading plasmid into needles)	Eppendorf	Z317047-1PAK	1	$135	Link
43	Glass bottom well plates (6 well, case of 20)	In Vitro Scientific	P06-1.5H-N	3	$210	Link
Biosafety Level 1 Lab Facilities & Equipment						
44	Orbital shaking incubator (refrigerated, worm recovery post-injection)	VWR USA	MAXQ4000, SHKE4000-8CE	1	$10,829	Link
45	Capillary needle puller	Sutter	P-97 Flaming Brown	1	$8,350	Link
46	Safety eye glasses (pack of 12)	ULINE	S-13390C	1	$30	Link
47	Temperature sensor with probe	Omega	HH147U	1	$315	Link
	Stable room temperature (cannot fluctuate above 25°C)	Electrical outlets & surge protectors	Air pressure source (wall source @ 100PSI)	Sink, Biowaste with sharps disposal	Deionized water	Ice

1b. Hardware assembly instructions (Timing: 2 days)

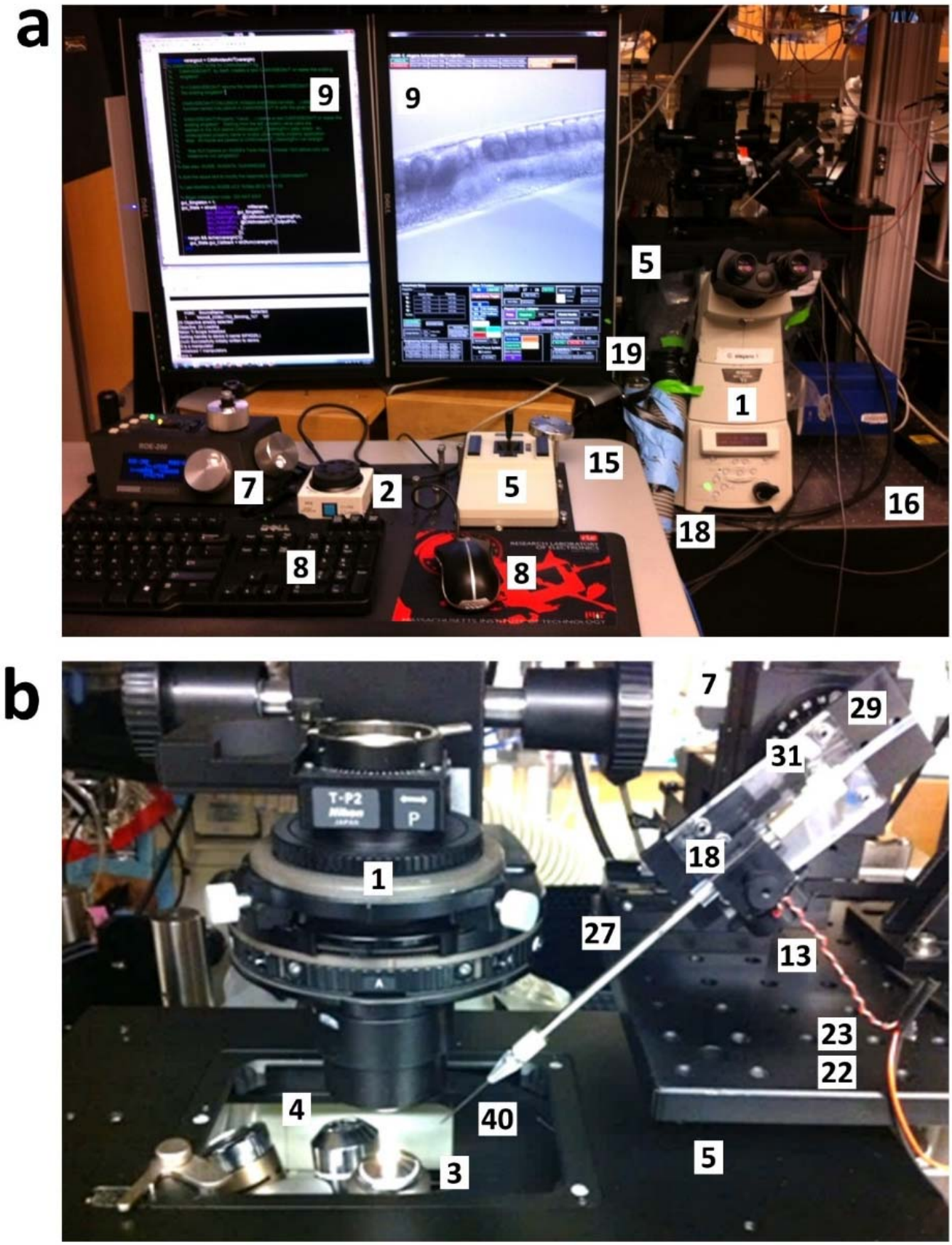


Figure S2. Precision instrumentation system and controls a) The unified software interface enables the user to interact and control the entire system largely by mouse control. The second screen (part#9) allows for simultaneous updating of the code to refine the system. The microscope (part#1) is secured to an anti-vibration air table (part#16) while the incubator heating unit (part#17) is placed on the ground to avoid vibration from the oscillating fan. A duct hose (part#18) supplies the warm incubator air to the plastic sheet (part#19) surrounding the base of the microscope and is connected loosely by tape to minimize any vibrations from the incubator fan. An adjustable height table (part#15) hosts the manual instrument controls to prevent vibration from user interaction and allows the user to sit or stand on demand to reduce fatigue. On the adjustable table: Sutter XYZ precision manipulator (part#7), perfect focus Z height offset adjustment (part#2), XY motorized stage joystick (part#5), keyboard and mouse (part#8). b) The Nikon Ti eclipse microscope is equipped with DIC optics (part#1), perfect focus laser system (part#2), 20X/2X objectives (part#3,4) on a rotating turret and mounted with an XY motorized stage. The manipulator hosts the micro-needle and capillary holder (part#27) with piezo vibration device (part#13). In this prototype we use a metal post and breadboard connected by an L-bracket. Commercially available mounting brackets are available for mounting the Sutter manipulator directly to the Nikon Ti microscope (part#7, Sutter MD-54-1/MUP). The automated Nikon Ti eclipse microscope enables rapid changing of objectives, filters, shutters and cassettes to quickly respond to imaging demands.

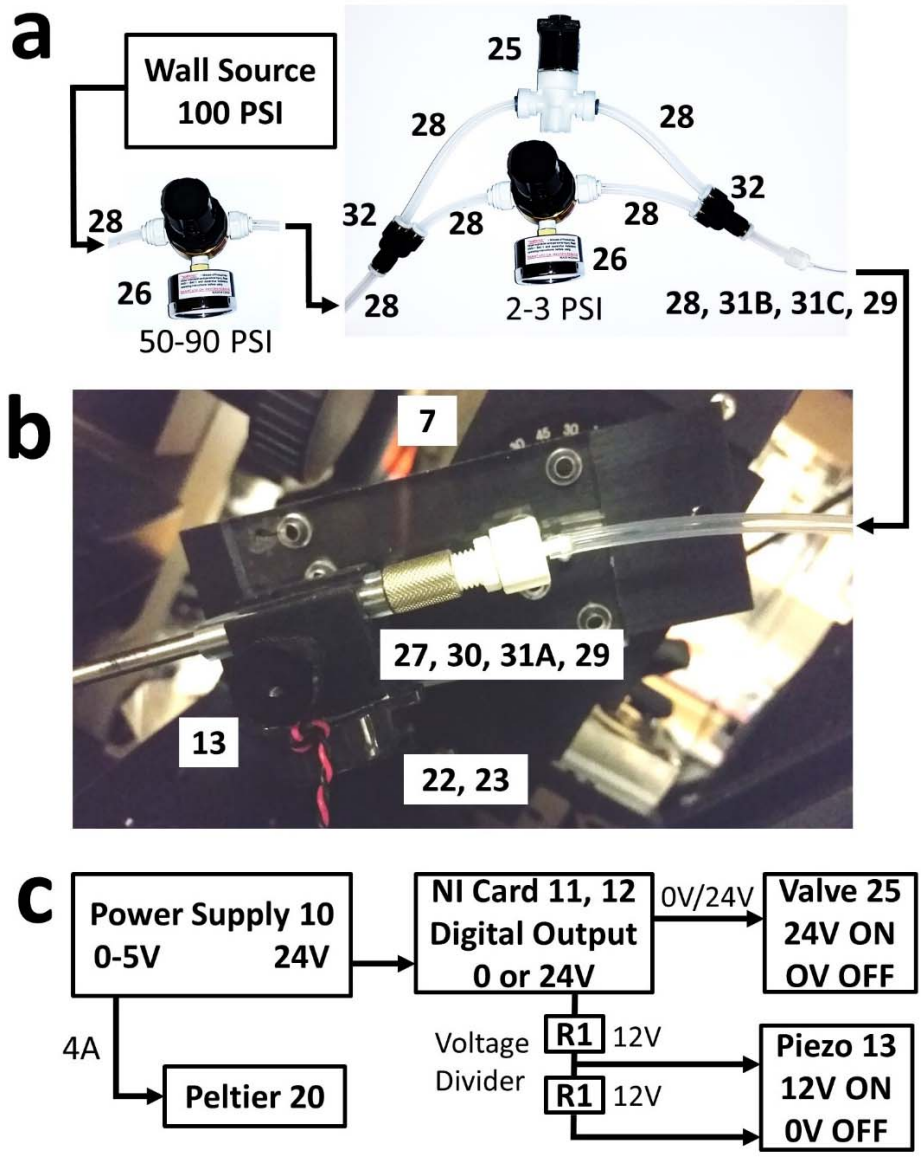


Figure S3. High pressure pneumatic connections and electrical diagram. a) The wall source (100PSI) is split between two pressure regulators (back pressure: 3 PSI, injection pressure: 50-90 PSI) and the injection pressure regulator is controlled by a digital pneumatic valve to control injection pressure duration. This results in a system that provides a constant back pressure (3 PSI) while allowing computer controlled pulses of high pressure for injection over a specified duration. b) The smaller 1/8" tubing is used closer to the needle to allow for fast pressure transitions from back pressure (3 PSI) to the injection pressure (90 PSI). The Eppendorf Universal Capillary Holder (part#27) is used to accommodate high pressures by firmly securing the micro-needle. c) The power supply is used to control the peltier unit to hydrogel immobilization (4A for 3 min) and also supplies 24V to the NI digital out card. The NI card is used to directly control the pneumatic actuator (24V) and then pass through a voltage divider to control the piezo actuator (12V). The resistors (R1) in the voltage divider should be of equal resistance to split the 24V into 12V sections over each resistor. The NI Card is controlled by software interface and then transferred to the 32-bit MATLAB program as described previously. The ground cables are omitted to simplify the diagram. Each component should also be attached to a ground cable.

