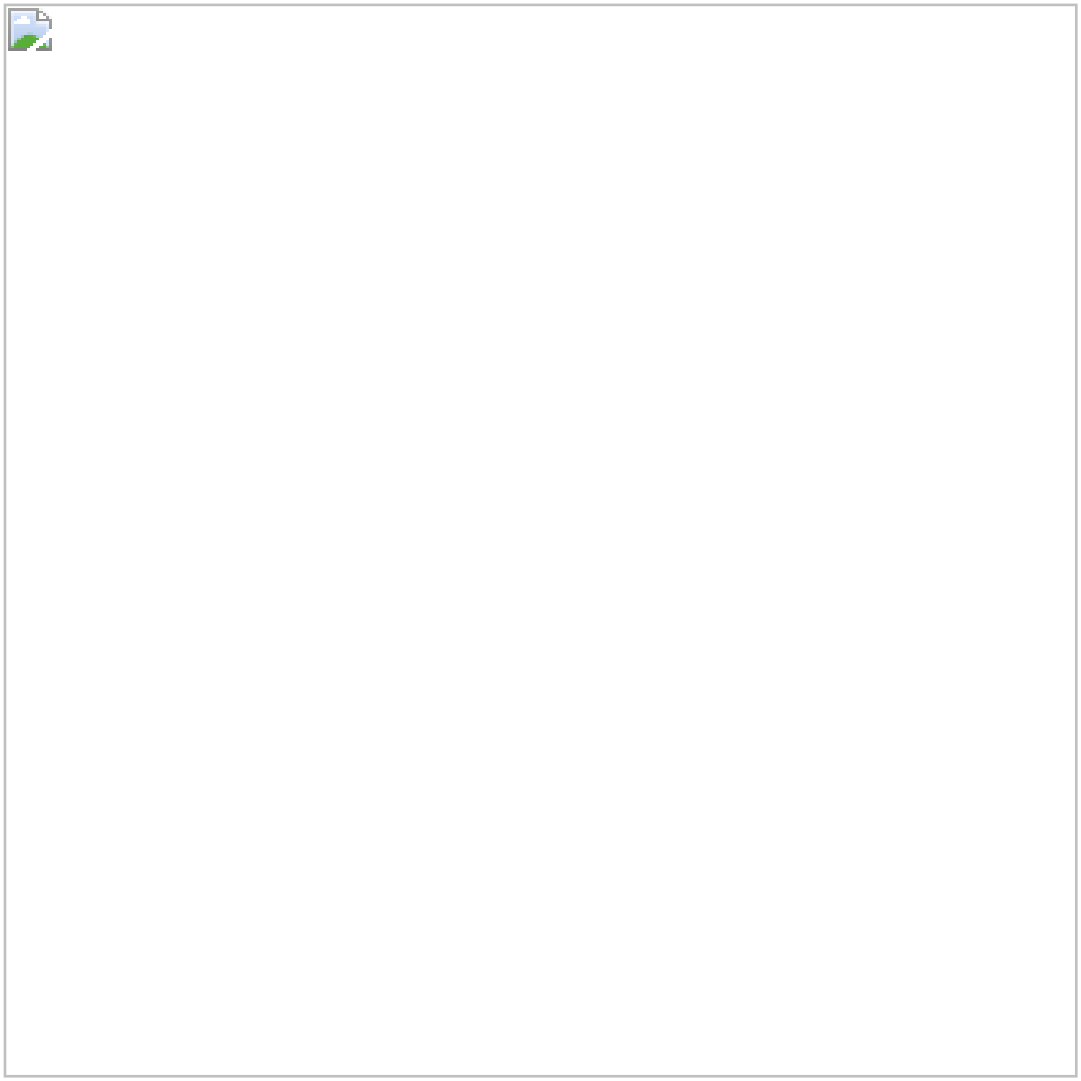

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A helium burst biolistic device adapted to penetrate fragile insect tissues.

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Abstract

To compensate for the extremely low penetration efficiency of the original PDS/1000-He Bio Rad biolistic® device and the deleterious blast effect, design modifications have been made to the launching module. These modifications were evaluated on *Bombyx mori* embryos and fragile tissues, such as oocytes and imaginal wing disks. The original floppy macrocarrier was replaced by a rigid macrocarrier to avoid the effects of the helium blast. The efficiency of the gene gun bombardment was reinforced by the addition of a focusing nozzle. The reduced blast effect allowed us to carry out high-pressure shootings to small organs with improved penetration. This system allowed potentially all the internal embryonic tissues to be transfected with optimal survival rates. The new module was effective on tissues that are difficult to transfect, such as the epithelial wing disk that is covered by a peripodial membrane, and the ovarian follicle cells that lie under the ovariole cell membrane. The new macrocarrier allowed both an aqueous delivery of particles and an ethanolic dry delivery. No significant differences were noted between these two modes of delivery. The major improvement is the possibility of high pressure shooting correlated with appreciable penetration and a weak blast effect.

Keywords: Biolistics, gene gun, *Bombyx mori*, insect, transfection, gene expression

Introduction

Biolistics has been a useful technique for testing expression of genes, particularly in plants. In our silkworm transgenesis program, we needed an efficient technique to evaluate the functionality of transgenes before their injection into eggs. We decided to use biolistics due to its documented success as a powerful transformation technique (Baldarelli and Lengyel 1990, Horard *et al*., 1994, Miahle and Miller 1994, Gendreau *et al*., 1995, Koster *et al*., 1996, Kravariti *et al*. 2001).

