Automating fruit fly Drosophila embryo injection for high throughput transgenic studies

E. Cornell
Engineering Division, Lawrence Berkeley National Laboratory, Berkeley, California 94720, USA

W. W. Fisher
Life Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, California 94720, USA

R. Nordmeyer and D. Yegian
Engineering Division, Lawrence Berkeley National Laboratory, Berkeley, California 94720, USA

M. Dong and M. D. Biggin
Genomics Division, Lawrence Berkeley National Laboratory, Berkeley, California 94720, USA

S. E. Celniker
Life Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, California 94720, USA

J. Jin
Engineering Division, Lawrence Berkeley National Laboratory, Berkeley, California 94720, USA

(Received 13 August 2007; accepted 26 November 2007; published online 15 January 2008)

To decipher and manipulate the 14 000 identified Drosophila genes, there is a need to inject a large number of embryos with transgenes. We have developed an automated instrument for high throughput injection of Drosophila embryos. It was built on an inverted microscope, equipped with a motorized *xy* stage, autofocus, a charge coupled device camera, and an injection needle mounted on a high speed vertical stage. A novel, micromachined embryo alignment device was developed to facilitate the arrangement of a large number of eggs. The control system included intelligent and dynamic imaging and analysis software and an embryo injection algorithm imitating a human operator. Once the injection needle and embryo slide are loaded, the software automatically images and characterizes each embryo and subsequently injects DNA into all suitable embryos. The ability to program needle flushing and monitor needle status after each injection ensures reliable delivery of biomaterials. Using this instrument, we performed a set of transformation injection experiments. The robot achieved injection speeds and transformation efficiencies comparable to those of a skilled human injector. Because it can be programed to allow injection at various locations in the embryo, such as the anterior pole or along the dorsal or ventral axes, this system is also suitable for injection of general biochemicals, including drugs and RNA*i*. © *2008 American Institute of Physics.*
[DOI: 10.1063/1.2827516]

I. INTRODUCTION

Our research goals are to understand gene function in the control of early development of the fruitfly, *Drosophila melanogaster*.[1–4] One technique for manipulating genes in Drosophila is to inject DNA into very young embryos.[5,6] In a percentage of these embryos, the injected DNA will be incorporated into the chromosomes of the germ line cells and thus will become a permanent part of the next generation of the fly. The embryo must be injected while still just a single ovoid cell, merely 500 μm in length and 200 μm in width. To produce transgenic flies, DNA must be deposited just inside the posterior tip of the embryo prior to the formation of germ-line pole cells. Following a 1 h egg lay, embryos must be collected, dechorionated, aligned, desiccated, and injected within 60 min. Hundreds of eggs must be injected to get a few transgenic flies.

Genome sequencing and annotation has identified over 14 000 Drosophila genes.[7–9] With the shear number of experiments proposed, the generation of transgenic flies becomes rate limiting and automation becomes beneficial.

Robotic systems have been extensively used in the life sciences and the pharmaceutical industry, where they outperform human operators in carrying out simple, repetitive tasks, for example, instruments we have developed to automate aspects of x-ray crystallography.[10,11] Still, the automation of Drosophila embryo injection presents a particular challenge because it must match a trained professional in an application that requires a high degree of hand-to-eye coordination, in real time and with microscopic precision. Recently, Zappe *et al.*,[12] have developed a high throughput microelectromechanical system capable of automatically injecting DNA and RNA into Drosophila embryos. It uses a custom design and a microfabricated needle. The needle is positioned vertically over each embryo and injects downward into the side of the embryo. The needle design, its physical dimensions and the curvature of the embryo at the termini make it difficult to inject DNA close enough to the posterior pole to achieve useful transformation rates. As a

result, this instrument is not suitable for producing transgenic flies. Using microfabrication and a fluidic microassembly, the same research group has reported the development of a tool for easy positioning of embryos in a two-dimensional (2D) array.[13] While it succeeds in arraying embryos in 2D, this system permits only vertical injections, because the estimated adhesion force obtained by the process is much less than the force required to penetrate embryos from a horizontal position.