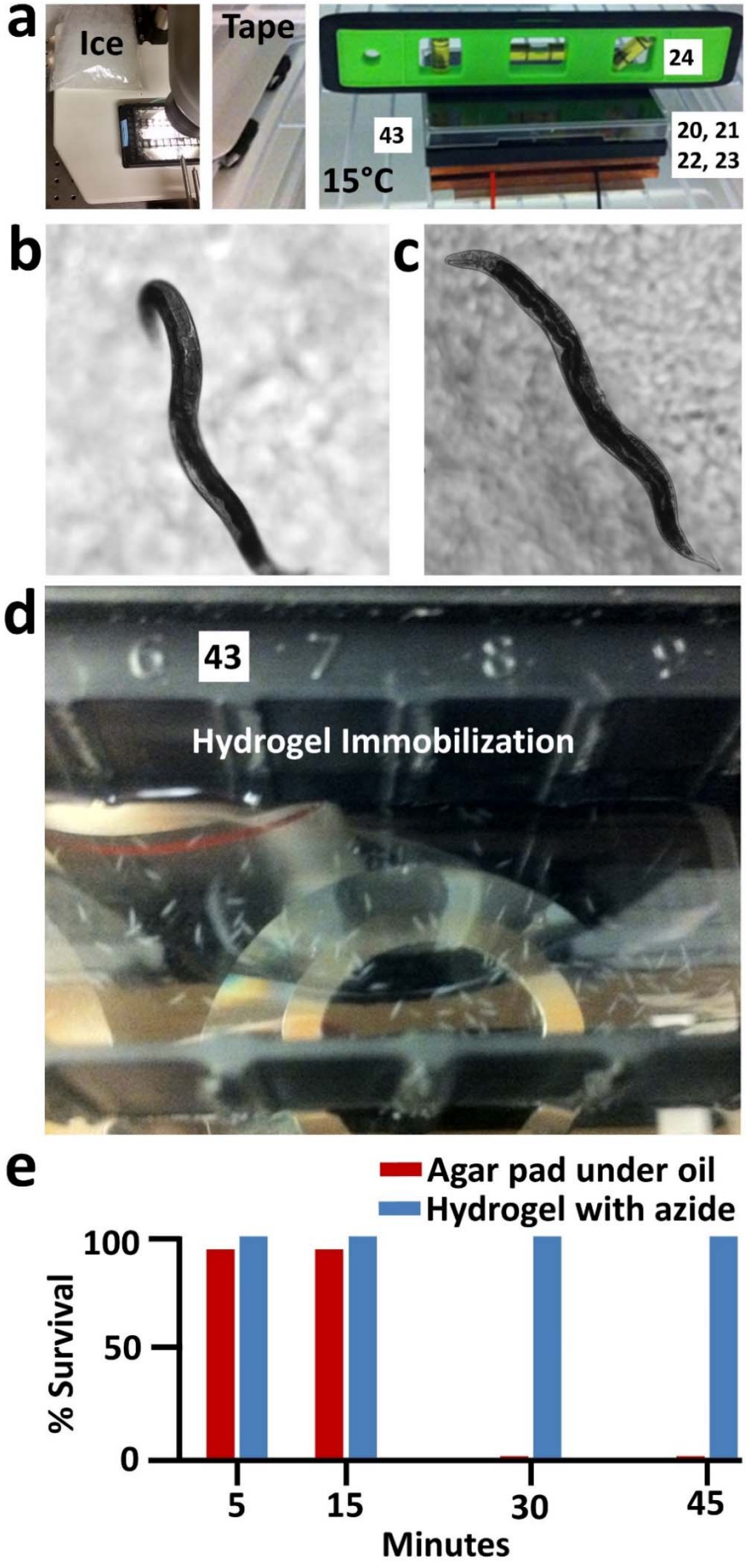


Figure S4. Hydrogel immobilization of *C. elegans* flat against the cover glass in uniform Z-plane a) To ensure that the hydrogel does not transition to the more viscous gel phase too quickly place an ice bag on the stage to cool it down before using the stereo-microscope to check worm positions. Do not use a stereo-microscope with a light source that is close to the sample to avoid premature heating of the hydrogel. Tape can be used to level the shelf inside the incubator by wrapping concentric layers around the shelf supports. The glass bottom well plate is covered and placed on top of the peltier unit and copper plate with level to ensure even thickness of hydrogel. The peltier unit is coated with thermally conductive paste and sandwiched between two thermally conducting copper plates to evenly transfer the heat to the glass bottom of the well plate. Notice the electrical tape is used to level the shelf. A plastic cover is placed over the well plate to retain heat and moisture while a bubble level ensures that the hydrogel is evenly coated to prevent uneven drying of the gel leading to desiccation of the animals. See Troubleshooting for gel temperature calibration and refinement of peltier current parameters. b) Worm immobilized out of Z-plane is demonstrative of hydrogel immobilization without peltier heating method or sodium azide. c) Worm in Z-plane flat against the cover glass using sodium azide and the quick peltier heating method that hardens the gel before the worms are able to crawl to the top of the gel away from the heated glass surface. d) Image of worms successfully immobilized in the hydrogel as the well plate is placed on the microscope stage above a 2X objective. This well plate is custom made to have long troughs for needle entry access over a large area and can be custom ordered at large scales. These custom plates were washed with ethanol and reused. e) Survival of young adult animals (one day after final molt) mounted using the conventional method for injection (agar pad covered with oil) or using the hydrogel mixture with sodium azide described here. N=20 per condition.

2. CAMI Software Platform

This custom software platform was built on the MATLAB software package and uses many of the MATLAB toolboxes for image acquisition and computer vision.

2a. MATLAB program/toolboxes and hardware driver installations (Timing: 1 day)

Operating System and Computer: Windows 7 Professional x64, 16GB RAM, Intel i7 processor

Software Programs	
MATLAB 2011B x64 bit for Windows Image acquisition toolbox Link Computer vision toolbox Link Mathworks.com>AccountLogin>MyAccount>DownloadProducts Note: The toolbox versions must match the MATLAB version since there are differences between toolbox releases	Link Link Link
MATLAB 2010A x32 bit for Windows Mathworks.com>AccountLogin>MyAccount>DownloadProducts Note: This x32 bit version is necessary for controlling Digital Output from the National Instruments card since it is not supported in the x64 bit version	Link
GigE Sample Viewer (Allied Vision)	Link
Hardware Drivers	
Allied Vision GX2300 hardware driver (camera)	Link
Nikon Ti Drivers (USB)	Link
Sutter Manipulator Driver (USB)	Link
Prior XY Motorized Stage Driver (Proscan II)	Link
National Instruments Driver (NI-DAQmx)	Link

2b. CAMI Software documentation

The CAMI software package is hosted on github and is available for download at the following link:

https://github.com/CodyLGilleland/CAMI_Gilleland_2015_GENETICS.git

Demo of gonad detection: Stand alone version with 6 example worm images
https://github.com/CodyLGilleland/CAMI_Gilleland_2015_GENETICS/tree/master/DemoGonadDetection

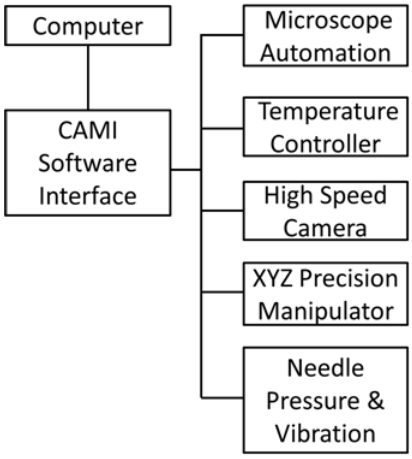


Figure S5. CAMI Software Integration. This diagram demonstrates how the hardware components are controlled with a custom software package. This custom software enables non-compatible hardware devices to work together with precise timing and robotic control. This platform also enables rapid software prototyping for new types of physical experiments and is highly adaptable to other applications.

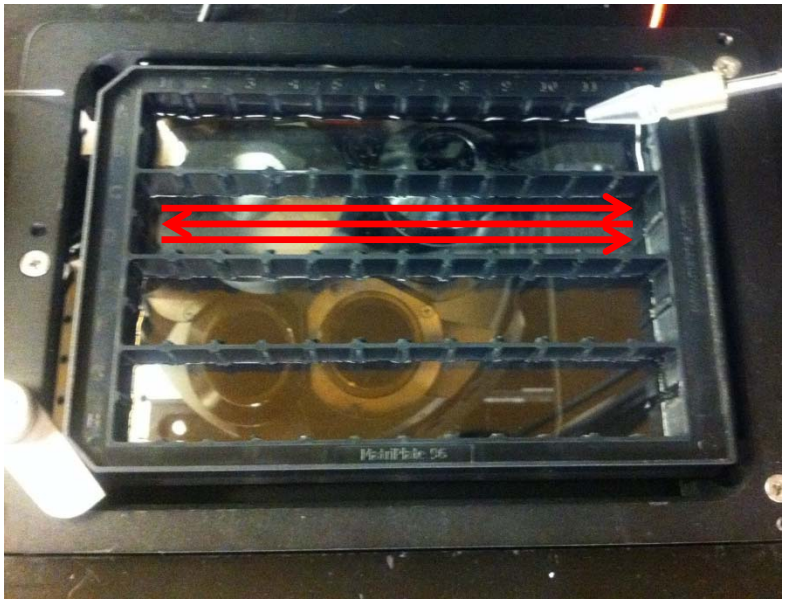


Figure S6. Rapid four well scanning. The custom well plate is scanned at maximum speed in one X-direction while the camera captures a video then repeats in the opposite X-direction after shifting down one row. The well plate was cut from a standard plastic well plate mold using a waterjet to ensure a smooth surface and allow the glue to adhere the plastic mold to the cover glass. A cover glass bottom was then super-glued to the bottom. This allows for optimal scanning and open access for needle intervention. For future applications custom plastic molds can be designed and fabricated with cover glass attached by multiple companies. These well plates were rinsed with ethanol and re-used. Tip: Do not use a saw to cut the well plates since the rough edges will prevent smooth contact from plate to the glass bottom and may cause leakage where the glue does not seal properly.