The principle behind biolistics consists of the high speed propulsion of DNA-coated micro-particles, currently tungsten or gold, into a wide range of biological samples from cellular organelles to *in situ* mammalian organs (Sandford *et al.*, 1993). There are various systems most of which use a burst of a gas, such as dry steam generated by an electric discharge on a drop of water (Christou *et al.*, 1990, 1995), a capacitive electric discharge through a wire electrode that instantaneously vaporizes creating a shock wave (Shigeru and Kimura, 1997), a gun powder explosion (Sandford *et al.*, 1987; Klein *et al.*, 1992), a burst of compressed helium (Sanford *et al.*, 1992) or a gas flow (Sautter *et al.*, 1991; Clarke *et al.*, 1994). In the case of a gas flow, particles are directly propelled whereas in the case of a gas burst the kinetic energy is transmitted to the particles that are loaded on an accelerated macrocarrier. The sudden stopping of the macrocarrier projects the micro particles by the inertia principle.

The Bio Rad PDS 1000/He system is a helium burst that uses a macrocarrier system device. The macrocarrier consists of a floppy, thin kapton disk (45 µm in thickness) that is accelerated by a compressed helium burst depending on the value of a rupture disk. The calibrated thickness of the rupture disk determines the value of the shooting pressure, which in turn determines the velocity and the penetration of the microprojectiles. However, the higher the shooting pressure, the higher the expanded helium volume correlated with the deleterious blast effect. In this system, high shooting pressure could contradict the expected penetration effect. The blast effect lies in the structure of the macrocarrier of the BioRad device, which crashes into the stopping screen at the end of its path. At this moment, a strong residual helium flow is generated, which destroys, or blows out, fragile target samples. Moreover, the particle impact is systematically decentered due to poor mechanical guidance of the fine macrocarrier, the flight of which is always off course.

During the preliminary experiments we encountered all of the aforementioned noticed problems concerning deleterious blast effect. In this paper we describe some design modifications which optimize the use of the PDS 1000/He on silkworm (*B. mori)* embryos,

and on some fragile larval or pupal organs. These modifications allowed us to significantly reduce the blast effect, while keeping a noticeable penetration effect, as well as a constant centered impact cloud. The main advantage of this transfection technique is that it allows a quick and powerful test to evaluate DNA constructs designed for transgenesis.

Materials and Methods

Bombyx mori *strain*

The Indian polyvoltine Nistari strain was used as a source of embryonic, larval and pupal tissues. This strain was obtained from a silkworm collection maintained at UNS/INRA (France). The silkworms were reared at 25 °C and were fed with mulberry leaves from spring to autumn and on an artificial Japanese diet during the winter.

Preparation of embryos for gene gun bombardment experiments

Eggs that had been newly laid on a sheet of paper were placed in an incubator at 25° C and 80% RH for 6 days and the occurrence of stemmata pigmentation (the first pigmented structures) was observed. The eggs were used after the appearance of stemmata pigmentation until mandibular pigmentation appeared. After which the cuticle becomes too hard for particle penetration. A constant supply of eggs was maintained by keeping them at 5° C for no longer than one week. This temperature stops development without killing the embryo. Eggs were collected by incubation for 5 minutes bath in cold water (approximately 20° C) and dried on paper towels before being attached to petri dishes with cyanocrylate glue ensuring that they laid flat. Eggs were disinfected with a 4% formaldehyde solution for 10 min, rinsed with distilled water and finally dried with absolute ethanol. Eggs were dissected in Grace's medium containing antibiotics (Sigma catalog # A-5955). For the shooting with the PDS-1000 He biolistic® (Bio Rad, Life Science Research, Marnes-la-Coquette, France) embryos were transferred to a 1% agarose plate and covered with a coverslip, the center of which was replaced by a fine netting with 120 µm mesh. The netting remained about 1 cm above the samples and was not in direct contact with them, thus avoiding a masking effect. After the shootings, the embryos were placed in Grace's medium containing antibiotics for 2 days at 25° C for subsequent development. It was possible to cultivate embryos in plates with wells of 16 mm or 35 mm in diameter, or in standard 1.5 ml microtubes filled with one ml of culture medium, which was more economical.

Preparation of ovarioles for biolistics

Ovarioles were dissected from 7-day-old pupae. Care was taken to ensure the entire ovariole was obtained. They were shot in their entirety or as segments, depending on the developmental stage of choriogenesis (Eickbush *et al.*, 1985). In the former case, the cell membrane covering the ovariole was maintained in place because it was very difficult to dissect it without breaking the ovarioles. In the latter case, it was very easy to eliminate the ovariole cell membrane and to get naked follicles. The second situation was better for the penetration of the particles. For the shootings, ovarioles or segments were placed into a Falcon® Cell Trainer (40µm Polylabo, catalog # 19425) which was then placed on a 1% agarose plate. After the shooting the Cell Trainer was placed on a plate with six 35 mm wells. The wells were filled with Grace's medium containing antibiotics. Ovarioles were cultivated for two days before Xgal staining.

Preparation of imaginal wing disks for biolistics

Larvae from the fifth instar (4 to 5 days old) were dissected and their wing disks were collected. A critical step was the sterilization of the outer surface of the larvae before dissection. For this purpose, larvae that had been killed with diethyl ether washed first in standard laboratory detergent (dilute working solution) for 2 min, then in sodium hypochlorite (3% in water) for 2 min, and finally in distilled water. The wing disks were shot and cultivated as for the embryos.

DNA vectors

We used two densoviral recombinant vectors as controls to establish experimental parameters for LacZ expression. pBRJZ vector was created by cloning the LacZ coding sequence as a fusion protein with the VP4 viral coding sequence and was used in most cases (Jourdan *et al.*, 1990). pBRJZΔNS3 is a derivative of pBRJZ in which a short coding sequence in the non-structural gene, NS3, had been deleted (Romane 1996). These vectors were very useful in Lepidoptera due to their strong, ubiquitous P9 promoter.