We have developed an automated Drosophila embryo injection system that is capable of injecting nearly as successfully and rapidly as an experienced human operator, but without the fatigue factor that limits the number of injections that an operator can perform per day. Since it injects horizontally, it is capable of depositing biological materials to the poles as well as along the length of the embryo. Therefore, it is applicable not only for high throughput generation of transgenic animals but also for RNAi screening.[12] A prototype alignment jig capable of positioning embryos on a slide ready for injection is also presented. We describe in Sec. II the injection procedure as manually performed; Sec. III, the instrument and methods employed in the automated system; Sec. IV, preliminary results using this instrument. Lastly, we give a summary of our present studies and briefly discuss our future development plans.

II. MANUAL INJECTION

DNA was prepared using a QIAGEN plasmid midi kit. The helper plasmid used in these experiments is the S129A P-element transposase.[14] The DNA solution injected was a mixture of 0.5 mg/ml transgene-containing plasmid and 0.1 mg/ml helper plasmid. The DNA solution was spun through a 0.45 μm filter (Costar Spin-X 8162) immediately prior to injection.

To prepare embryos for injection, flies with the genotype yw were kept in Plexiglas cages in the dark and allowed to lay eggs on a stiff gelatin surface of agar and molasses with a daub of yeast paste. Embryos were collected every 60 min, immersed in a 50% Clorox solution (3% sodium hypochlorite) for 90 s to remove the chorion, and thoroughly rinsed. Dechorionated embryos were visualized at 25× magnification, manipulated with a sewing needle into a line on a 0.9% agarose slab and affixed to a glass cover slip coated with adhesive. The adhesive is obtained by dissolving it from tape backing (Tesa 4124 Beiersdorf AG) in high-grade heptane (Burdick and Jackson, catalog No. 207-1, 99% *n*-heptane for high performance liquid chromatography). This adhesive/heptane mixture has been shown not to damage Drosophila embryos. Embryos were desiccated in a Drierite-filled chamber (Drierite.com) for 10–15 min, and then covered with a thin layer of halocarbon oil (Series 200, Halocarbon Products Corp) before injection.

Aluminosilicate capillary tubes (Sutter Instrument Co., catalog No. AF100-68-10) were pulled in a needle/pipette puller (model 720, David Kopf Instruments, Tujunga, CA) to produce a needle with a very sharp point. DNA solution was back loaded into each needle using Eppendorf Microloaders (order No. 5242 956.003). Loaded needles were connected via tubing to an injector device (Harvard Medical Systems PLI-100 Pico-Injector) and locked into a Narishige micromanipulator (model No. MN-151). Pulled needles have sealed ends, so the tips were broken against a glass slide to create an opening of 0.1–0.5 μm. Alternatively, a preformed μ TIP™ needle with an opening of 0.3 μm (WPI catalog No. TIP03TW1F, World Precision Instruments, Inc., Sarasota, FL) was used in place of the pulled capillary tube.

Desiccated embryos were viewed on a Carl Zeiss Axiovert microscope at 200× magnification. The micromanipulator was used to position the needle tip into the approximate center of the visual field. After positioning, it was used primarily to move the tip up or down to match the z coordinate of the center plane of each embryo. Needle tips that strike the embryo off center are typically deflected by the curvature of the posterior pole of the embryo.

Embryos are moved by the microscope stage into the needle until the membrane is penetrated. The distance that a needle tip must be advanced into a given embryo to penetrate the membrane is a function of needle sharpness and embryo desiccation. A relatively stiff embryo, subjected to little desiccation, will give only a small amount before needle penetration occurs. A flaccid embryo, one that has been desiccated a great deal, may form a large indentation before needle penetration occurs. Likewise, a sharper needle will penetrate a given embryo more easily than will a blunt needle. After penetration is detected, the embryo is backed off until the needle tip is just inside the posterior pole, where the pole cells, the future germ-line cells, will form. A small volume of DNA is injected, and the needle withdrawn. Ideally, no cellular material will leak from the embryo, which should look virtually the same before and after the injection.