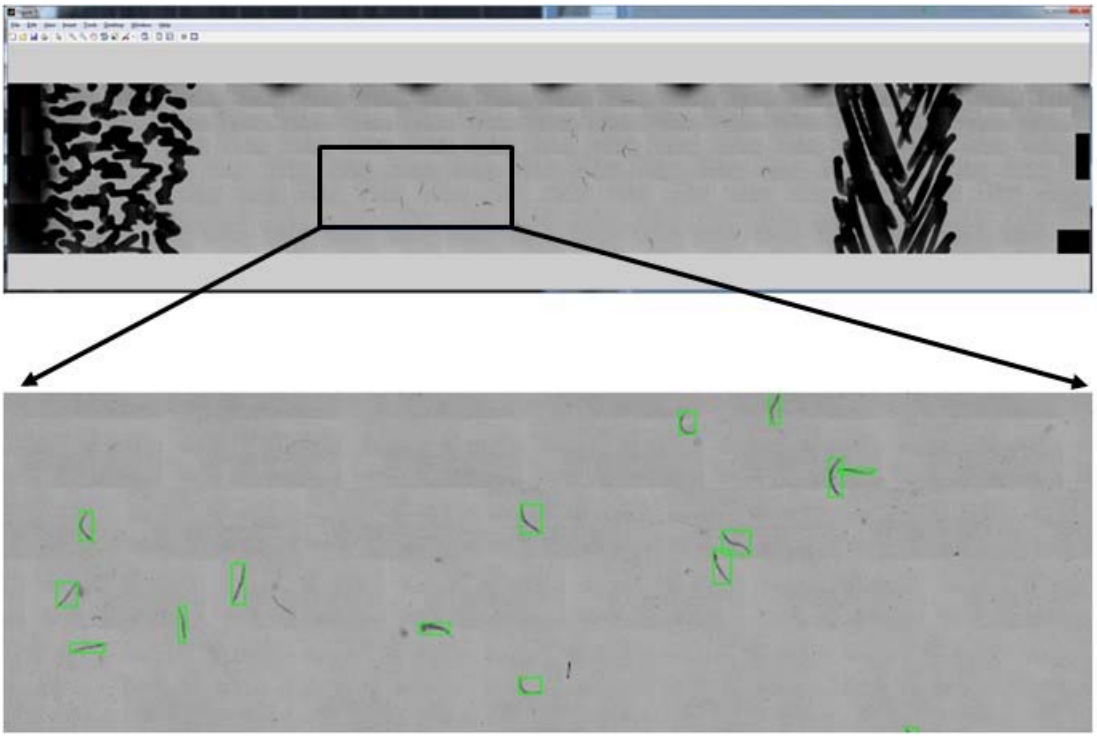


Figure S7. Image stitching to create the large montage and worm selection. Fiducial markings provide location references when stitching together each of the rows. Using a simple intensity threshold and object area function the worms are quickly selected for analysis at full pixel resolution. This allows us to perform precision processing on a subset of this larger image in the next step.

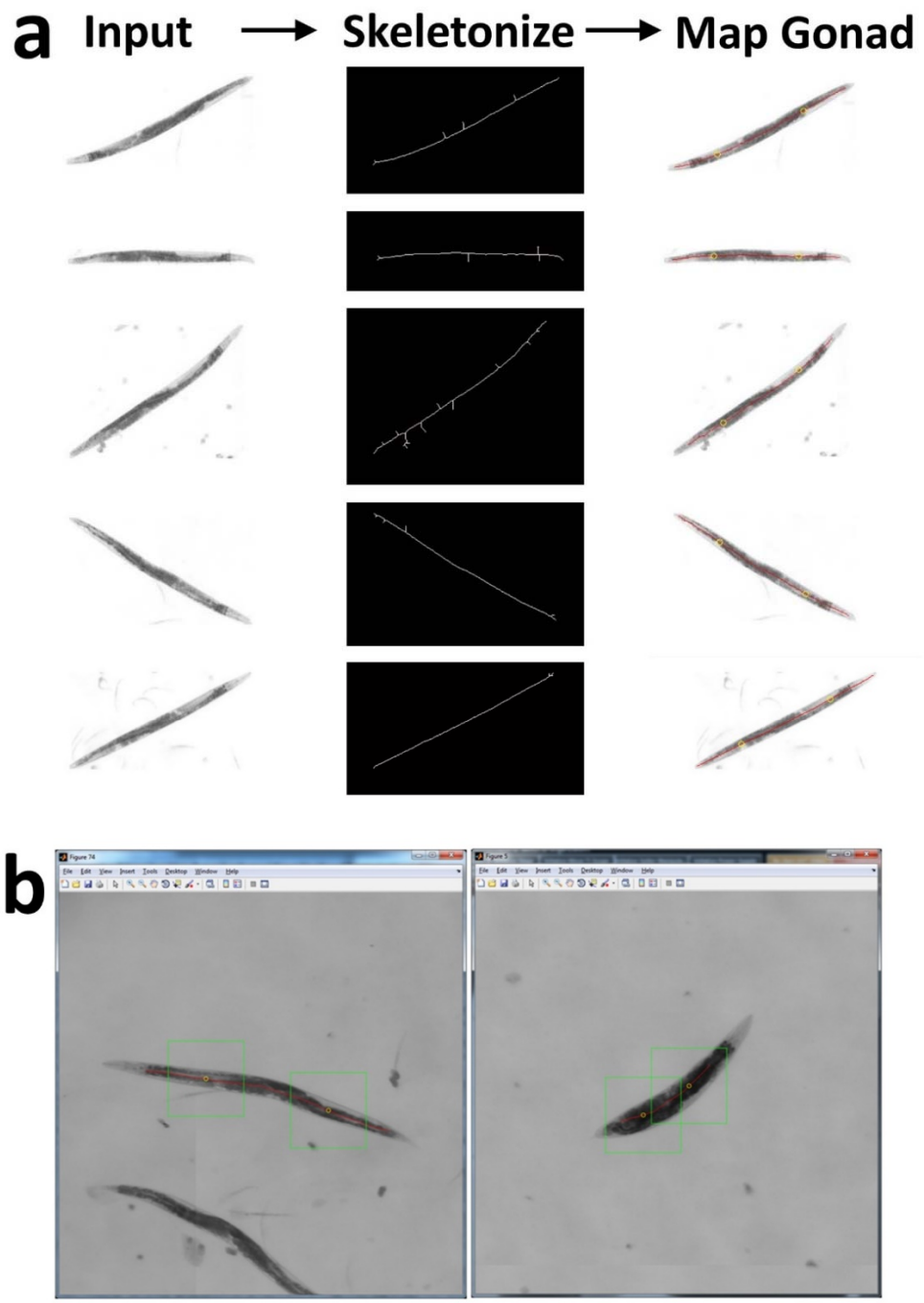


Figure S8. Worm selection and spline-based gonad mapping. a) The worm is selected using intensity thresholding and object area detection. The resulting objects are then skeletonized. Using the skeleton as a template a spline is then drawn through the center of the animal and distances are measured along the spline (25% and 75% of length) that correspond to the gonad regions of interest. If more than one worm is located in the field of view the worm closest to the center is selected for processing. b) (Left) A region of interest is then determined for gonad location. This is the approximate field of view in the 20X magnification. (Right) The robust algorithm automatically adapts to body morphology phenotypes like the dumpy mutant shown here with a short body. The parameters for initial worm selection can be adjusted for worm size (area) and the standard deviation can be set to adjust the selection criteria.

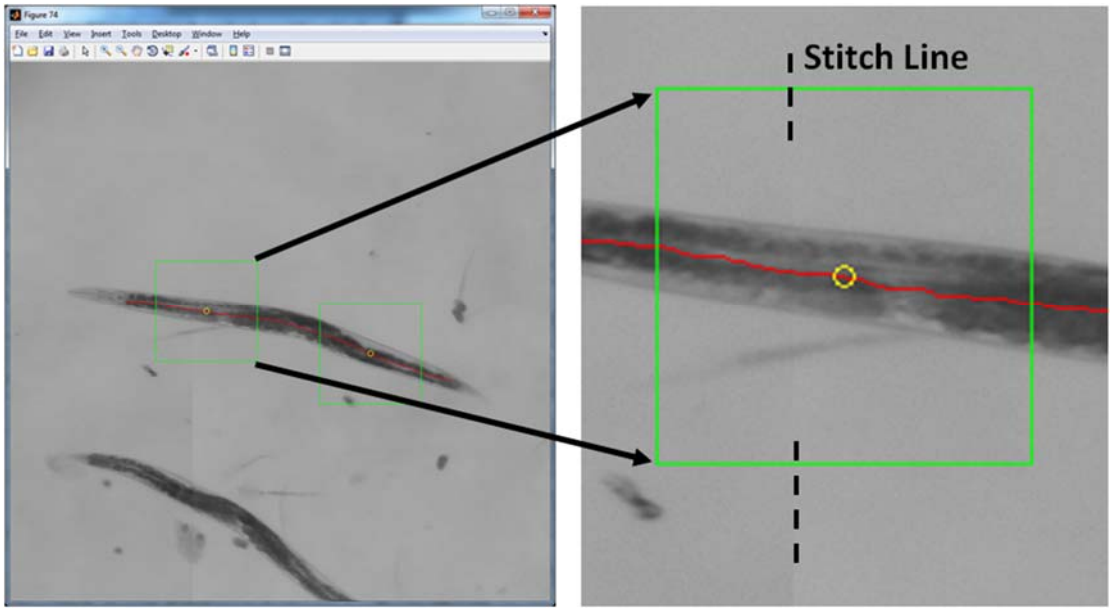


Figure S9. Precision image stitching. The stage is run at full speed scan while the camera records a video of the passing frames. Our algorithm then stitches these frames together with the accuracy of a single pixel. The algorithm uses MATLAB computer vision toolboxes for corner detection and mapping then estimates a best fit approximation. The animals are approximately 1 mm in length. Notice the stitch line provides relatively seamless continuity.