Xgal staining

Every sample type was treated in the same manner. They were fixed with 4% formaldehyde solution in 10 mM PBS (phosphate buffered saline), pH 7.4 (Sigma catalog #1000-3) for 10 min and washed with PBS for 5 min. They were subsequently incubated in Xgal solution (1 mg/ml 5-bromo-4-chloro-3-indolyl-βD-galactopyranoside; 15 mM potassium ferrocyanate; 15mM potassium ferricyanate; 2 mM MgCl2 in 10 mM PBS, pH 7.4) for 15 hours (overnight) at 37° C.

Preparation of DNA-coated particles

The preparations of gold or tungsten particles for the wet and dry procedures were the same. The particles were weighed and resuspended to a concentration of 50 mg/ml in 25 ml absolute ethanol before being sonicated for at least 10 min. This working particle suspension was kept at room temperature until use. Before use the particle pellet was resuspended by vigorously vortexing. All subsequent operations were done under continuous agitation (pipetting or vortexing). The required quantity of particles was loaded with DNA as follows. After resuspension, the desired aliquot of particles was removed and washed three times with distilled water in a Treff Lab microtube (catalog # 96-7246.11) as recommended by Sandford *et al.* (1993). DNA, spermidine-free base and calcium chloride were sequentially added. For a 10 µl shooting (0.5 mg of particles) 4 µl of DNA solution (generally 1 µg/ µl), 4 µl of 0.1 M or 1 M spermidine-free base (see results) and 10 µl 2.5 M $CaCl_2$ were added. For a series of several shootings, the corresponding volume of each reagent was scaled up. When the loaded particles were dry shot the above procedure was followed by three successive rinses in absolute ethanol. Most of the ethanol was then removed leaving a minimum volume necessary for deposition of the particles on the macrocarrier. After deposition on the macrocarrier, the ethanol

was totally evaporated. Total drying is important to insure the quality of the particle dispersion and penetration into the samples. When particles were shot in aqueous suspension, we allowed the particles to sediment at the extremity of the pipette tip to pool them in a minimum volume of water. After the deposition of the particles into the small cup of the rigid macrocarrier (Fig. 1 E), care was taken to remove the maximum amount of aqueous volume. This was particularly important when using the smooth Bio Rad kapton macrocarrier. In general it is harmful to shoot too much water as it results in a great decrease of penetrability. Spermidine must be either fresh (0.1 M) or frozen (1 M solution at - 70 to -80° C). We froze 1 M spermidine at -70° C and thawed aliquots just before dilution. The new aliquots were kept at -20° C for no longer than one week and a new aliquot was used on each day.

Results

New design of the shooting module

As specified, some shootings were carried out using the Bio Rad equipment and others with our modified shooting module. The modifications are presented in Figure 1 and are described in detail in the following paragraph.

The focusing nozzles were made of brass or stainless steel. Our macrocarriers were made of a thermoplastic cast in the mould shown in Figure 1E. Briefly, a 1.5 g pellet of thermoplastic that had been boiled in water was put into the base of the mould and flattened with the pestle by rotating movements. The resultant macrocarriers were cooled in cold water and due to slight retraction they were easily removed from the mould. Due to the cutting edge of the internal stainless steel ring of the mould the excess thermoplastic was easily cut off. For the shootings, the thermoplastic macrocarrier was placed in the upper ring of the shooting module. On the periphery of this ring we use an awl to create some bumps which maintain the macrocarrier in place by its superior part. After a series of shooting, all equipment was washed with sodium hypochlorite, rinsed with distilled water and dried with absolute ethanol. The insides of the nozzles were swabbed. Moreover, if several plasmids were shot in the same day, a new shooting module (i.e. barrel and nozzle), a new protective netting and a new macrocarrier were used for each plasmid, to avoid cross contamination.

Role of a rigid macrocarrier and a focusing nozzle

The first trials were performed with the Bio Rad device PDS 1000/He, which is known to be one of the most powerful gene guns. The results of the shootings, carried out with different distances between the target and stopping screen, were irregular and it was not possible to obtain satisfactory results based on the penetration capability without a strong blast effect, due to the crashing of the macrocarrier on the stopping screen. This crashing led to nearly all of the shootings being decentered (Fig.1 A and B) and leakage of helium flow causing a strong blast effect (Fig. 1A). The blast had a deleterious effect on the quality of the shootings because it blew the small biological samples out of the target area. This problem was circumvented by decreasing the value of the rupture disk, resulting in the loss of the penetration capability of the particles due to the loss of their velocity and, therefore, of their kinetic energy. This was correlated with the quasi absence of transgene expression in internal embryonic tissues. Thus, we made some design modifications (Fig. 1C). First, we replaced two sections of the cylinder of the Bio Rad gun barrel with a unique smooth cylinder. Secondly, we replaced the initial stopping screen with a smooth stopping ring. Thirdly, we added a focusing nozzle (Fig. 1C). The main modification was the use of a thick, rigid macrocarrier that allowed ethanol dried particles or wet particles to be shot. The principal advantage of the rigid macrocarrier was the nearly total valve effect obtained when the macrocarrier stops on the stopping ring at the end of its flight. With such a modified shooting module, the shootings were always centered and the particle impact clouds were very regular in shape for the gap distances tested (Fig. 1D).

