Customized Microinjection Glass Capillary Needles for P-Element Transformations in *Drosophila melanogaster*

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ABSTRACT

Here we describe how to generate customized microinjection needles from glass capillary tubes. Controls demonstrate the range of variables and effects on needle tip shape using a standard Flaming/Brown micropipet needle puller. Needles generated with two-cycle pulls provide a wider range of needle shapes in a predictable fashion. We used the needle puller's ramp function for multiple-cycle programs to determine the useful range of heat settings inherent to the glass capillary tube. This article focuses primarily on the preparation of injection needles utilized for P-element-mediated germ-line transformation in *Drosophila melanogaster* that do not require the dechorionation of the egg. However, these types of needles can be useful for numerous other types of injections, such as RNA interference, homologous recombination mutagenesis, morpholinos, transient gene regulation, drug delivery, and the transfer of cytoplasmic factors that are useful in a wide range of biological systems ranging from plants to vertebrates. Using our standard needle, we correlate the survival of injected *D. melanogaster* embryos with transformation efficiencies and plasmid construct characteristics.

INTRODUCTION

Microinjection techniques are used in vivo with numerous research strategies, such as transposase-mediated germ-line transformation, Gal4-mediated gene regulation, RNA interference, homologous recombination mutagenesis, P-element mutagenesis, antibody inhibition, morpholino antisense oligomers, transient gene expression and regulation, targeted gene regulation with *engrailed* fusion proteins, drug delivery, and transfer of biologically active cytoplasmic factors (2–16, 18, 19, 21–34, 36, 38, 39, 41–52). Many of these techniques were initially characterized in *Drosophila melanogaster*. However, the wide range of organisms in which others utilize microinjection techniques includes numerous arthropods, vertebrates, stem cells, plants, yeast, paramecium, and cultured cells.

Here we demonstrate a method for generating customized microinjection needles from glass capillary tubes. The technique involves a two-cycle pull in which the capillary tube is stretched during the first cycle and then separates during the second cycle. We utilize a standard Flaming/Brown micropipet puller (model P-87; Sutter Instrument, Novato, CA, USA). Alternative techniques used for the preparation of microinjection needles have been previously described (1, 7, 19). This analysis describes how to alter the shape of your injection needle in a predictable manner to produce one that is suitable to most techniques and organisms. Previously, we had difficulty producing a needle that had both durability and functionality for injections through the tough

chorion of the *D. melanogaster* embryo during germ-line transformations. The methods described here explain how to alter the parameters of the pipet puller to produce predictable changes in the needle tip shape. We also describe how to calibrate the heat settings, based on the nature of the capillary tube, using the preprogrammed ramp function with some modifications.

The investigation includes Table 1, which summarizes the effects of each first- and second-cycle parameter on the tip shape of the needle. We will also present data that describe the effectiveness of our standard needle for doing P-element-mediated gene transformation in the *D. melanogaster* germ-line cells. We examine some limitations of microinjection techniques associated with plasmid sizes, increased viscosity, and needle tolerances.

MATERIALS AND METHODS

Pulling Injection Needles

All needles are pulled with a model P-87 Flaming/Brown micropipet puller. The heating element is the box type (cat. no. FB220B; Sutter Instrument). The distance between the bottom of the heating element and the air jet is set at 5 mm. The needles used for this study are borosilicate glass 1 BBL with filament, 1 mm diameter, 0.058 mm i.d., and 6 in long (World Precision Instruments, Sarasota, FL, USA). The ramp value (heat units) for the first pull cycle (ramp-1) is determined by the ramp program included in the manufacturer's instructions. The ramp value for the

Table 1. Summary of Needle Puller Parameters and Effects on Needle Tip Shape

Parameter	Effect on L	Parameter Description
Ramp	NA	Heat value determined by the glass characteristics.
Heat-1 (H1)	~(1/L)	The H1 setting works best at the ramp-1 value. Not a good variable for changing L. (Use 25 U increments.)
Heat-2 (H2)	~L	The H2 setting works best at 25 U over the ramp-2 value. (Use 25 U increments.)
Pull-1 (P1)	~(1/L)	P1 determines pull in cycle 1. (Use 25 U increments.)
Pull-2 (P2)	~L	P2 determines pull in cycle 2. Not a good variable for changing L. (Use 50 U increments.)
Vel-1 (V1)	~(1/L)	V1 sets the velocity of the separating arms in cycle 1. (Use 5 U increments.)
Vel-2 (V2)	~(1/L)	V2 sets the velocity of the separating arms in cycle 2. (Use 5 U increments.)
Time-1 (T1)	~L	T1 sets the duration of the air pulse in cycle 1. (Use 50 U increments.)
Time-2 (T2)	~L	T2 sets the duration of the air pulse in cycle 2. (Use 50 U increments.)
Pressure (Pr)	~(1/L)	Pr determines the strength of the cooling air pulse. (Use 100 U increments.)