Slides with injected embryos were placed in 100 mm Petri dishes filled with agar-molasses medium and allowed to develop for 24 h at 21 °C followed by 24 h at 25 °C. Hatched larvae were collected, placed into vials filled with standard Drosophila media, 40–50 larvae per vial, and allowed to develop at 21 or 25 °C. Newly eclosed adults were collected, put into individual vials, and mated to *yw*; Sco/CyO flies. The progeny of this cross were scored for transgenic animals, i.e., flies that have incorporated the injected DNA into their chromosomes.

III. AUTOMATED INJECTION

In this section, we describe how the injection system and the control software were designed and constructed to reproduce the steps detailed above. The robot must recognize the anterior and posterior poles of each embryo, insert a needle into the center of the posterior pole, recognize when penetration has occurred, and deposit DNA as close as possible to the posterior pole, all before the embryos on a given slide have become too old to be transformed.

A. Alignment

To inject embryos quickly using a system with a single needle, they need to be closely aligned in an evenly spaced

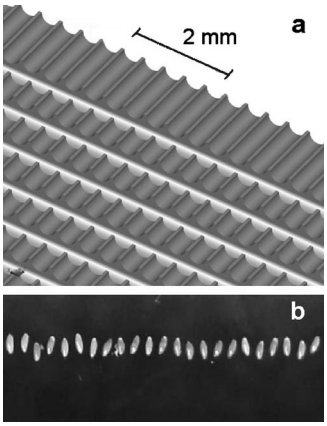

FIG. 1. Embryo alignment jig. (a) A computer drawing of a portion of the jig showing the groove structure and (b) an example of jig-aligned embryos that have been transferred onto a coverslip.

row and oriented for injection. Manual alignment requires considerable practice in working with dechorionated embryos that are small, fragile, and sticky.

We tested a number of tools and methods to address this alignment challenge, including microfabricated arrays similar to those designed by Bernstein *et al.*[13] The most practical device we developed was a jig that contained a dense array of precisely machined U-shaped grooves on a block of stainless steel [see Fig. 1(a)]. To use it, embryos are brushed into prewet grooves with a fine grade paintbrush. Embryos settle deeper into the grooves as the water evaporates and require subtle adjustments with the paintbrush or a metal needle to improve alignment. The embryos are monitored under the microscope until their surfaces are sufficiently dry to be transferred onto a coverslip. The width of the U-shaped groove is slightly wider than the embryo diameter, but the depth slightly shallower, allowing sufficient exposure of the embryo for contact with the coverslip. An example of a set of aligned embryos is shown in Fig. 1(b).

This alignment jig provides an alternative to the traditional alignment method. It has the potential to allow higher throughput in alignment as many rows can be aligned at once. There are, however, two drawbacks to using the jig. First, embryos are aligned without visual inspection, so embryos cannot be screened to reduce the number of embryos that have developed too far to be transformed. Ensuring that embryos are well staged in advance minimizes this problem. Second, the embryos are not oriented with regard to the anterior posterior axis. This could be overcome by either a second injection pass with the slide manually rotated 180° or by modifying the robot to use two injection needles to inject from both sides. In the latter scenario, all embryos will be injected in a single pass

B. Injection instrument

The automated Drosophila embryo injection instrument, as shown in Fig. 2, was built on a Nikon TE200 inverted microscope, with a 75 W top illuminator and a 2 Mpixel camera (model-LE275C, Lumenera Corp., Ottawa, ON, Canada). The magnification obtained by a 4× objective lens provides a field of view of about 1.6×1.2 mm^2, and four to