Preliminary test shootings, with an 8 cm gap distance, on agarose plates, revealed that the focusing nozzle improved the particle penetration for high shooting pressure without adding blast effect. All test and experimental shootings were therefore done with an 8 cm gap between the macrocarrier stopping ring and the targeted tissues. We found two advantages to covering the samples with a plastic netting. It disrupted some DNA/particle aggregates protecting against sample damage, and the residual blast effect was reduced. The netting was placed 1 cm above the sample and we did not observe any masking effect. Table 1 shows the comparative results between shootings on 6-day-old embryos (1.5 mm long, 0.25 mm wide) with the Bio Rad device using the floppy kapton macrocarrier and shootings using the new rigid macrocarrier and the short focusing nozzle. The mean number of stained blue spots per shot embryo was low at 1800 psi with Bio Rad's conditions. This was mainly due to decentering and the blowing out of embryos from the target area (Table 1, experiment 1). By reducing the pressure from 1800 to 1100 psi the percentage of positive embryos increased, but the mean number of blue spots per shot embryo remained low (Table 1, experiment 2). In this more favorable situation, only superficial staining was obtained, i.e. into the integument. The addition of a focusing nozzle led to an increase in the mean number of blue spots per shot embryo even though the percentage of positive embryos was comparable (Table 1, experiment 3).

The association of a focusing nozzle and a rigid macrocarrier gave the best results and significantly increased the mean number of blue spots per shot embryo (Table 1, experiment 4). In this case the relatively low difference in the percentage of positive embryos was a constant result, strictly correlated with the use of the rigid macrocarrier. Another remarkable trait, linked to both modifications, was the improved penetration of the particles inside the internal organs of the embryos (Table 1, experiment 4; Fig. 2). Use of the focusing nozzle alone slightly improved the penetration, but we obtained the main penetration effect by using the rigid macrocarrier. This could be explained by a decrease in the blast effect, which allowed us to shorten the gap between the stopping ring or the nozzle tip and the target. As an anecdote, our first rigid macrocarriers were disks the diameter of which was similar to that of the barrel. We now cast the macrocarriers from a thermoplastic in a mold (Fig. 1E). The thermoplastic has the advantage of being very resistant and can be remolded when fissures appear. The plan of our shooting module is shown in the Figure 1E indicating the dimensions of our nozzle. We used several nozzles that were identified by their length (from the upper face of the stopping ring to the tip of the nozzle) and the internal diameter. Therefore the

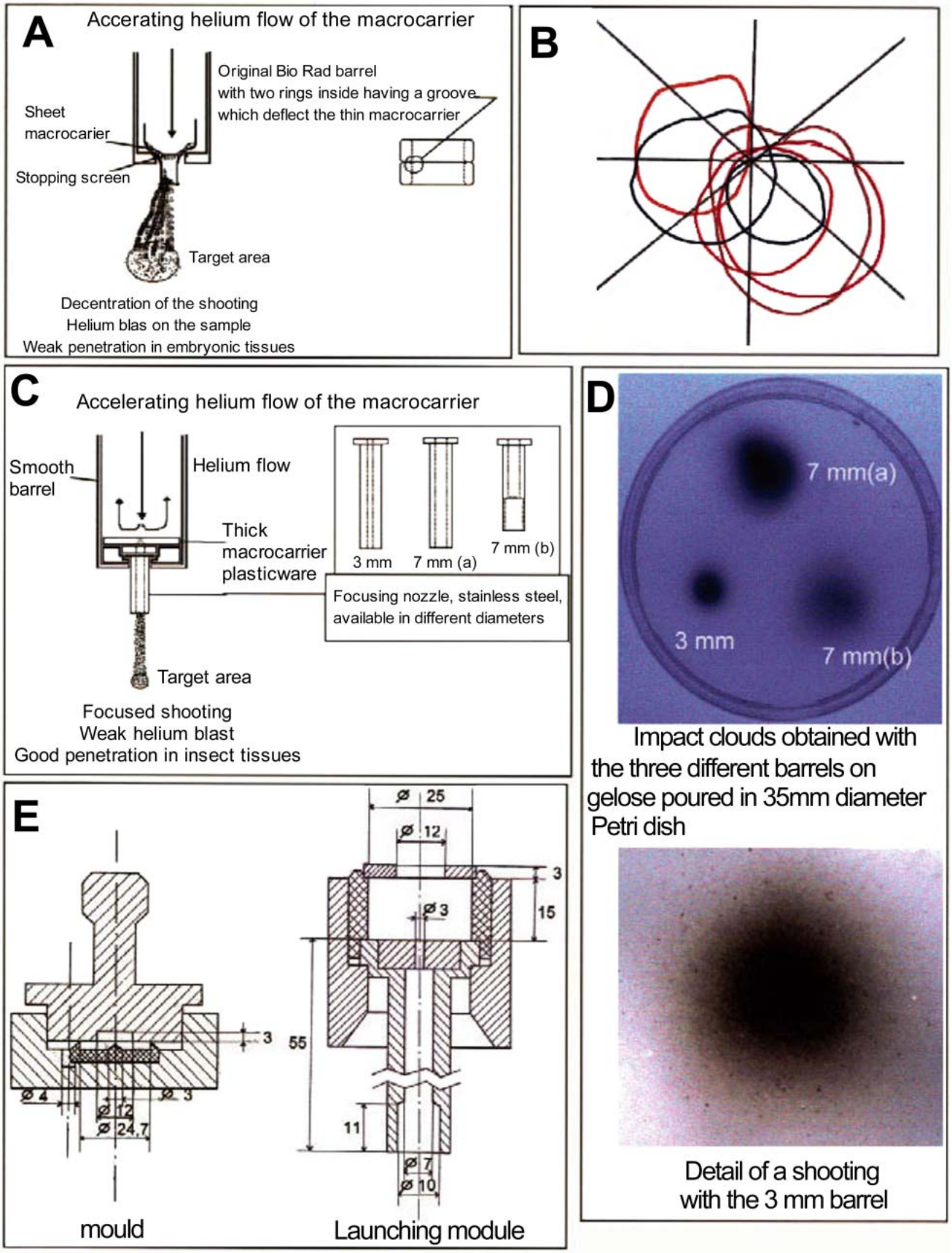

Figure 1. Mechanical modifications on the Bio Rad PDS/1000 He biolistic device shooting module. **A**: Schematic illustration of the problems encountered with the original shooting module. **B**: Decentering of the shootings with the original shooting module. **C**: Schematic illustration of our mechanical modifications. **D**: Focusing the shootings with our shooting module. **E**: Plan of our macrocarrier mould (left) and the shooting module (right).