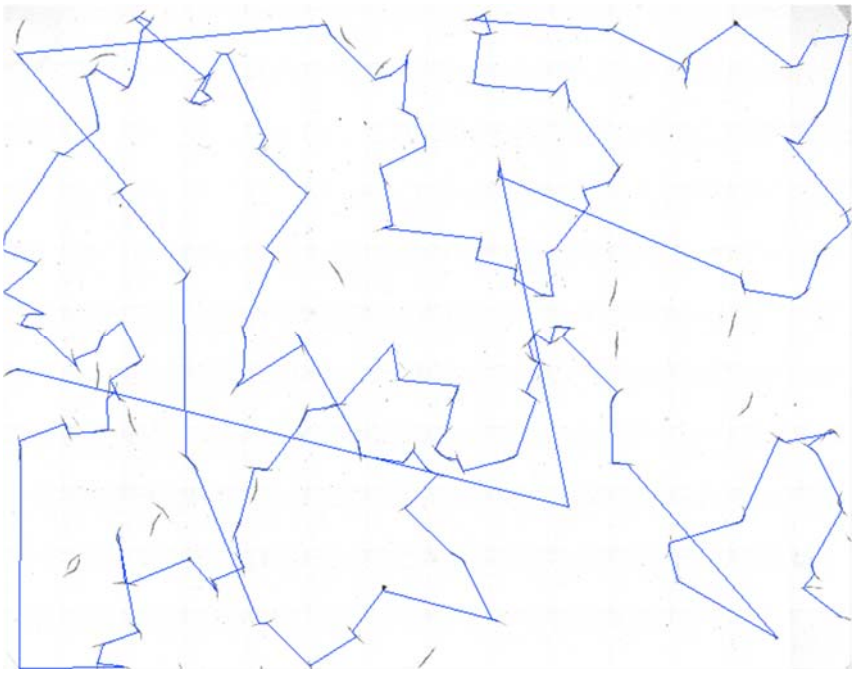


Figure S10. Traveling algorithm for nearest neighbor path optimization. The waypoints are formed and incorporated into a greedy nearest neighbor traveling algorithm to find the most efficient path to include each waypoint. Since the target locations are mapped in XY stage coordinates this allows the user to remain in 20X as they move from worm to worm without the need for changing objectives and allows the perfect focus unit to track the bottom of the glass and keep the sample in Z-focus. This helps to streamline the microinjection process by quickly presenting injection targets.

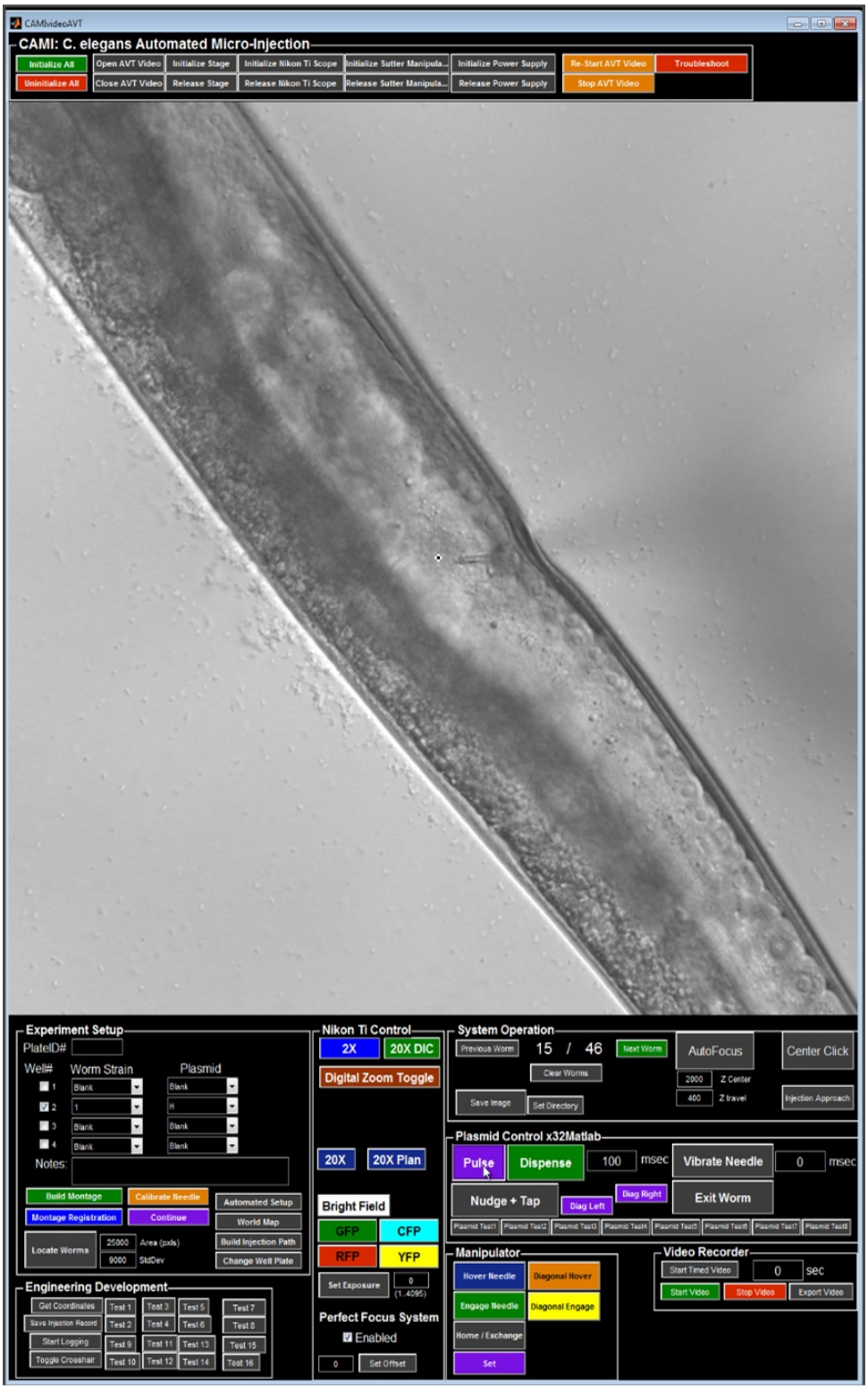
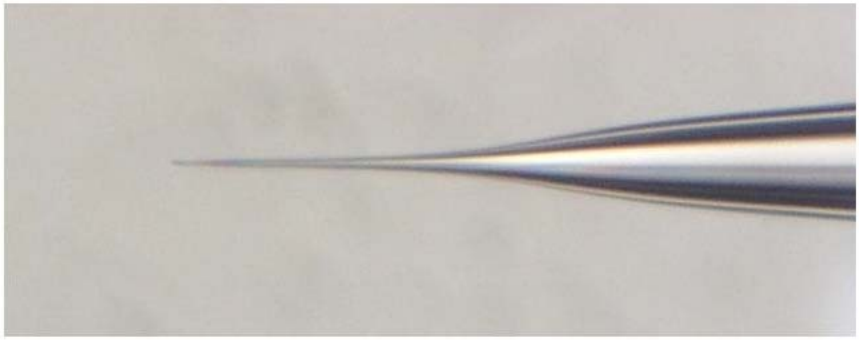


Figure S11. Screenshot of the CAMI software interface during microinjection. The image shown here is in 20X magnification DIC of a young adult hermaphrodite worm during the microinjection process. The needle enters the worm from the right side at a 45° angle above the cover glass surface then pressurizes to dispense the reagent into the gonad arm forcing it to expand. The software controls allow the user to iteratively adjust the needle position and apply short pulses (~100 ms, ~60 psi) to ensure the needle is in the correct position and adequate vector delivery is occurring.

Microinjection Needle Parameters

a



b

Step	Heat	Pull	Velocity	Delay	Pressure	Air Time at Start
1	Ramp	100	14	90	500	60
2	Ramp + 10	50	20	90	500	60
3	Ramp + 10	20	20	60	500	60
4	Ramp + 11	0	18	75	500	60
5	Ramp	120	25	1	500	60

Figure S12. Custom needle design and refinement. The needles are adapted from the bee-stinger shape in the Sutter P-97 Pipette Cookbook with a box filament (Link with video tutorials). The broad shaft allows for rigidity to move through the viscous hydrogel while the thin taper enables delicate penetration of the worm cuticle and gonad sheath without excessive damage. Our needle design is highly sensitive to room humidity. We include a pre-programmed air purge for 60 sec before beginning the pulling procedure. We find that our needle pulling is more successful when the weather outside has low humidity which influences our lab conditions. Troubleshooting of micro-needle variability can also be provided by Sutter Instruments (Link and video tutorial). Tip: Perform the ramp test to attain the proper ramp heating value and avoid exceeding this value by more than 15 to 20 degrees to prevent damage to the filament. Perform multiple ramp tests and use the average ramp value in your program.

3. Detailed Step-by-step protocol

Summary: The animals are grown on standard agar plates seeded with OP50 bacteria. The agar plates are then washed with a pluronic gel solution (25% pluronic, 10 mM sodium azide) and poured onto a dish with a cover glass bottom and placed into a 15°C incubator allowing the worms to settle to the bottom (Figure S4). The near perfect Z-planarity enables image processing algorithms to quickly detect animal locations and features to map injection targets. The well plate is scanned to map the injection targets and the needle filled and calibrated. Microinjection is then performed using the software interface and the animals are released by diluting the hydrogel with cooled M9 medium and gentle shaking in a cooled incubator. The animals are pipetted onto agar plates for recovery and then transgenic progeny are selected in the next generation. In all images the young adult worms are roughly 1mm in length and 60-100 µm in diameter.