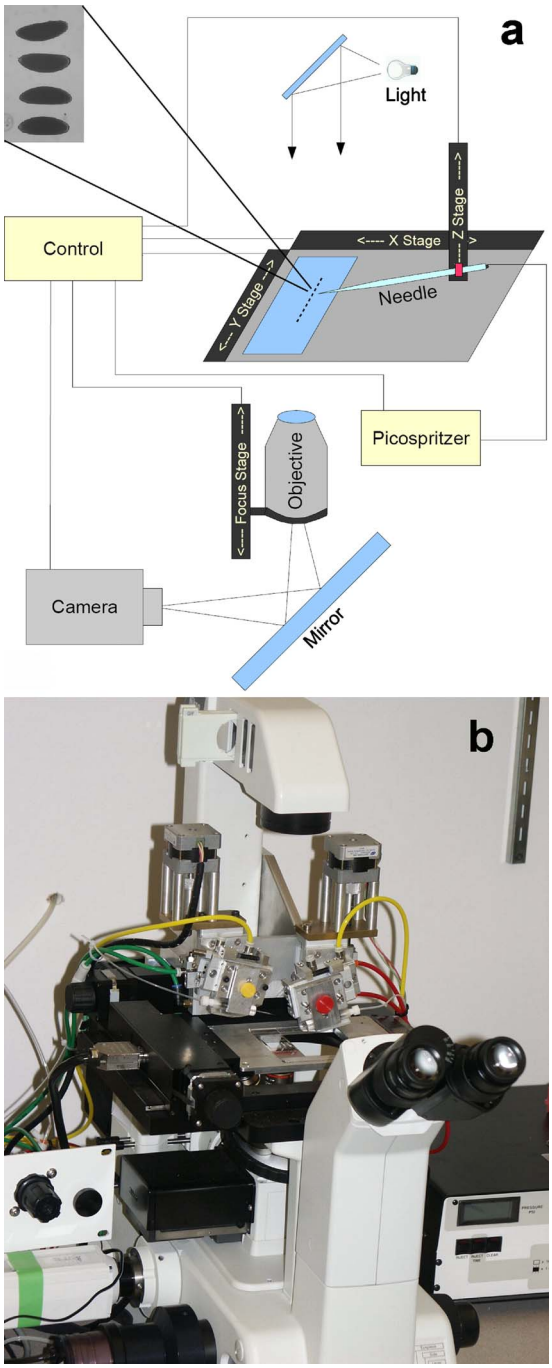

FIG. 2. (Color online) The robot injector microscope. (a) An illustrative sketch of instrument layout. (b) A photo of completed instrument. The motorized stage includes a mount that accommodates three slides. The stage has calibrated movement in the x and y planes, while the needle holders have calibrated movement in the z direction. Needle holders on the left and right allow for injection regardless of embryo orientation. (Note: The second Needle holder on left side has not yet been integrated into the software). The charge coupled device camera is visible at the left side port.

five embryos spaced 0.15–0.2 mm apart can be seen in each frame. The microscope has a Prior stage (OptiScan II, Prior Scientific Inc., Rockland, MA) for motorized *XY* movement of the slide. The same Prior control system provides motorized control of microscope focus. We built a custom slide holder, allowing up to three slides of embryos to be loaded at a time. The loaded needle was inserted into a precision holder connected to a gas pulse generator (Picospritzer III,