Table 1. Biolistic results : effect of the use of a focus nozzle in *Bombyx mori* embryos

Shooting conditions	µg DNA/ mg of particles	Number of shootings	Number of positive shootings	Embryo number per shooting	Number of shot embryos	Number of positive embryos	Number blue spots	Mean number of blue spots per embryo	Percentage of positive embryos
Bio Rad 1800 psi	2/0.5	9	5	10	90	8	18*	0.2	8.8
Bio Rad 1100 psi	2/0.5	4	3	4	16	9	10*	0.625	56
Brass nozzle 17-∅ 7 floppy macrocarrier 1800 psi	2/0.5	3	3	10-10-4	24	12	48**	2	50
Brass nozzle 17-∅ 7 rigid macrocarrier 1800 psi	2/0.5	5	5	6-8	36	24	590***	16.38	66.6

The vector used was pBRJZ

For experiments 1 and 2 the distance between the stopping screen and the target area was 101 mm. Nearer was not possible because of the strength of the blast effect.

For experiment 3, the distance from the nozzle tip and the target area was 80 mm.

For experiment 4, the distance between the tip of the nozzle and the target area was 30 mm. With a distance of 1 cm the blast effect is too strong and blows out the embryos. With a distance of 20 mm effect was intermediate but not so good as with a distance of 30 mm (data not shown). The reduced distance of experiment 3 was possible due to the use of rigid macrocarrier and the associated valve effect.

* All staining was superficial on the integument.
** Staining was superficial and internal
*** Almost all staining was internal

nozzle designated 69-∅7 means 69 mm in length and 7 mm in diameter. Our 55-∅7∅10 nozzle has two successive internal diameters as shown in Fig.1E. The Bio Rad outer brass piece, which screwed on the supplier's Teflon shooting plate, was kept, and all the other new stainless pieces were adjusted to this piece. The initial stopping ring had a small aperture (3 mm in diameter), however due to the good resistance of the macrocarrier, a 5 to 7 mm in diameter aperture can be used that allows greater amounts of particle suspension to be deposited onto the macrocarrier if necessary. The volume of the shot particle suspension can be determined by the projection of the exchangeable part of the mold (Fig. 1E).

Comparisons of the Bio Rad shooting module and the modified device for wet shot particle suspension

As Miahle and Miller (1994) obtained better results on mosquito embryos using a wet DNA/particle suspension than with ethanol dried DNA/particles, we tested the wet condition. As it was not possible for us to use a helium flow shooting module as these authors did, we used the macrocarrier system for all the trials. We tested the DNA/particle ratio parameter. In the literature, several DNA/particle ratios were used, which roughly ranged between 0.4 and 5µg. We chose to test 0.5 µg and 4 µg of DNA per mg of particles. Results are shown in Table 2. For the first series of experiments carried out with the Bio Rad device, the percentage of positive embryos were comparable with those obtained in experiment 2 in Table 1, using the same 1100 psi pressure. But in the latter case, there was an increase in the mean number of blue spots per embryo from 0.2-0.625 to 4.2-5.8. Furthermore, the mean number of blue spots per embryo were comparable in the two DNA/particle ratios, with a slight advantage observed for the percentage of positive embryos when the DNA/particle ratio of 4 µg per 0.5 mg was used. For some experiments (data not shown), this difference was greater and always favored the highest ratio. Thus, we routinely used the ratio of 4 µg per 0.5 mg particles. This experiment suggests that the aqueous protocol works better than the ethanol protocol. However, comparative assays showed that this was not always the case.
Use of two different conditions, the rigid macrocarrier and the focusing nozzle, significantly improved the mean number of blue spots per embryo to a greater extent than the percent positive embryos.

To determine if the nozzle geometry had an influence on the results we tested several nozzles of differing sizes and we observed a constant definitive improvement of the particle penetration, as observed for the previous experiments (Table 1). In