3a. Pull micro-needles and prepare all reagents (Timing: 1 day)

1. Prepare the hydrogel mixture as described in the methods section in the main text (1 day). The bottle of hydrogel is stored at 4°C until ready for use. It can then be kept in a bucket of ice on the bench or quickly returned to the refrigerator between uses. Tip: Transfer the hydrogel to a smaller 50 mL tube to allow easier pipet access. Close the lid on the hydrogel after use to prevent dehydration.
2. Prepare the micro-needles using the Sutter P-97 needle puller (15 min). Check the needles under stereo-microscope to ensure that they have the proper long-taper shape shown in Figure S12. Store the needles on clay. Tip: ensure that the back of the needle where it enters the needle holder is not touching the clay to prevent clogging the capillary holder.
3. Prepare a population of age-synchronized young adult animals (one day after final molt).
4. Prepare the plasmids by thawing them and centrifuging to remove debris (10 min, 25,000 rcf). Tip: This step is to be done each day the plasmid is used. Ensure that the initial plasmid cleaning procedure has been completed to clear the plasmid of debris and prevent clogging (See Methods section of main text).
5. Turn on the incubator under the microscope stage and allow it to reach 28°C. Check that room temperature is below 24°C. Tip: The temperature sensor is placed beneath the stage and should be slightly higher than 25°C to ensure that the heat from the warmed air below the well plate results in a steady 25°C temperature in the hydrogel. Ensure that the air hose is providing indirect flow to the well plate to prevent temperature gradients. See Troubleshooting for gel temperature calibration using external handheld thermometer.

3b. Hydrogel immobilization of *C. elegans* (in parallel with recovery, Step 3h)

6. Remove any condensation on the agar with a pipet or a paper wipe. Pipet 3 ml of hydrogel (depends on plate size) onto an agar plate with worms, swirl the plate to allow the animals to be suspended in the hydrogel, and then use the same pipet tip to aspirate the gel with animals (1.8 ml for 2 x 12 well trough area, 1.16 ml for 6 well plate). Pipet the gel onto the agar plate directly into the glass bottom well plate. Ensure an even coating by gently tilting the well plate to distribute the hydrogel. Tip: Removing moisture from the agar plate avoids dilution of the hydrogel. Troubleshooting: If the agar plate is at room temperature the hydrogel may harden too quickly. Place the agar plate in a 15°C incubator to lower the temperature of the agar.
7. Inspect the well plate under a stereo-microscope to check an even distribution of animals. Use a pick or pipet to redistribute the animals if necessary. Tip: If the hydrogel solidifies too quickly place a bag of ice on your stereo-microscope base to lower the temperature surrounding the plate (Figure S4a). Also check the room temperature to ensure it is below 24°C.
8. Place the well plate onto the peltier unit inside a 15°C incubator. Place a plastic cover on top of the well plate to retain moisture and temperature. Use a level to ensure the plate is not tilted to achieve a uniform hydrogel distribution (Figure S4). Tip: Use electrical tape to level the shelf in fine increments as shown in Fig S4a. See Troubleshooting if using a thermal cycler unit (PCR).
9. Wait for 6min to allow the animals to settle to the bottom of the glass plate as the hydrogel remains in liquid form for 6 min. Tip: This is the time when the user should fill the needle with plasmid and install it into the needle holder. Turn on the CAMI stage incubator unit and place an empty well plate over the stage opening to allow it to reach an internal temperature of 25°C.

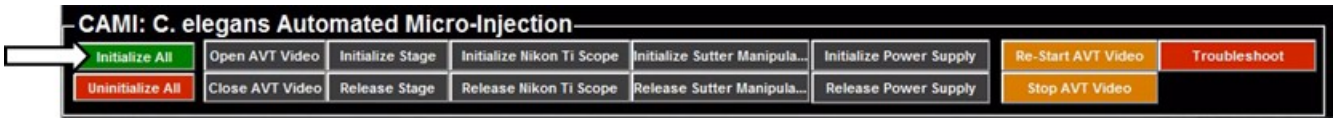
10. Apply heat using the peltier unit by setting the power supply to the calibrated current (in our case, 4 A) for 3 min to warm the hydrogel and cause it to harden to the gel state and immobilize the worms. Tip: The peltier method allows the hydrogel to solidify quickly and trap the animals against the glass bottom of the well plate before they crawl away from the heat source. If the animals are moved to the microscope without peltier warming then the movement will cause them to move out of the Z-plane. A heated air incubator alone does not provide the quick temperature change necessary to harden the gel before the animals crawl out of plane. See Troubleshooting section if using a thermal cycler unit (PCR) as alternative.

11. Turn off the peltier power supply and remove the well plate from the 15°C incubator. Place the plate on the CAMI microscope stage that provides uniform heating from warm air source below the stage. Tip: Ensure that the stage incubator temperature is at 28°C (in our case, measured below the stage providing the desired 25°C in hydrogel). See Troubleshooting for temperature calibrations.

3c. Scan and stitch montage of well plate (in parallel with recovery, Step 3h) (Timing: 6min)

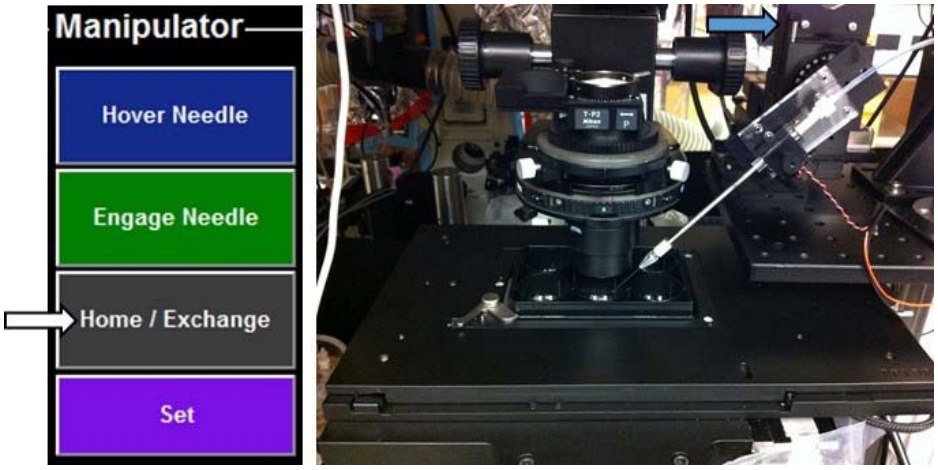
12. Click the 'Initialize All' button (Arrow) to establish communication with the hardware components. The buttons to the right control the ability to initialize or release control for each individual hardware component. Each button is labeled accordingly. (Timing 5 sec)

    Troubleshooting: If the live camera view freezes then click 'Stop AVT Video' then click 'Re-Start AVT Video' to refresh the camera. If the camera is still having issues then click 'Stop AVT Video' then open the Unicam Viewer Software and view live video feed to ensure the camera is working properly and then close the Unicam Viewer. Click 'Re-Start AVT Video' to refresh the camera. Otherwise, unplug the camera at the power outlet and restart the camera as above. Do not unplug power cable from camera port to avoid unwanted tilting of the camera orientation.

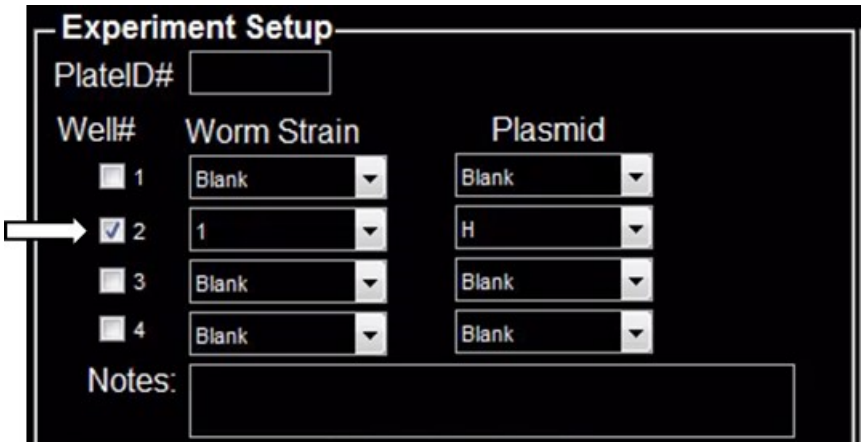


13. Load a new well plate of worms immobilized in the hydrogel (after peltier warming step). Ensure that the manipulator has moved to a safe position above the well plate area by clicking the 'Home/Exchange' button (white arrow). Move the micro-needle holder to the side by unscrewing the release mechanism (blue arrow) and manually moving it by hand. Place the well plate into the XY motorized stage holder and secure it with a holding lever. Insert a new micro-needle into the capillary holder and screw it down tightly by hand. (Timing 1 min)

    Caution: To prevent clogs at the back end of the needle ensure that any clay used to hold the needles during storage does not enter the needle holder.



14. Enter information about the experiment. Check the box for the current ‘Well#’ (arrow) to indicate the correct well to scan for worm locations. Select the worm strain and plasmid from the drop-down lists (Strains are labeled with numbers and Plasmids mixtures are labeled with letters. As an example here we denote the resulting strain as ‘1H’.) Enter ‘PlateID#’ and any special ‘Notes’ about the experiment in text box.

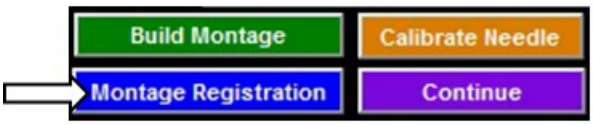


15. Run the mapping procedure to map the positions of the worms in 2X magnification. Click the ‘Build Montage’ button to initiate collection of the large montage of worm locations. The automated microscope will switch to the 2X objective and adjust the Z height of the objective to a region close to the focal plane of the worms. The user will be prompted to finely adjust the Z height of the objective to bring the worms into focus using the 2X objective.

16. Scan and stitch the montage. Click the 'Continue' button (arrow). The system will then build the montage by scanning and stitching as described in Figure 3A of the main manuscript. This will result in a large image of the well at high resolution. Inspect the montage image to ensure that the stitching is properly aligned. (Timing 4min) See Troubleshooting section if images are not properly aligned.