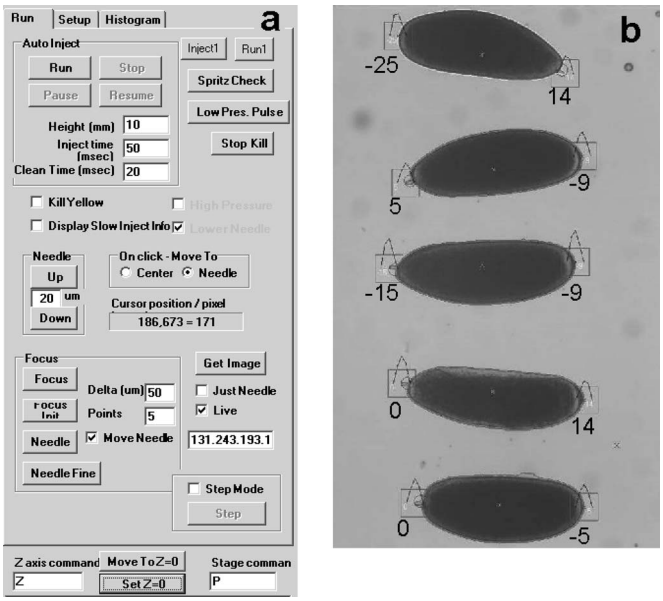

FIG. 3. The injector control GUI. (a) The "Run" tab. In the "Auto inject" box, the "Inject time" defines the volume of biological material to be deposited, the "Clean time" determines the needle flushing volume following each injection, and by pushing the "Run" button a whole slide will be injected automatically. The buttons on the right hand side allow injection of a single embryo or all in the current field of view, and manual tests of injection volume and needle flushing. The "Needle" box controls the vertical movement of the needle. The "focus" box defines autofocus parameters and provides controls of autofocus on embryos and needles. (b) An example that displays the result of autofocusing. Embryos in this field of view were marked accept or reject (e.g., the one on the top because its micropyle was on the wrong side) for injection. The relative z offsets (in μm) of center plane heights of each embryo were also clearly indicated.

Parker Hannifin Corp., Cleveland, OH). Pressure and pulse length are used to control the speed and amount of DNA dispensed. Each needle is calibrated to dispense 40–80 pl of DNA by determining the size of the droplet in halocarbon oil dispensed by 20–200 ms pulses at a typical injection pressure of 30 psi. The needle holder is mounted onto a custom motorized stage and the height (z axis) of the needle automatically adjusted with high speed and high precision ($\sim$1 μm). The needle is tilted 15° relative to the horizontal plane, to avoid collision with the motorized stage. This system can be built on any optically appropriate microscope. The only modifications made to the Nikon TE200 were a few tapped holes on the support beam of the top illuminator to allow attachment of the motorized stage for needle movement. The total cost for parts and components used was \$25–30K. Part list and engineering drawings are available at "http://bdtnp.lbl.gov/Fly-Net/."

C. Control software

We have developed software for integrated instrument control, image acquisition and analysis (available at http://bdtnp.lbl.gov/Fly-Net/). It was coded in the C# language of Microsoft VISUAL STUDIO.NET and used a MySQL database for data management and archiving. Figure 3(a) shows the main user interface of this software. Using the Run tab, a user is able to initiate injection of a row of embryos, inject all the embryos in the current field of view, or control the components individually. The Setup tab (not shown) provides access to parameters for injection and image analysis.

D. Measuring embryos

For each embryo in a field of view the position, size, angle, orientation, age, and height of the center plane of the termini were determined. An autosegmentation algorithm found the boundary of the embryo, and an anterior embryonic structure called the micropyle was identified and used to determine orientation. The embryonic length, width, area, perimeter, and angle relative to the normal were calculated and compared to the values for a defined embryo. (The accepted values are length of 430–584 μm, width of 170–250 μm, area of 65 000–85 000 μm^2, perimeter of 875–1460 μm, age factor of 0–50, and angle of −45° to +45°.) These values were empirically determined. Those that fell outside the accepted range for any of these values were not injected. Embryos at the blastoderm stage of development, too old to be transformed, have a contrasting light band along their external edge, caused by the migration of nuclei to the perimeter of the embryo. The magnitude of this contrast was measured and used as a criterion for rejecting embryos.

The height of the termini varied by about 50 μm. Since it was important for the inserted needle to be within 10 μm of the embryonic center, it was necessary to measure the z coordinate of the posterior pole of each embryo so the needle could be properly positioned. We developed an autofocusing procedure which moved the focal plane of the microscope through five points, separated by a chosen step size (typically 50 μm), and used the contrast in the images from the 2 Mpixel camera at each of these five points to determine the z coordinates of the center plane of both termini of each embryo in the field of view. The three points with the best contrast were fit with a quadratic equation. The point at which the equation reaches a maximum was used as the estimate of the z coordinate. In Fig. 3(b), the five-point curves for the termini of each embryo are shown and the z coordinate calculated by the fit is printed below the curve. This procedure took about 15 s to complete and the z coordinates were reproducible to about 10 μm.