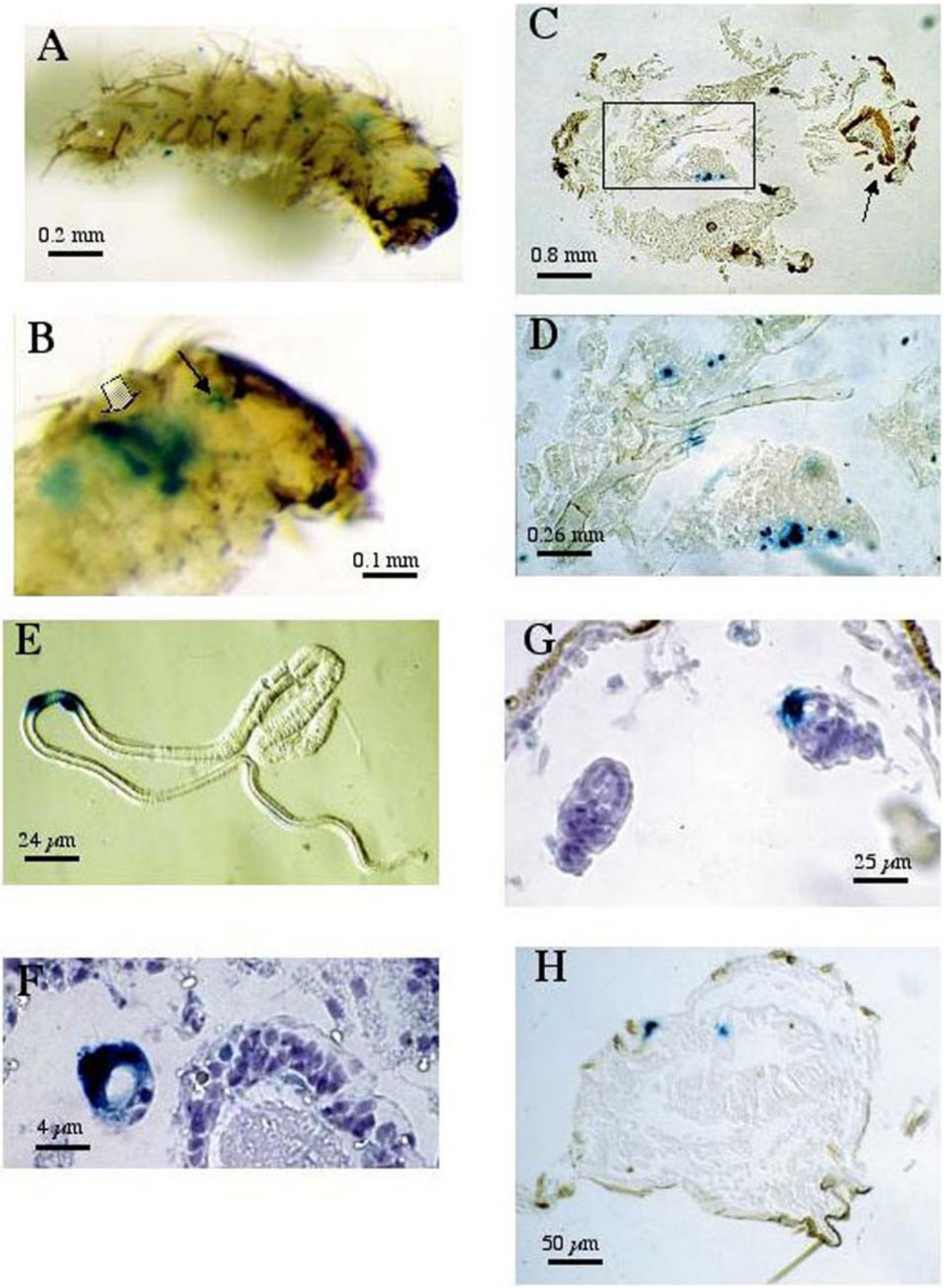

Figure 2. *LacZ* gene expression in embryonic tissues after bombardment with the pBRJZ densonucleovirus vector by our shooting module. **A**: *LacZ* gene expression in all potential parts of the embryo. **B**: Expression is often observed in the brain with the pBRJZ vector (arrow). **C**: Histological section of the area shown by the arrow in B. **D**: Detail of the brain *LacZ* gene expression framed in C. **E**: Expression in the posterior silk gland. **F**: Histological section of an expressing silk gland. **G**: LacZ expression in the gonad (probably not in the germ cell). **H**: LacZ expression in the gut and integument.

experiments 2 and 3 (Table 2), we used two different nozzles. The 69-∅3 nozzle with its small internal diameter was constructed to attempt to improve the main focusing andpenetration effect. It was necessary to reduce the shooting pressure because the valve effect of the rigid macrocarrier was not absolute. At 1800 psi there was a deleterious blast effect, which could be solved in two ways. The first possibility was to remove the target while keeping the shooting pressure constant. Alternatively, the shooting pressure could be lowered while maintaining the target distance. In the first situation, part of the focusing was lost because the particles were dispersed. Therefore, we favored focusing and the reduction of the pressure. We tested a second nozzle of the same length, but 7 mm in diameter, which allowed shooting with higher pressure associated with a negligible blast effect, probably due to the reduction of the pressure of residual helium flow in the largest section of the nozzle. As a general rule, this remarkable penetration effect was most likely due to the residual leakage of helium flow, which participates in accelerating the particles a little more than the acquired kinetics alone with the macrocarrier. The nozzle reinforced this effect. After assays undertaken to make a zero blast effect-shooting module (with spouts to allow the residual helium flow to escape) by use of a rigid cylindrical macrocarrier, we determined that a residual helium flow was necessary to retain the penetration capability of the particles. With a zero blast effect (tested on shaving cream), particles did not have any penetration strength (tested on plastic petri dishes) and the recovery of the penetration capability was strictly correlated with the admission of a certain level of helium leakage (data not shown). Nevertheless, Tables 1 and 2 show that too much helium leakage at high pressure was not an advantage, as shown by the Bio Rad equipment alone (Table 1, experiment 1).

Another aspect of the use of focusing nozzle was the ability to reduce the amount of shot particles. As there was nearly no diffusion of the particles along their flight from the nozzle tip to the target the amount of particles shot must be reduced to retain a reasonable density of particles at the impact. This conserves DNA, for example for shooting with the 69-∅3 nozzle we could reduce the quantity of particles to 0.3 mg per shooting which is a five-fold decrease compared to the Bio Rad equipment (1.5 mg per shooting). This reduction was three-fold when the 69-∅7 nozzle was used (0.5 mg per shooting).