Parameter descriptions: the ramp value is the heat setting at which the capillary tube melts enough to cause the separation of the arms at the factory preset velocity with the ramp program. Ramp-1 is for the original capillary, while ramp-2 is for the unseparated needle generated by the first cycle only. The H1 setting regulates the melting rate of the glass capillary in the first cycle. The H2 setting regulates the melting rate of the glass capillary in the second cycle. P1 determines the pull applied when the V1 of the separating arms reaches the set value and trips the cycle. P2 determines the pull applied when the V2 of the separating arms reaches the set value and trips the cycle. V1 is the velocity of the separating arms at which the trip engages the P1 and T1 air pulse at the set Pr. V2 is the velocity of the separating arms at which the trip engages the P2 and T2 air pulse at the set Pr. T1 is the timed air pulse when the V1 trips the cycle. T2 is the timed air pulse when the V2 trips the cycle. The Pr determines the strength of the cooling air pulse given at the end of each cycle, which occurs when the V1 or V2 of the separating arms reaches the set value, and the trip engages the P1 or P2 and T1 or T2 air pulse. L indicates the length of the distal tip (see Figure 1), and ~L indicates proportionality to L (i.e., increasing parameter increases L). U, arbitrary units indicated by the instrument read-out for each parameter.

second pull cycle (ramp-2) is determined by completing the first cycle of a pull program with the user-defined settings (without the complete separation of the capillary tube) and immediately stopping the program with the stop button. The ramp program is now executed with the partially pulled needle (first cycle completed) still in place. All test needles are collected from the right side of the puller to minimize variation.

P-Element Transformations

P-element transformations are performed using a modified version of the previously reported technique (38, 42). Injections are done into *D. melanogaster* w[P_{ry+}, Δ 2-3](99B)/TM3 or TM6B syncytial embryos less than 2 h old. This fly line provides the stable source of transposase required for the mobilization and germ-line transforma-

tion of P-element DNA (35). All plasmid DNAs used for injections are prepared with plasmid DNA purification kits (Plasmid Midi Kit; Qiagen, Valencia, CA, USA). The DNA is then precipitated with one-half volume 7 M ammonium acetate, pH 7.0, and 3 volumes of ethanol, washed with 70% ethanol, and dried completely. The DNA is suspended at 1 mg/mL injection buffer (0.1 M sodium phosphate and 5 mM potassium chloride, pH 6.8, filter-sterilized). All DNA samples are centrifuged at 14 000× g for 10 min and transferred to a sterile tube before being loaded into the injection needle. Injection needles are pulled according to the description for the standard injection needle (Figure 1) and back-loaded with an Eppendorf® microloader tip (Brinkmann Instruments, Westbury, NY, USA) or hand-pulled glass capillary. The injection needles are usually ready for injection after loading. Injections are typically done with 30 psi nitrogen pressure and 5 psi balance pressure to prevent back flow. Occasionally, needles must be carefully sharpened on the frosted side of a microscope slide under halocarbon oil (HC-400; Halocarbon Products, North Augusta, SC, USA) with nitrogen pressure at 80 psi. Sharpening increases sample flow when necessary and can also be used to remove clogs caused by cytoplasm from the egg. Sharpening correctly produces no visible change in the appearance of the needle tip, except that the DNA solution flows at a reasonable rate. Embryos are collected at 1-h intervals on yeast paste and then thoroughly washed in deionized water. Embryos are staged, sorted, and aligned in the same orientation (in pairs) with controlled amounts of deionized water on a microscope coverslip that will fit into a soft agar egg collection plate. The aligned embryos are dried for 5 min to fix them to the coverslip and covered with a minimal amount of halocarbon oil before injection. Embryos are injected into the posterior end where the germ-line pole cells will form during cellularization. The injections are performed on a Diaphot-TMD inverted microscope (Nikon, Melville, NY, USA) equipped with an MN-188 3-D micromanipulator with NS-188 adaptor, an MO-188 joystick hydraulic micromanipulator, an