E. Calibration of needle positions

To accurately penetrate the embryo and deposit DNA, we manually calibrated the x, y, and z coordinates of the needle tip. The needle was lowered until it was focused and its x and y coordinates were manually set. The z coordinate of the needle was calibrated relative to one of the embryos in the field of view. Specifically, the needle was lowered incrementally in 10–20 μm steps. At each step, the embryo was brought into contact with the needle tip by moving the xy stage, creating a dent (see Fig. 4) on the embryo. When the height of the needle tip was within 10 μm of the height of the center of the posterior pole, a clear and maximum indentation was observed on the tip of the embryo and the operator set the z position to zero. This process has recently been automated by a software function carrying out the needle and embryo movements described while analyzing the contact to

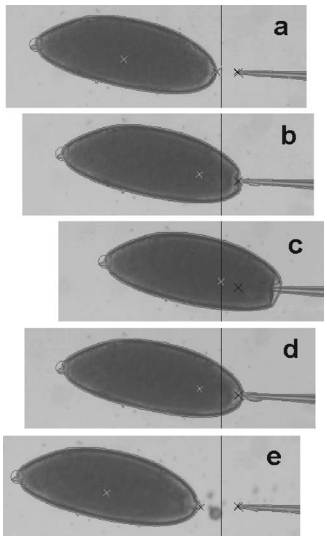

FIG. 4. A typical injection sequence. (a) The starting positions of needle tip and point of contact were marked with an *X* and the stationary reference point with a vertical line. (b) When the embryo makes contact with the needle, a clear indentation was observed. (c) Just prior to penetration, the indentation enlarges. (d) Penetration was complete and the membrane recovered (as shown, the needle is at dispensing position and the DNA has been deposited). (e) The needle has been pulled out the embryo and flushed (the droplet between the embryo and the needle, which had a volume equivalent to the DNA deposited).

determine where the maximum indentation was observed. The test results presented later were generated before the calibration of the needle was automated.

F. Detecting embryo penetration

As in the manual protocol, our system moves the embryos toward the needle for injection. It is important that the robot recognizes when the needle has entered an embryo, since pushing it further onto the needle than necessary increases the size of the holes in the vitelline and plasma membranes and results in sterility or lethality. Before the outer membrane is pierced it is displaced inward, forming a measurable indentation, which gets larger as the embryo is advanced, and disappears upon penetration. By comparing the initial and current images while driving the embryos into the needle (see Fig. 4), the software was able to detect both the indent and the recovery in real time.

G. Automated injection

Having developed methods for imaging the embryos and detecting needle penetration, we performed automated embryo injections. Embryos were mounted, 60–70 per slide, a needle loaded, and its position calibrated. The injections started with the autofocus routine that located all embryos and determined their heights. Each embryo that was suitable for injection based on its age, length, width, area, perimeter, and angle was injected. Once the embryo was moved to the starting position (~40 μm away from the tip of the needle), the needle was lowered to the center plane of the embryo. The stage was then moved 100 μm in the x direction. If penetration was not detected, the stage was then advanced in 10 μm steps until the images showed evidence of embryonic

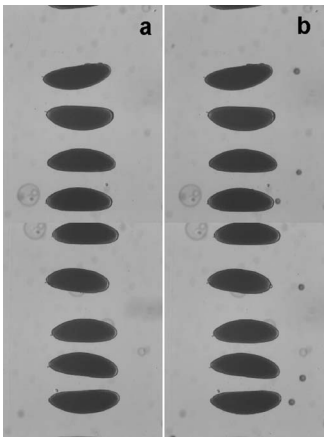

FIG. 5. Automated embryo injection. Shown are 9 of approximately 60 embryos (a) pre- and (b) postinjection. Neither leakage of embryonic fluids nor any visible damage is observed, demonstrating the sensitivity of the robot. Flushing the needle following each injection generates the small droplets seen on the right hand side of the embryos in (b).

penetration or until a maximum allowed depth of 150 μm had been reached. Once penetration was detected, the stage was moved back to position the needle tip 15 μm inside the embryo. The Picospritzer III was then activated to deposit a droplet of DNA solution.