17. Register the montage to XY stage coordinates. Click the 'Montage Registration' button (arrow). The system will take a live camera image and perform a normalized cross-correlation of the current image with the montage to determine the location of the current photo within the larger montage. The montage with then be calibrated to the current XY stage coordinate.

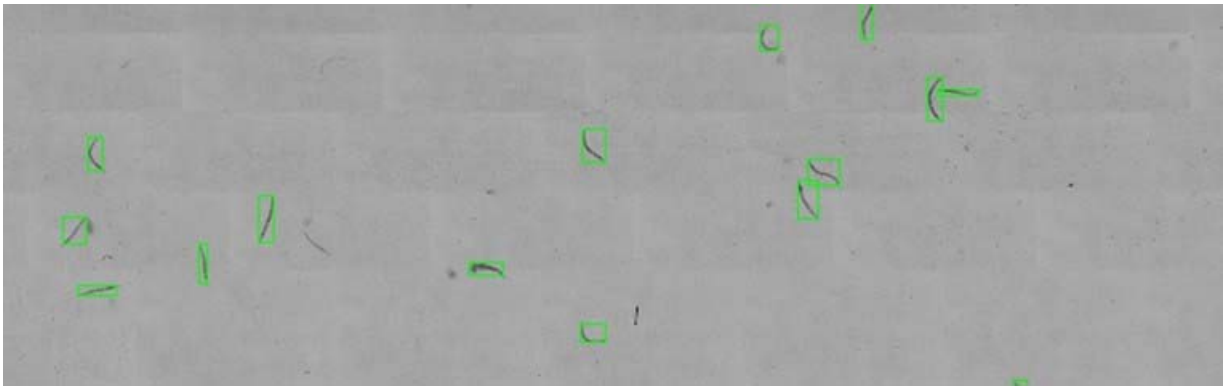
    Tip: Ensure that the current image is positioned over the scanned area and has a unique feature with contrast to enable detection.



3d. Map worm locations (Timing: 30 sec)

18. Click the 'Locate Worms' button to process the large montage image for coarse detection of worms.

The algorithm will select worms (green bounding boxes) by area only for rapid detection. The image below is a subset of the larger montage image. See **Troubleshooting** (Section 3j) if worms are not detected or if there are image non-uniformities due to a misaligned condenser.

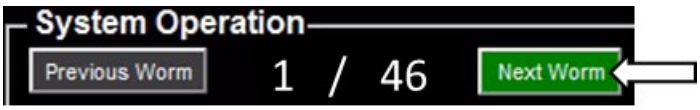


3e. Map gonad target locations (Timing: 4min, 5 sec/worm)

19. Gonad detection. After animal positions are located, an interactive procedure to confirm each gonad target will automatically be initiated. A sub-image of the large montage will be displayed. If the object presented is a valid worm then left-click anywhere on the screen. If the object is not a worm (debris, unhealthy worm) then press the spacebar to skip to the next object. The gonad detection algorithm will detect the worm outline with an intensity threshold, draw a spline down the center of the worm and then measure a distance along the spline to determine the XY location (region of interest) of the gonad. These XY coordinates will be stored with each corresponding worm number. If more than one worm is in the image then the algorithm will select the worm closest to the center of the image.

20. Click the ‘Next Worm’ button (arrow) to move (XY stage) to the first worm and use it as a Z-height reference for needle calibration.

    Tip: The hydrogel and glass are transparent and it is not easily determined if the objective is focused above or above the glass. By focusing on the worm we ensure that we do not overshoot and break the needle against the glass.

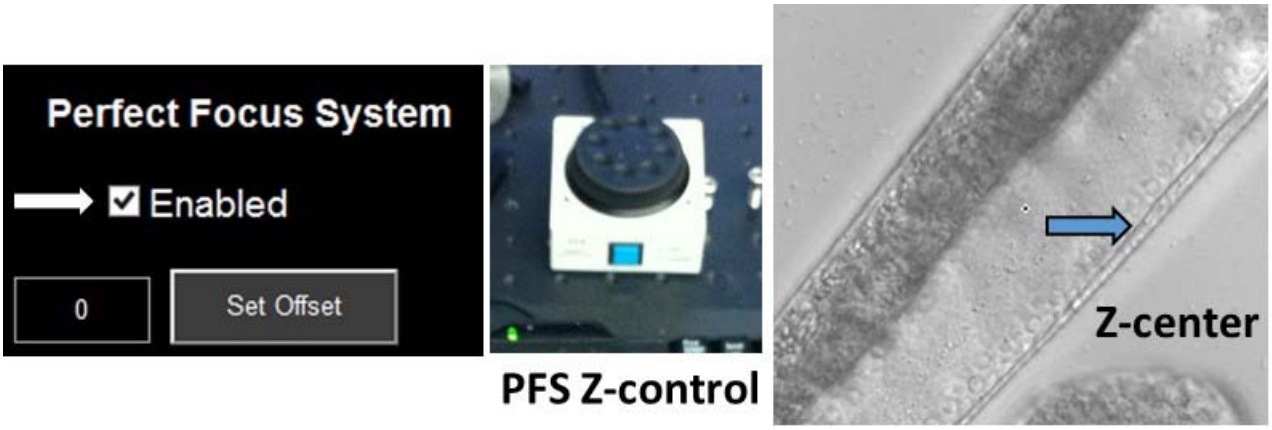


21. Turn on the Perfect Focus Unit. Check the box ‘Perfect Focus System: Enabled’ and then use the knob of the Perfect Focus System (PFS) joystick to adjust the Z height of the objective and bring the middle of the animal into focus (blue arrow). Click the ‘Set Offset’ button. The PFS system will

now automatically adjust the Z height of the 20X objective to this Z-level above the glass as the XY stage translates to new locations. The PFS uses a laser to track the glass surface and adjusts the Z-height of the 20X objective automatically.

Tip: The age synchronized animals should have very similar body widths so subsequent worms should be very close to this original setting.

Troubleshooting: See online tutorial If DIC image is poor and requires re-alignment (Link).



3f. Load and calibrate needle (2 min)

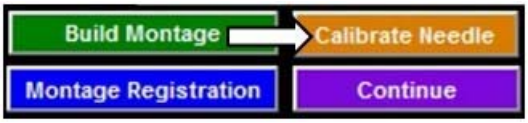
22. Fill the micro-needle with the plasmid from the back side using a long thin pipette tip and ensure that the plasmid is loaded into the tip of the micro-needle with no air bubbles.

    Tip: If air bubbles are present then gently flick the micro-needle to disperse the bubble. To prevent debris from clogging the needle load the micro-needles in a positive pressure air hood and use latex gloves. Store about 10 glass capillaries in independent plastic zipper bags to prevent exposure to debris as the original larger package (cardboard) is opened each time.

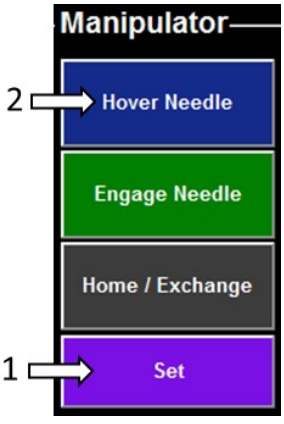
23. Turn off the main air pressure source at the wall and **put on safety eye glasses** since the micro-needle could become a dangerous projectile if broken or not properly secured in the capillary holder during a high pressure pulse. Unscrew the release mechanism on the Sutter manipulator

(See Figure in Step 13 above, blue arrow) and pull the capillary holder toward the user. Load the micro-needle into the universal capillary holder and tighten firmly. Return the capillary holder to the original secure position and tighten the release mechanism on the Sutter manipulator by hand.

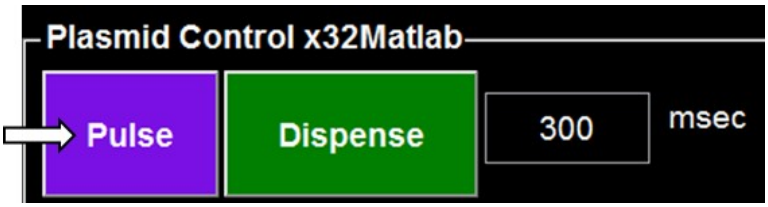
24. Calibrate the micro-needle position within the 20X image plane (Z height of the 20X objective). Ensure that the worms are still in focus with the 2X objective (adjust them into focus if necessary). Click the 'Calibrate Needle' button. The manipulator will move the needle into the field of view of the 2X objective (preset XYZ). Manually adjust the needle position to be centered over the crosshairs of the screen and Click the 'Continue' button. The system will automatically switch to the 20X objective.



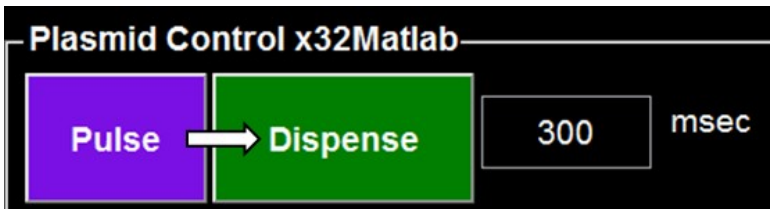
25. Use the Sutter ROE controller to bring the tip of the needle into view of the 20X objective. Click the 'Set' button (arrow 1) then immediately click the 'Hover' button (arrow 2) to calibrate the needle height with the Z height of the manipulator (The 'Set' button is active for 3 seconds).



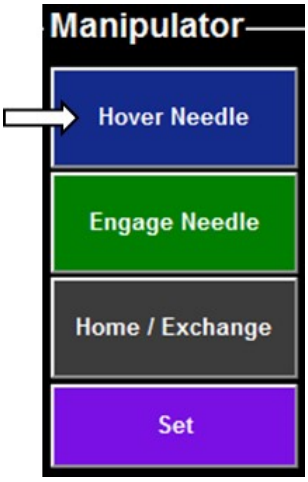
26. Click the ‘Pulse’ button to push out any air bubbles or gel from the tip of the needle. The ‘Pulse’ duration is preset for 100ms. The backpressure should be adjusted to ~3 PSI to enable a constant stream of plasmid that displaces a 3 µm diameter sphere of gel surrounding the tip of the needle.