IM 300 microinjector (all Narishige; Fryer Company, Warrensville Heights, OH, USA), and an Eppendorf capillary needle holder. To prevent anoxia, the embryos and coverslips are cleared of halocarbon oil and drained as much as possible without perturbation. The coverslip, with injected embryos, is transferred to a soft egg collection plate, where they are allowed to hatch without being disturbed. Hatched larvae are then transferred to a freshly yeasted vial after two days. Survival frequency is determined by monitoring the number of adult survivors following the injection of the P-element DNA. Constructs used for the injections contain cDNAs for the *centrosomin* (*cnn*) gene (20) that are inserted into the Gal4 responder P-element vector pUAST (5). P-element transformation into germ-line cells is monitored by the rescue of the *w¹¹¹⁸* eye phenotype in the F1 generation by the encoded mini-*white* gene present in the pUAST vector.

RESULTS

Figure 1A shows the standard needle used for injections into *D. melanogaster* w;P[ry+, Δ2-3](99B) embryos. The machine settings used to generate this needle are tabulated in Figure 1B. This needle is durable and penetrates easily through the chorion of the embryo. A two-cycle pull is used to generate this type of needle when all attempts fail in a single cycle, even when several other heating elements and capillary tubes are used. Typically, this needle is formed with an open tip that will accommodate DNA concentrations of 1–2 mg/mL for P-element transformation. However, it is sometimes necessary to sharpen the tip slightly (see Materials and Methods). The thinly tapered tip is long enough to penetrate well into the posterior of the embryo but sturdy enough to withstand the stress required to penetrate the tough chorion surrounding the entire embryo. Failure to wash off residual yeast results in damage to the needle tip.

We monitor the viability, fertility, and transformation efficiency of the flies following injections to assess the effectiveness of our standard injection needle. The viability of the injected

embryos is determined by counting the adults of the injected generation. Viability is 10% after injections ($n = 6738$). Fertility is also affected by the injection procedure because we obtain only 68% fertility of the viable adults in this generation. The transformation efficiency of the total embryos injected is 1.7%, as determined by the rescue of the *white w¹¹¹⁸* eye phenotype. This actually represents 17% of the viable adults or 25% of the fertile adults. These results are similar to those previously reported (35,38). We find that, by using higher (visible) injection volumes, we can maintain transformation efficiencies of the fertile adults near 25% to minimize the labor. This is important because the bulk of the effort in generating stable transformants, following clone construction, involves the screening for *w¹¹¹⁸* mutant rescue. Cellularized embryos (incapable of being transformed) are terminated by over-injection to minimize screening efforts. The injection time and pressure required to obtain a visible injection volume are dependent on minor variations with the needle and the viscosity of the DNA solution and therefore must be adjusted by changing the pressure and/or holding time of the injection button. The construct that we use for the control contains a cDNA clone for *cnn* that is 4 kb. The total size of this mobile DNA fragment (to be incorporated into the genome) is 10 kb, including the mini-*white* gene with a total plasmid size of 12 kb. We find significantly lower transformation efficien-

cies for constructs of larger sizes. Our efforts to transform an *Scr* regulatory element reporter, with a mobile genomic element of 30 kb (32 kb total plasmid size), gives reduced transformation efficiencies of nearly 10-fold.

Needle puller parameters are adjusted one at a time to establish how they affect the shape of the injection needle tip. We will use the term proportional ($\sim$) to describe the general relationship of the parameter change to the change in length (L) of the distal taper of the needle tip (Table 1 and Figure 1A). In other words, if the increasing parameter X increases L , then X is proportional to L ($X \sim L$). Figure 1 shows the shape and parameters we use for our standard injection needle. This needle is shown in panel A of all figures for comparison. Figure 2 demonstrates the effects of changing the heat, pull, velocity, and time parameters. Increasing cycle 1 heat (H1) by 25 U generates a blunter needle tip (Figure 2B), while decreasing H1 by 25 U has a subtle reverse effect (Figure 2C). Therefore, H1 is inversely proportional to the length (L) of the distal taper ($H1 \sim 1/L$). However, the H1 parameter is not a good choice for altering L . Changing cycle 2 heat (H2) has the reverse effect on L . Figure 2D shows a needle generated by increasing H2 by 25 U, which exhibits an increase in L . The needle in Figure 2E is generated by reducing H2 by 25 U and has a reduced L . Consequently, H2 is proportional to L ($H2 \sim L$). Figure 2F shows the result of increasing P1 by 25 U, where we find that L is reduced. Figure

Figure 1. Standard needle shape and parameters for *D. melanogaster* injections. (A) Standard needle (shown in panel A for all figures). (B) Parameters used to generate the standard needle. Scale bar = 150 μ m.