After injection, the needle is withdrawn, residual debris in the needle cleared with a high-pressure (~100 psi) pulse of 20 m and the needle was raised slightly above the oil surface. The practice of bringing the needle in and out of the oil between injections helped prevent buildup of viscous embryonic fluid on the needle tip. The stage is then moved to the next embryo and the injection process is repeated. Figure 4 illustrates an injection run and Fig. 5 shows examples of embryos pre- and postinjection. After the robot injected all acceptable embryos, or had reached the user-defined end of the slide, the remaining embryos were robotically destroyed, using an algorithm that directs the needle to tear the vitelline and plasma membranes. The "kill subroutine" does not affect the size or shape of the needle tip. The next slide was then processed. Recalibration of the needle height between slides was not usually required as the variation of slide thickness was small and within the range of the vision system.

IV. RESULTS

To evaluate the robotic injection instrument versus the traditional manual method, we performed identical automated and manual transgenic injection experiments. The comparative injection results are summarized in Table I.

A. Comparison of manual and robot injection results

The success of a given injection experiment can be measured by the transformation efficiency, defined as the number of transformed flies obtained per 100 embryos injected. Using P-element based constructs, transformation efficiencies typically range from a few to 15%. Many factors significantly affect this efficiency, including the fly strain used, time window for injection, degree of desiccation, location and amount of DNA deposited, as well as the specific nature

TABLE I. Comparison of manual and automated injections based on survival, fertility, and transformation rate.

Experiment	Injected	Larvae[a]	Adult[b]	Fertile[c]	Transformed[d]	Overall rate[e] (%)
Initial injection algorithm (four injections/min)[f]						
Manual 1	392	250 (64%)	181 (72%)	147 (81%)	36 (25%)	9.2
Robot 1	184	113 (61%)	87 (77%)	69 (79%)	7 (10%)	3.8
Manual 2	404	288 (71%)	221 (77%)	164 (74%)	42 (26%)	10.4
Robot 2	113	75 (66%)	57 (76%)	44 (77%)	4 (9%)	3.5
Manual 3	101	76 (75%)	61 (80%)	41 (67%)	13 (32%)	12.9
Robot 3	106	66 (62%)	45 (68%)	30 (66%)	6 (20%)	5.7
Manual 4	114	72 (63%)	48 (67%)	22 (46%)	4 (18%)	3.5
Robot 4	152	83 (55%)	34 (41%)	22 (65%)	2 (9%)	1.3
Improved algorithm (eight injections/min)[f]						
Manual 5	334	209 (63%)	122 (58%)	88 (72%)	25 (28%)	7.5
Robot 5	116	69 (60%)	49 (71%)	30 (61%)	6 (20%)	5.2
Manual 6	172	141 (82%)	122 (87%)	72 (59%)	13 (18%)	7.6
Robot 6	341	250 (73%)	187 (75%)	166 (89%)	14 (8%)	4.1
Manual 7	236	168 (71%)	136 (81%)	102 (75%)	10 (10%)	4.2
Robot 7	249	146 (59%)	109 (75%)	67 (62%)	7 (10%)	2.8
Manual 8	201	168 (84%)	137 (82%)	98 (72%)	25 (26%)	12.4
Robot 8	341	216 (64%)	162 (75%)	101 (62%)	5 (5%)	1.5
Manual 9	179	128 (72%)	105 (82%)	72 (69%)	5 (7%)	2.8
Robot 9	327	196 (60%)	153 (78%)	100 (65%)	7 (7%)	2.1

[a]Number of embryos that hatch to become first instar larvae.
[b]Number of larvae that become adults.
[c]Number of adults that are fertile.
[d]Number of fertile adults that are transformed.
[e]Percentage of embryos injected that generate transformants.
[f]Speeds quoted have a nominal error of 0.5 embryos/min.

of the DNA to be inserted and the helper plasmid chosen. We therefore tested robot versus manual injection efficiency using several different DNA constructs. Each pair of manual and robot injections used the same construct/helper mix and fly strain.