Comparison between wet and ethanol-dried DNA particle preparations

After our initial evaluation of biolistics on fragile *B. mori* organs, using the wet procedure described by Miahle and Miller (1995), the wet and the ethanol-dried procedures were compared (Sandford *et al*., 1993). The ethanol-dried procedure gave almost 3 times more internal staining per shot embryo than the wet procedure and 100% positive embryos compared to 74% (Table 3). Nevertheless, we usually used the wet procedure in biolistics and it

Table 2. Comparison of the Bio Rad device and UNS modifications of the biolistic on *Bombyx mori* embryos

Shooting conditions	µg DNA/ mg of particles	Number of shootings	Number of positive shootings	Embryo number per shooting	Number of shot embryos	Number of positive embryos	Number of blue spots	Mean number of blue spots per embryo	Percentage of positive embryos
Bio Rad conditions 1800 psi**	0.5/0.5	31	20	2	62	34	142*	4.2	54.8
1100 psi	4/0.5	4	4	12-13	50	44	256*	5.8	88.0
UNS conditions 69-∅3 nozzle	0.5/0.5	104	85	2	208	118	604	5.1	56.7
1100 psi	4/0.5	81	76	2-3	178	148	1163	7.85	83.14
UNS conditions 69-∅7 nozzle	0.5/0.5	18	18	10-15	201	124	404	3.25	61.7
1800 psi	4/0.5	19	19	9-11	199	179	1793	10.01	89.9

The vector used was pBRJZ

The distance from the nozzle tip to the target area was28 mm.

* All staining obtained with the Bio Rad method and gun was superficial and exclusively in the integument. Following the UNS modifications, staining was mainly inside the embryo body, especially with the 3 mm ∅ nozzle.

** Microcarriers were shot in aqueous suspension.

Table 3. Comparison between aqueous and ethanol biolistic conditions.

Shooting Conditions	µg DNA per mg of particles	Number of shootings	Number of positive shootings	Embryo number per shooting	Number of shot embryos	Number of positive embryos	Number of blue spots	Mean number of blue spots per embryo	Percentage of positive embryos
UNS conditions 55-∅7 ∅10 nozzle** 1800 psi, aqueous	4/0.5	5	5	6-7	31	23	137	5.95	74
UNS conditions 55-∅7 ∅10 nozzle 1800 psi, ethanol	4/0.5	5	5	5-6	28	28	>441*	>15.75	100

The vector used was pBRJZ

* For two embryos in one shooting, the amount of staining was too high to be counted because of overlapping.

** The numbers describe the nozzle dimensions. See the results.

was applied to many experiments with satisfactory results. Even though the results shown Table 3 were favorable for the ethanol-dried procedure, biolistic transfection is extremely sensitive to variations and can occasionally give variable results. For this reason it was difficult to reach a definitive conclusion and to choose between these two procedures, which gave very comparable results on *B. mori* embryos.

Application to fragile tissues

To confirm that the modified biolistic device could be used efficiently on fragile organs and tissues, we tested our materials on two kinds of soft organs, pupal ovarioles and imaginal wing disks of fifth instar larvae. The tissues of these organs are very fragile, but are protected by a very resistant outer cellular sheet that should be kept in place to conserve their integrity. However, in the biolistic context these envelopes constitute a barrier to transfection and particularly to the launched particles during shooting. Thus, the particle velocity must be as high as possible to penetrate the cellular barrier but must have no more damaging effects other than at the very localized particle impact site.

To demonstrate this effect we used the densoviral vector, pBRJZΔNS3 (Romane, 1996), derived from pBRJZ (Jourdan *et al.*, 1990) with a deletion in the nucleic acid sequence of the non-structural protein 3. This vector constitutes a good positive control, for LacZ gene expression, in almost all *B. mori* tissues. For the imaginal wing disks, initial experiments on the preliminary and prototype mechanical modifications were not successful. This was mostly due to a blast effect that was too great and a lack of the particle penetration. Our definitive mechanical modifications allowed the efficient transfection of wing disk epithelial cells (Fig. 3 A and 3 B). This success encouraged us to test our launching module on ovarian follicular cells, and the correct shooting conditions allowed us to transfect numerous ovarian follicular cells (Fig. 3 C and D).

Discussion

The standard commercial method of biolistics (PDS 1000/He) has successfully introduced DNA into *B. mori* embryos, but the level of introduction measured by the Xgal staining of the LacZ gene activity is low. Although it was possible to obtain more than 50% positive embryos, it was not possible to obtain internal staining. During the shooting the main problem was a deleterious blast effect and the poor delivery of DNA to the internal organs of the embryos.

Design modifications were made based on an analysis of the mechanical process of the shootings. The main problem was the structure of the floppy kapton macrocarrier which was decentered on the stopping screen resulting in a constant decentering of the particle impact cloud. As the macrocarrier crashed on the stopping screen, no valve effect occurred, allowing a strong helium blast proportional to the shooting pressure.

Miahle and Miller (1994) showed that shooting wet particles gave better results on mosquito embryos. Horard et al. (1997) showed that this was also the case on silk gland. The design of our macrocarrier was adapted to wet conditions by the presence of a mini well in its center which can receive the water droplet particle suspension. Ethanol-dried particles were also tested with equivalent success. These results were inconsistent with those obtained by Miahle and Miller (1994). The shooting module used by Miahle and Miller (1994) worked very well on silk glands because they are relatively big organs that are easily maintained along the circular edge of a petri dish. These authors had to maintain mosquito eggs

in place with netting to avoid the blast and embryo blow out. The necessity to maintain small samples in place with fine netting leads to a masking effect. On the masked locations no gene expression was apparent *in situ* with the *LacZ* reporter gene.