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2G shows a decrease in P1 by 25 U and an increase in L. Thus, P1 is inversely proportional to L ($P1 \sim 1/L$). Changes in P2 by 50 U show only subtle effects on L but suggest that P2 is directly proportional to L (Figure 2, H and I). However, the P2 parameter is not a good choice for altering L. Figure 2J shows the standard again for comparison. Increasing V1 by 5 U decreases L significantly (Figure 2K), while decreasing V1 by 5 U increases L (Figure 2L). Therefore, V1 units are inversely proportional to L ($V1 \sim 1/L$). Changes in V2 by 5 U show similar effects on L (i.e., $V2 \sim 1/L$). However, the proximal

taper of the needle (away from the tip) is oppositely affected (Figure 2, M and N). Changing the time parameters (T1 and T2; cycles 1 and 2, respectively) also alter needle morphology. The time setting for the standard (Figure 1 and Figure 2, A and J) is set at 250 U. This is the maximum setting for this parameter. Figure 2O shows a needle that is generated by decreasing T1 by 50 U. The needle in Figure 2P is generated by reducing T1 by 100 U. Clearly, T1 units are directly proportional L ($T1 \sim L$), as we observe decreases in L. The needles in Figure 2, Q and R, are generated by reducing the T2 parameter by 50 and

100 U, respectively. Reducing this parameter has a subtle effect of reducing L and appears to be directly proportional to L ($T2 \sim L$). However, reducing T2 seems to alter the proximal taper of the needle more significantly when we detect an increase in this length.

Figure 3 shows that cooling air pressure (Pr) also affects the shape of the injection needle. This parameter cannot be changed independently for each cycle. The maximal Pr setting is 600, which is the setting used for the standard (Figures 1 and 3A). Reducing the Pr setting by 100 U (Figure 3B) and 200 U (Figure 3C) produces needles with an increased L. Reducing the Pr setting by 300 U causes the capillary to separate during the first cycle (data not shown). Therefore, P1 was reduced by 50 U, and P2 was reduced by 90 U when Pr was reduced by 300 U. This allows a two-cycle pull for the lower Pr setting (Figure 3D). Reducing Pr by

Figure 2. Effects on needle shape by altering parameters. (A) Standard needle (settings in Figure 1). (B) Increase H1 by 25 U from standard. (C) Decrease H1 by 25 U. (D) Increase H2 by 25 U. (E) Decrease H2 by 25 U. (F) Increase P1 by 25 U from standard. (G) Decrease P1 by 25 U. (H) Increase P2 by 50 U. (I) Decrease P2 by 50 U. (J) Standard (Figure 1). (K) Increase V1 by 5 U compared to the standard. (L) Decrease V1 by 5 U. (M) Increase V2 by 5 U. (N) Decrease V2 by 5 U. Note that the maximum settings for T1 and T2 are 250 U (standard settings). (O) Decrease T1 by 50 U. (P) Decrease T1 by 100 U. (Q) Decrease T2 by 50 U. (R) Decrease T2 by 100 U. Scale bar = 150 μ m.

Figure 3. Effects on needle shape by altering cooling air pressure (Pr). (A) Standard needle (settings in Figure 1). (B) Decreased Pr by 100 U compared to standard. (C) Pr was decreased 200 U below the standard. (D) Pr was decreased 300 U (along with adjusted pull settings below). It should be emphasized that the capillary becomes separated during the first cycle, when Pr is reduced by 300 U. Consequently, the pull settings in both the first and second cycles adjusted to demonstrate alternative settings necessary for shifting the range of allowable Pr settings for two-cycle pulls. P1 was reduced by 50 U, and P2 was reduced by 90 U. (E) Pr was decreased 400 U (with adjusted pull settings in Figure 3D). Scale bar = 150 μ m.

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400 U, compared to the standard (along with the new P1 and P2 settings; Figure 3D), increases L even more drastically (Figure 3E). These data indicate that Pr is inversely proportional to L ($Pr \sim 1/L$) and the proximal taper of the needle.