Injection quality was measured at four different stages: survival to larvae (hatch rate), survival to adult (eclosion rate), fertility, and transformation (rate that fertile adults incorporated the injected DNA into their germline DNA). The overall efficiency is captured in the final column of Table I, represented by the number of transformant fly lines obtained per 100 embryos injected. In the first four experiments listed in Table I, the robot was roughly one-third as efficient as the manual control. We noticed that the hatch, eclosion, and fertility rates were comparable, but the transformation rates of the automated injections were roughly half that of the manual injections. The robot was not damaging the embryos, but neither did it seem to be placing the DNA in the right place at the right time. One possibility was that the robot was not fast enough to inject each embryo on a given set of slides before many had developed too far to be transformed. Subsequent adjustments in the injection algorithm and optimization of the image acquisition speed greatly improved the injection speed. This change had an immediate effect on the transformation results as seen in exps. 5–9. Overall efficiency varied from 50% to 75% of the manual control for all but experiment 8, a marked improvement over the previous version of the algorithm.

It takes us approximately 30 min to prepare 150–200 embryos for injection including collections, dechorionation, alignment, transfer to a coverslip, and dessication. Once a slide is in place and the needle position calibrated, the robot can inject 60 embryos in 8 min. After injection, it takes a few minutes to prepare the embryos for hatching. So a complete cycle for embryo preparation and injection is 60–75 min. Since it takes the robot 8 min to inject each slide, and the robot, as configured, holds three slides, it is possible for the robot to operate without intervention for stretches up to 24 min. The operator can use this time to prepare embryos. In the sixth experiment 341 embryos were robotically injected in less than 2.5 h, resulting in 14 transformants.

V. SUMMARY AND OUTLOOK

We have developed an automated instrument for high throughput transgenic injection of Drosophila embryos. It has proven ability in transgenic experiments, as it reliably and consistently delivered biomaterials just inside the posterior poles of Drosophila embryos. Different algorithms could be written that would allow the robot to inject at other locations in the embryo, such as the anterior pole or along the dorsal or ventral axes, thus this system is also suitable for injection of general biochemicals, including drugs and RNAi.

Once the injection needle and embryo slide are loaded, our system automatically locates and characterizes all embryos on a set of slides and injects all qualified embryos while destroying those that are unwanted. With its 2 Mpixel vision capability and specialized injection algorithm, it injects nearly as accurately and gently as a human operator.

The capabilities of programed needle flushing and monitoring of needle status after each and every injection ensures reliable delivery of biomaterials. Our data suggest that the robot can achieve transformation efficiencies similar to those of a skilled human operator. Given its speed and success rate, it is now possible for the robot to handle this technically demanding task, with the human operator mainly handling embryo alignment in parallel to the automated injection. In principle, the robot could inject five constructs, or around 1000 eggs in 5–6 h, which is more than a manual injector is likely to do in a day.

We are in the process of integrating a second needle into the automated injection protocol so that the benefit of the alignment jig can be fully realized. We will continue to improve injection speed by reducing the image acquisition time and explore better ways to align embryos. The ability to quickly exchange needles (and therefore biomaterials) will become increasingly important with the advent of new technologies that improve the biological efficiency of transformation, so that mere dozens instead of hundreds of embryos per compound need be injected. We plan to improve the needle position calibration method and design and develop a rotary mechanism that can hold an array of needles, so that needles can be rotated in and out of the injection position and calibrated quickly.

ACKNOWLEDGMENTS

We thank Barret Pfeiffer for preparing the DNAs for injection and Caitlin Barale for preparing some of the embryos used in this study. The manual injections were done by WWF. The software was written by EC. This work was supported by NIH grant R01 (GM070444) to MDB. E.C. and W.W.F. contributed equally to this work.

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