27. Dispense plasmid to calibrate pressure and duration. The ‘Dispense’ duration can be set in the field to the right in msec. Tip: The suggested dispense duration is 300-500 msec at ~60 PSI depending on needle tip. The ‘Dispense’ pressure should be adjusted to ~60 PSI to enable a constant stream of plasmid that displaces a ~10 µm sphere of gel surrounding the tip of the needle.

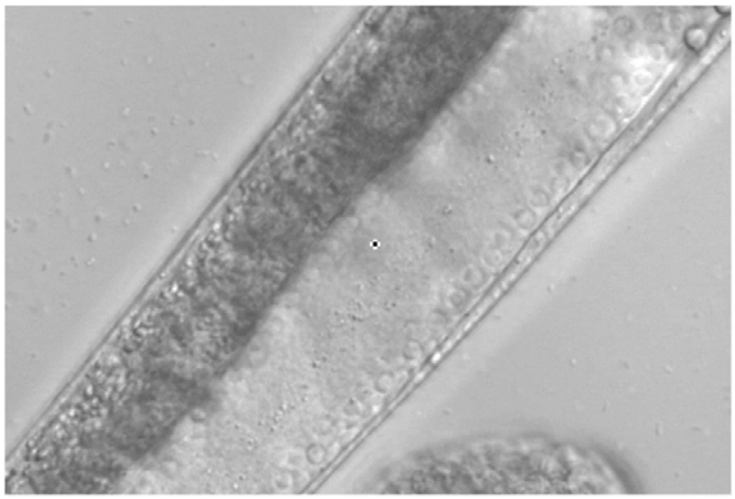


28. Click the ‘Hover Needle’ button to retract the needle at a 45° angle to a Z-height just above the hydrogel. This is the staging position for the needle. The software automatically performs a ‘Dispense’ after the needle exits the gel to remove any gel from the tip and prevent clogging.

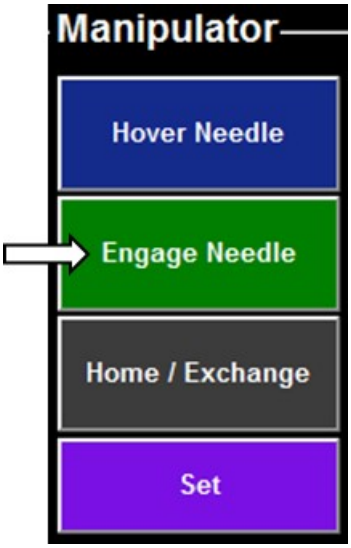


3g. Computer-assisted microinjection with complete software user guide (Timing: 25 sec / worm)

29. Perform microinjections: Switch to 20XDIC and click 'next worm' button to move to the first gonad position. Tip: Do not leave the worms in the gel for more than 1 hour since the gel may dry out and desiccate the animals. We show 100% animal survival after being immobilized in the hydrogel for 45 min (Figure S4e).
30. Bring the gonad into focus and left-click on the center of the gonad to perform a small XY alignment. The XY stage will translate to bring the center of the gonad in to the crosshairs.



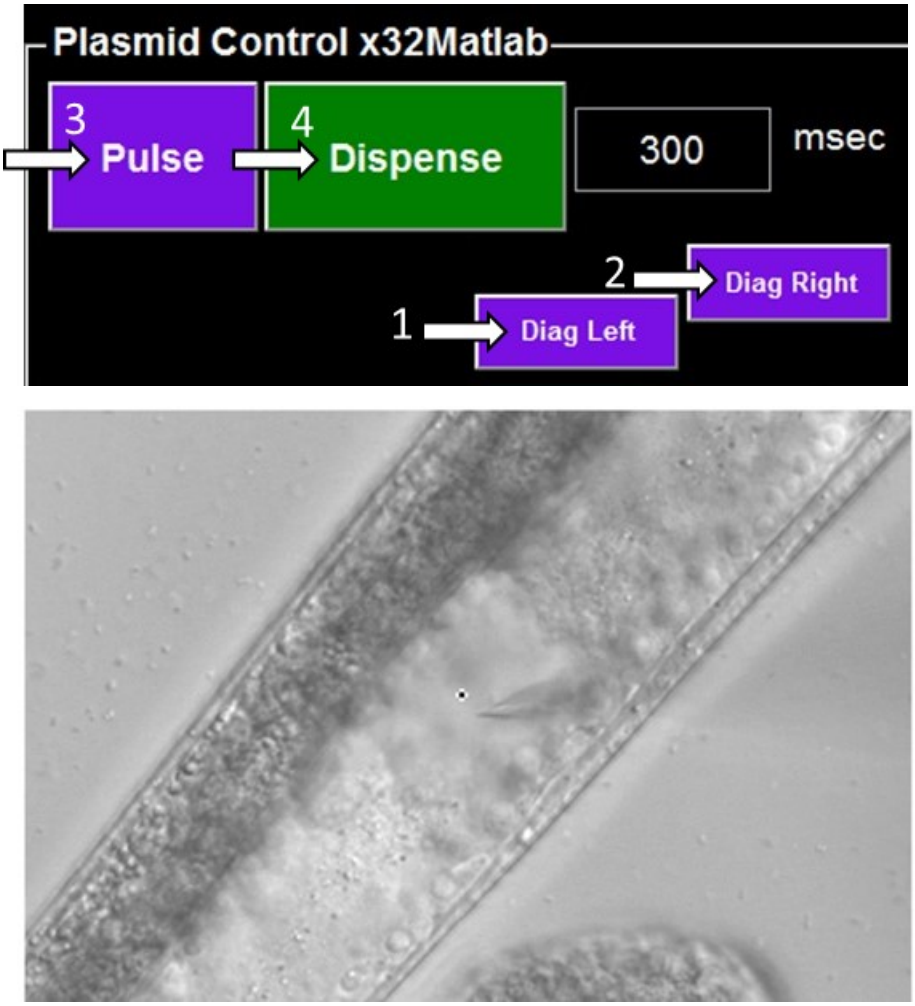
31. Click the 'Engage Needle' button to bring the needle into the image plane penetrating the worm cuticle. Before the needle begin its descent into the hydrogel the software automatically performs a 'Dispense' to prevent clogging and adjusts the XYZ position of the manipulator to enable a 45° angle of approach into the hydrogel.



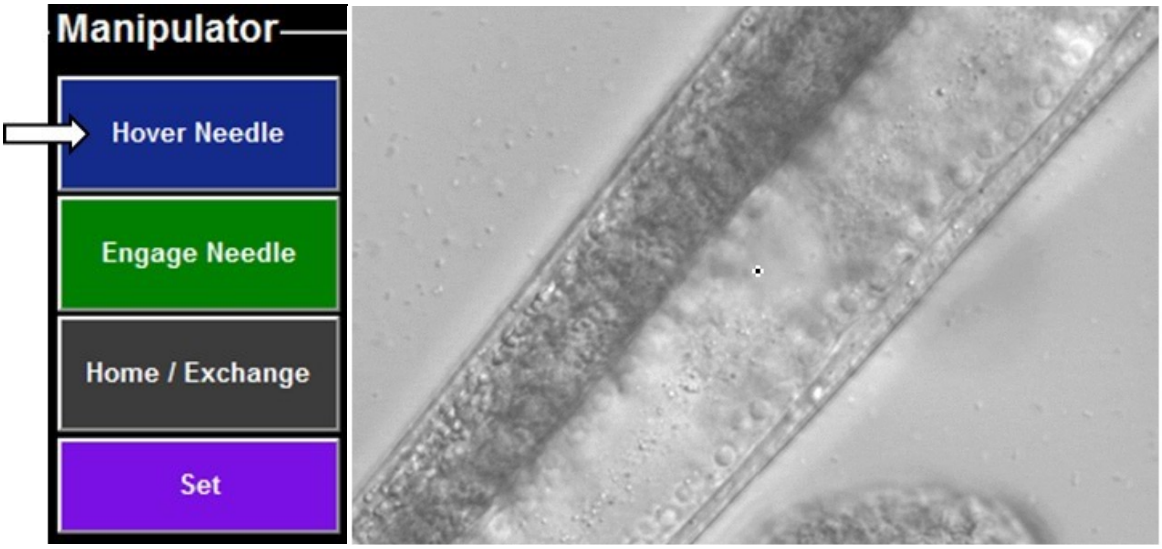
32. Adjust the needle position by clicking the ‘Diag Left’ and ‘Diag Right’ buttons (arrows 1 and 2, respectively) to ensure that the needle has penetrated the gonad sheath (with each click the needle will move 4.24 µm, 45° along the needle axis in the respective direction). The software automatically activates the piezo actuator (vibration) to assist with penetration of the gonad sheath. The back pressure flow of the needle can help the user to see the position of the needle inside the worm as the needle dispenses a very small amount of fluid. Click the ‘Pulse’ button to expel a small amount of plasmid and check that the gonad arm is being filled. If the needle is in the proper location then click the ‘Dispense’ button to fill the gonad arm until it acquires a fully

"inflated" appearance. Depending on needle tip opening, pressure, and duration the system should be calibrated to completely fill the gonad with ~2 clicks of the 'Dispense' button. The software automatically activates the piezo vibration during the 'Pulse' and 'Dispense' buttons to free the tip from debris to enable flow of plasmid.

Tip: If the animals burst (internal organs spill out) then use less pressure and less duration of the 'Dispense' button (the animals are being compressed by the hydrogel which creates an increased internal pressure). If the needle tip is against the opposite side of the gonad wall then it can block the flow of plasmid. Click the 'Diag Right' button to back away from the gonad wall and click the 'Dispense' button to fill the gonad.



33. Click the 'Hover' button to exit the worm. The needle will reverse out of the worm along its axis and into the 'Hover' position just above the hydrogel. The software automatically performs a 'Dispense' when the needle reaches its final position to clear any hydrogel that may dry on the tip and cause clogging.



34. Click the 'Next Worm' button to proceed to the next gonad target. The XY stage will translate to the next stored XY location for gonad region of interest. The worm number will update to the next worm number after both gonad locations have been visited.

35. Repeat Steps 29-34 until all desired microinjections are completed. Do not perform microinjections for more than 1 hr to ensure that the animals will be healthy enough to produce progeny.