DISCUSSION

The increasing number of biological techniques that require microinjection prompted us to investigate new methods for generating customized needles. Additionally, these techniques are being applied to a wide range of organisms that include representatives from several animal and plant groups. We have developed a two-cycle pull protocol that allows more control over the shape of the injection needle. Our attempts to generate a durable tip shape with one-cycle pulls were unsuccessful with a wide range of heating elements

and capillaries. However, by using a two-cycle pull, we pre-stretched the capillary tube in the first cycle to allow more control over the tip shape in the second cycle. The box-heating filament produced a more uniform needle shape with a wider range of control. Using the preprogrammed ramp function of the Flaming/Brown micropipet needle puller in a unique manner (see Materials and Methods), we determined the proper heat parameter values for the capillary tube being used. The ramp value is a heat setting that generates the necessary amount of capillary melt-down to cause the puller arms to separate at the preset factory velocity. Typically, the first-cycle heat value was set at ramp-1. The second-cycle heat value usually worked best 25 U above the ramp-2 value. We first established settings for the needle puller that generated the standard needle for P-element germ-line transformations in *D. mela-*

nogaster embryos (Figure 1). We then altered each parameter of the needle puller, one at a time, and compared the needle product with our standard to determine the effect on needle tip shape. Table 1 summarizes all the effects of all independent parameter changes on L.

Most first-cycle parameters (e.g., pressure) used to generate new needle shapes have a limited range of values. Otherwise, the capillary tube will be separated in the first cycle, resulting in an unsatisfactory needle. Preventing separation in the first cycle can usually be accomplished by readjusting other parameters, as demonstrated in Figure 3. This was true for each of the parameters in which we saw a limited range of useable values, contingent on all other settings. This was determined empirically each time. Another limitation was the capillary tube. It could not be too thick or the ramp test would melt the heating filament. We were able to pull

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useful needles with three-cycle pulls; however, there appeared to be no advantage to this approach for the capillary tubes we used. Three-cycle pulls may be useful for thicker capillary tubes to pre-stretch them enough before the final separating pull. The 5-mm distance between the heating element and the air jet is often reduced to 3 mm (personal communication, Adair Oesterle, Sutter Instruments) and may be responsible for the high-pressure settings (Pr = 600) necessary to obtain a functional tip in our assay (Figure 1).

The standard needle (Figure 1) was developed for P-element DNA injections through the tough chorion enclosing *D. melanogaster* embryos. This technique required a needle with a fine tip to minimize trauma to the animal. The thin distal portion of the tip also needed to be relatively short to avoid flexing during penetration, yet long enough to avoid penetration of the embryo with the wider proximal region. For example, the proximal portion of the tip could not be too thin or the needle would flex when trying to penetrate the embryonic chorion (Figure 2, D, M, and R, and Figure 3, C–E). Alternatively, needles with shorter distal tip tapers (L) create a larger opening and reduce viability due to cytoplasmic leakage (Figure 2, B, E, F, K, and P). We have successfully utilized the standard needle for injections of dsRNA in vivo (RNA interference), rhodamine-tagged tubulin and actin, colchicine, and cytosolase B into *D. melanogaster* embryos (unpublished data). We found that our two-cycle needles were useful for RNA-interference injections into other insects as well (21). These injection needles are versatile tools for studying the evolution of developmental processes across broad phylogenetic groups of organisms. Understanding the effect of each parameter on needle shape permitted the development of customized needles with the desired characteristics dictated by the material being injected and the organism/tissue. Other studies have described additional techniques for analyzing needle characteristics, such as inner diameter (40). Our injection tests demonstrated typical survival and transformation frequencies (35,38). However, these results were somewhat variable and depended on the expertise of the

user as well as the nature of the DNA being injected. The construct design of the 4-kb *cnn* cDNA in the pUAST vector should not have affected the data because transcriptional activation (expression of the *cnn* transgene) requires Gal4 co-expression. Similarly, the 32-kb *Scr* regulatory construct only expresses Gal4, which has no effect on viability. Some Hox (e.g., *Scr*) regulatory regions repress mini-*white* expression and may have reduced our transformation efficiencies for this larger construct (17, 37), although this repressive effect seems to exhibit only patchy mini-*white* repression in the eye when heterozygous. Presumably, the limitations associated with the larger construct were higher sample viscosity, lower molar concentrations of the DNA, and reduced genomic insertion rates. In summary, the standard needle was found to be useful for injecting plasmid DNA sizes ranging from 12 to 32 kb. We also found this needle useful for DNA concentrations as high as 2 mg/mL. Given the relatively large sizes of these plasmids, this needle should be useful for most microinjection techniques.

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