As the shooting module used by Miahle and Miller (1994) was not completely convenient for our study we decided to overcome the problem of the blast effect. The Bio Rad floppy macrocarrier was replaced by a thick, rigid macrocarrier and the two rings of the original barrel were replaced by a smooth barrel of the same size. Under these conditions, the thickness of the macrocarrier ensures good guidance. These biolistical parameters allowed constant focusing and, by a sufficient valve effect, a very weak blast effect even with a shooting pressure of 1800 psi. It was not necessary to put netting in contact with the embryos. Placing the netting 1 cm above the samples was sufficient and ruled out any masking effect. The stopping screen was initially not replaced and we observed that after several shootings with the same macrocarrier, micro fragments were detached that damaged the biological samples. The Bio Rad stopping screen was therefore replaced with a smooth stopping ring with a small aperture of 3 mm in diameter. Under these conditions, the helium blast was almost totally reduced and it was possible to add a focusing nozzle to the barrel. The first nozzle used measured 3 mm in diameter and 69 mm in length. This nozzle had high penetration efficiency with a residual blast effect that was not deleterious. In this configuration we significantly improved the number of internal penetrations of DNA into embryos or imaginal wing disks, but the absence of a focusing nozzle did not give satisfactory results for imaginal wing disks. As the 3 mm diameter nozzle only permitted 2 to 3 embryos to be shot we decided to use a 7 mm in diameter nozzle of the same length. This nozzle did not have the same strength of penetrability but no blast effect could be detected on biological samples. Furthermore, a nozzle of 7 mm in diameter and only 17 mm in length with a very short shooting distance could be used without problem.

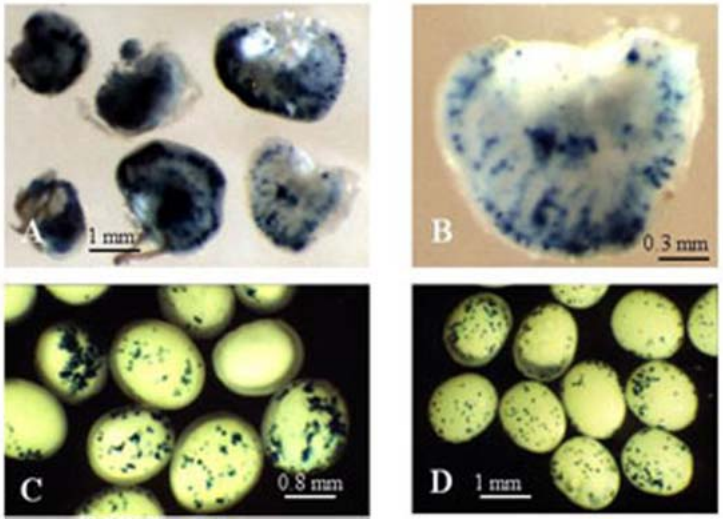

Figure 3. LacZ gene expression in a soft organ after gene bombardment with our shooting module. **A** and **B**: Expression in epithelial cells of imaginal wing disks of fifth instar larva. **C** and **D**: Expression in ovarian follicular cells of seven-day-old polyvoltine pupae.

A new barrel was built to provide 2200 psi without a blast effect (data not shown). A cylindrical titanium macrocarrier was used, the length of which equaled its diameter (1 cm) for good guidance. Six spouts were pierced into the circumference of the barrel which were placed just above the top of the macrocarrier at its end of flight. The result was that particles did not penetrate (data not shown). This design eliminated allresidual helium flow. It was expected that this barrel would benefit from the highest penetrability without the disadvantage of the blast effect. In fact, it was not successful in relation to the lack of helium flow. Penetration of the particles was retrieved if a minimum residual helium flow was allowed by filling some of the spouts. This study demonstrated that a minimum leakage of helium was necessary to ensure particle speed and penetration. The acceleration of the particle due to the acquired speed of the macrocarrier was not sufficient. Under these conditions the nozzle with the 3 mm diameter aperture had the most efficient penetrability, which was probably an effect of Bernoulli's law as applied by Sautter *et al.* (1991) in their microtargeting device. The restriction of the diameter between the barrel and the nozzle creates an acceleration of the helium flow that was accentuated because the difference in diameter was high. This shooting module was abandoned because the quality of the particle impact cloud was not as satisfactory as that obtained with the module described here.

This technique was successfully used as a transfection method to enable the study of regulation of chorionic gene promoters, in a reasonably short time (3 days) for an experiment, given that several repetitions were necessary to ensure an accurate statistical evaluation of the results (Kravariti *et al*., 2001). It was also used successfully in experiments on imaginal wing disks using DNA constructs carrying the 5' non-coding sequence of the Urbain gene (Chareyre *et al*., 1993; Besson *et al*., 1996). In this case the weakness of the studied promoter was the main difficulty compared to the densoviral vector used as the positive control. However, numerous repetitions confirmed the tissue specificity of the promoter (Besson *et al*., in preparation).

With our system, which comprised the rigid discoidal macrocarrier and the focusing nozzle, we were able to work on very fragile organs for which there was a necessity to introduce DNA deeply into internal cellular layers. We used *B. mori* embryos to estimate that particles could penetrate to the depth of about their whole thickness of 300 µm. For embryos, it was possible to express the foreign gene in all principal organs, including the gonads. Unlike Miahle and Miller (1994), we have no definitive preference for the wet or ethanol-dried procedure for the launch of the particles, as with our system and conditions the results were comparable.

We therefore feel that this technique will be useful for studies of gene regulation in *B. mori*, other insects, and fragile vertebrate tissues.

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