3h. Post-injection recovery and culture (in parallel with hydrogel immobilization, Step 3b)

(Timing: 10 min)

36. Recover the worms: pull the XYZ manipulator arm out to access the well plate. Remove the well plate and fill the well with 5 ml of chilled (4°C) M9 medium. Place the well plate into a chilled

shaking incubator at 13°C for 10 min @ 20 rpm. Alternatively, the user can place the well plate in an incubator and agitate periodically by hand.

Tip: The hydrogel should be completely diluted and the worms should be floating. The worms will be motionless at this point due to the remaining sodium azide.

37. Use a glass pipet to transfer the animals from the well plate to an agar plate and place into a 20°C incubator for recovery. After about 5-10 min the worms will recover from the sodium azide and begin to crawl on the agar plate.
38. Clean the multiwell plate for reuse by rinsing remaining hydrogel out with water and then soaking in ethanol.

3i. Follow-up screening of transgenic animals

For our screening purposes we placed 5 animals on large agar plates and directly selected transgenic animals from the F2 generation based on expression of the fluorescent reporters. If you are selecting independent lines then place each injected P0 worm on an individual plate and then pick transgenic F1s to individual plates to isolate each independent line.

3j. Troubleshooting section with figures

<u>Section 1 Hardware Assembly:</u> Use of thermal cycler unit in place of custom peltier system

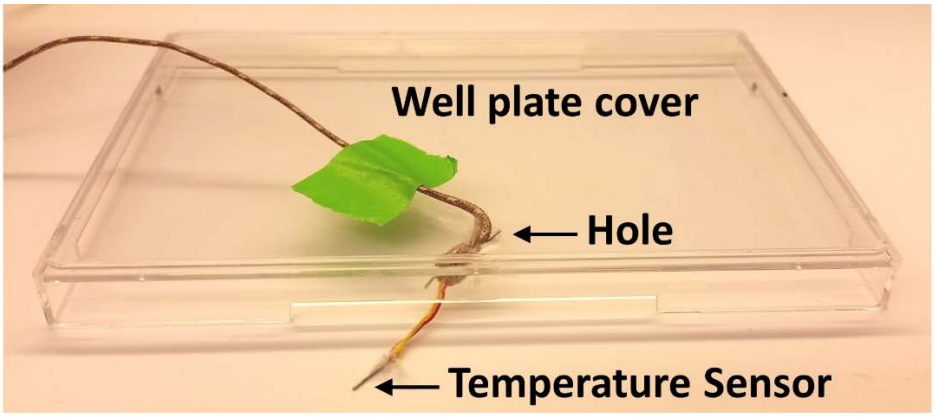
Problem: The user has a readily available thermal cycler unit (many models available: Link) and/or does not have the equipment or expertise to build the low cost custom peltier system with copper block and electrical circuitry (See Figures S3c and S4a).

Solution: A thermal cycler commonly used for PCR can be used for hydrogel immobilization in place of the custom peltier system. The temperature program should be set to 15°C for 6 min then quickly ramp up to 25°C for 3 min. The well plate can then be moved to the microscope for microinjection. Tip: Leave the top lid of the machine open as shown and ensure that the top lid heater is turned off to avoid overheating the plate. If the heating unit contacting the well plate is composed of metal cylinders commonly used for small tubes then the respective temperature program values should be adjusted to ensure adequate thermal transfer (estimate: 15°C to 13°C, 25°C to 27°C). See additional Troubleshooting section below to calibrate temperatures.

Section 1b Hardware Assembly, Fig. S4a, Step 5: Gel temperature calibration with handheld sensor

Problem: The user must measure the gel temperature to calibrate the parameters for the heating units (peltier heating unit, thermal cycler unit, stage incubator). The well plate cover must remain on the plate during the calibration experiment to retain heat and moisture limiting access to a temperature sensor.

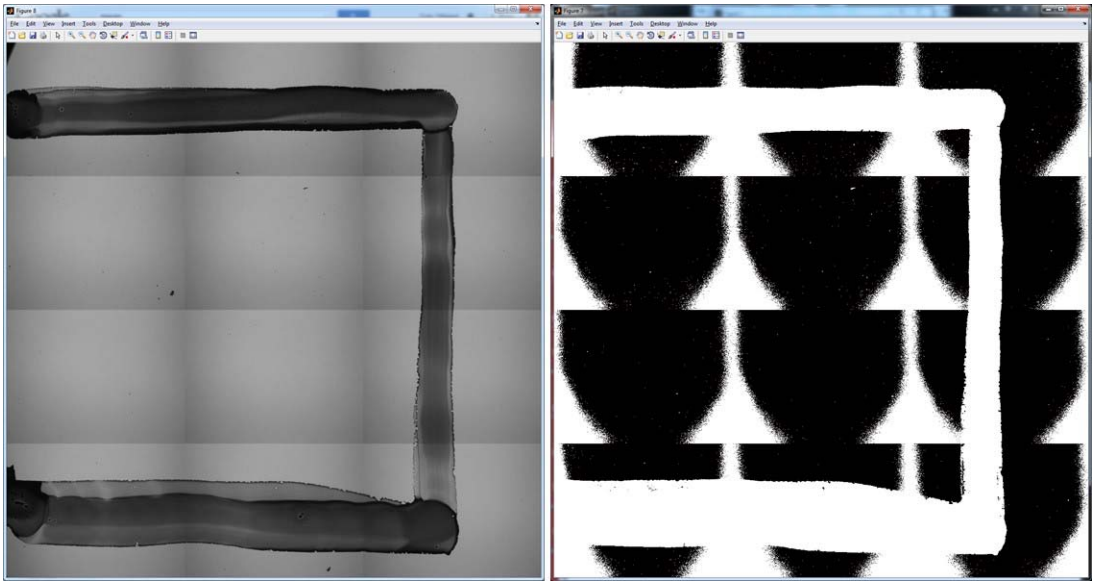
Solution:
(Left) Use a handheld thermometer (Omega #147U or similar model) with thermistor wire as temperature sensor. (Right) Drill a small hole in the plastic well plate cover over the desired sensing location. Thread the sensor wire through the hole to access the gel and leave enough slack to allow the thermistor to reach the surface of the glass. Use tape (green) to secure the wire in place. Place the cover on top of the well plate and keep a timed record of the temperature during the calibration to refine the input parameters for the peltier heating unit (current), thermal cycler unit (temperature) and stage incubator (temperature).



Step 16, 18

Problem: If the montage is not aligned properly or the worms are not detected then a misaligned light condenser may be causing too much variation in the background intensity. These non-uniformities in intensity may exceed the intensity threshold during image processing.

Solution: This can be corrected by re-aligning the condenser and creating a new condenser image to normalize the acquired images to remove the background non-uniformities.



Step 18

Problem: If a mutant worm is larger or smaller in area than the wild-type strain then they may not be recognized by the system.

Solution: Adjust the size selection criteria in Step 18 (area and standard deviation).

Table S2 Plasmids used in this study

Plasmid	Reporter	Neurons labeled
pCY32	*srh-142*pro:CFP	ADF
pMH419	*dat-1*pro:CFP	CEP
pMH421	*flp-8*pro:CFP	URX
pMH424	*flp-3*pro:YFP	IL1
pMH428	*ser-2*prom3:YFP	OLL
pMH429	*gcy-33*pro:CFP	BAG
pMH434	*tol-1*pro:YFP	URY
pMH445	*klp-6*pro:mCherry	IL2
pMH458	*ocr-4*pro:mCherry	OLQ
pMH459	*odr-1*pro:YFP	AWC
pMH461	*gcy-8*pro:mCherry	AFD
pMH470	*mec-3*pro:mCherry	FLP

Table S3 Transgenes used in this study

Transgene alleles	Plasmids
hmnEx353, hmnEx720, hmnEx708, hmnEx711, hmnEx713, hmnEx716, hmnEx728	pCY32, pMH459, pMH461
hmnEx371, hmnEx707, hmnEx709, hmnEx723, hmnEx714, hmnEx726, hmnEx717	pMH419, pMH428, pMH458
hmnEx719, hmnEx721, hmnEx710, hmnEx724, hmnEx764, hmnEx727, hmnEx729	pMH421, pMH434, pMH470
hmnEx705, hmnEx722, hmnEx763, hmnEx712, hmnEx765, hmnEx766, hmnEx718	pMH424, pMH429, pMH445

Table S4 Strains used in this study

Strain	Genotype
CHB776	*hmnEx353*
CHB794	*hmnEx371*
CHB1431	*hmnEx719*
CHB1417	*hmnEx705*
CHB1432	*him-5(e1490) V; ram-5(bx30) X; hmnEx720*
CHB1419	*him-5(e1490) V; ram-5(bx30) X; hmnEx707*
CHB1433	*him-5(e1490) V; ram-5(bx30) X; hmnEx721*
CHB1434	*him-5(e1490) V; ram-5(bx30) X; hmnEx722*
CHB1425	*cut-1(tm1126) II; hmnEx713*
CHB1426	*cut-1(tm1126) II; hmnEx714*
CHB1483	*cut-1(tm1126) II; hmnEx764*
CHB1484	*cut-1(tm1126) II; hmnEx765*
CHB1423	*cut-5(ok2005) X; hmnEx711*
CHB1435	*cut-5(ok2005) X; hmnEx723*
CHB1436	*cut-5(ok2005) X; hmnEx724*
CHB1424	*cut-5(ok2005) X; hmnEx712*
CHB1420	*cut-6(ok1919) III; hmnEx708*
CHB1421	*cut-6(ok1919) III; hmnEx709*
CHB1422	*cut-6(ok1919) III; hmnEx710*
CHB1482	*cut-6(ok1919) III; hmnEx763*
CHB1440	*cutl-14(gk533155) I; hmnEx728*
CHB1429	*cutl-14(gk533155) I; hmnEx717*
CHB1441	*cutl-14(gk533155) I; hmnEx729*
CHB1430	*cutl-14(gk533155) I; hmnEx718*
CHB1428	*T23F1.5(gk255820) V; hmnEx716*
CHB1438	*T23F1.5(gk255820) V; hmnEx726*
CHB1439	*T23F1.5(gk255820) V; hmnEx727*

File S2

Supplementary Video

Available for download as a .mov file at
www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.179648/-/DC1