Published in final edited form as:
Nat Protoc. 2007 ; 2(12): 3153–3165. doi:10.1038/nprot.2007.471.

Preferential delivery of the *Sleeping Beauty* transposon system to livers of mice by hydrodynamic injection

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Abstract

Nonviral, DNA-mediated gene transfer is an alternative to viral delivery systems for expressing new genes in cells and tissues. The *Sleeping Beauty* (SB) transposon system combines the advantages of viruses and naked DNA molecules for gene therapy purposes; however, efficacious delivery of DNA molecules to animal tissues can still be problematic. Here we describe the *hydrodynamic delivery* procedure for the SB transposon system that allows efficient delivery to the liver in the mouse. The procedure involves rapid, high-pressure injection of a DNA solution into the tail vein. The overall procedure takes <1 h although the delivery into one mouse requires only a few seconds. Successful injections result in expression of the transgene in 5−40% of hepatocytes 1 d after injection. Several weeks after injection, transgene expression stabilizes at ~1% of the level at 24 h, presumably owing to integration of the transposons into chromosomes.

INTRODUCTION

Gene delivery in mice

The mouse is the most used model system for developing tools and techniques for human gene therapy. Several types of genetic disease are caused by a lack of enzymes that either normally exist in the blood or can be supplied to various tissues through the circulatory system. For expressing genes whose protein products can be secreted into the circulatory system, the liver is a particularly favored organ because of its large size and its ability to secrete many polypeptides.

There are two approaches to introduce genes into organs and tissues in mice. The first method employs viral vectors that either may have preferences for specific organs or tissues, for example, hepatitis viruses that infect hepatocytes in the liver, or may have greater ranges of infectivity, for example, adenoviruses and adeno-associated viruses (AAVs) that have multiple serotypes and lesser preferences for various cell types. The second method of gene delivery is using plasmids to carry DNA into cells. Viral vector preparations from cultured mammalian cells come with the risk of contamination through a variety of different infectious agents, including replication-competent virus generated by recombination between virus vector and packaging constructs[1]. Moreover, the viral vector can be toxic; for example, adenoviruses can

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COMPETING INTERESTS STATEMENT The authors declare competing financial interests (see the HTML version of this article for details).

stimulate acute immune/inflammatory response following transduction in the liver[2]. The risks of DNA-mediated delivery, by comparison, are limited to those associated with plasmid preparation from bacterial extracts (endotoxin, etc.) and whatever chemical component is conjugated with the DNA for the purpose of delivery. A further complication in the use of retroviruses, lentiviruses and AAV may come from their preference for integrating into promoters and transcriptional units, where they may have increased chances of causing adverse effects[3–11]. In contrast, DNA-mediated delivery systems are likely to be more stable than viral vector preparations, more amenable to pharmaceutical formulation and do not target genes for integration. However, introducing plasmids that carry transposons into tissues of live animals is difficult.

This protocol is based on previously validated methods[12–18] and describes hydrodynamic delivery of *Sleeping Beauty* (SB) transpo-sons to mice and means for determining the extent of gene delivery. In mice, rapid, high-volume infusion of naked plasmid DNA, called *hydrodynamic delivery* by the practitioners, is the most effective method for *in vivo* gene transfer. The method is well-established in mice, in which up to 40% of the hepatocytes in test animals take up the transgenic DNA. The liver accounts for >95% of the expressed transgenic DNA following hydrodynamic delivery. The mechanism of DNA uptake is poorly understood but appears to involve expanding liver endothelium, which in mice heals within 24–48 h[19–25]. The method has been adapted to larger animals[15], including porcine[26,27] and rabbit liver[28] as well as muscles of larger animals[29]. The DNA should be ‘naked’, that is, not complexed with condensing polymers or other agents[30].

SB as a vector for gene delivery

The SB transposon system is a nonviral vector system designed to deliver specific genetic cargos to vertebrate chromosomes[31,32]. The SB system consists of two components: a transposon (*T*), which carries a DNA sequence that is often comprised of a gene of interest (GOI) behind a promoter (the combination is referred to as a gene cassette), and an SB transposase that can transpose the transposon from a carrier plasmid (or other donor DNA) to a target DNA such as a mouse chromosome (Fig. 1). The SB system has been used for two primary purposes—gene delivery and gene discovery[32–35]; here we discuss only its role in gene delivery. Transposons containing a transcriptional regulatory cassette and a transgene of interest within a plasmid carrier are delivered to target cells, after which SB transposase cleaves the transposon from the donor plasmid and reinserts the transposon at a site in a chromosome for long-term (often the lifetime of the cell) expression of the transgene. In mice injected with SB transposons and transposase encoded on a plasmid, transposition occurs over a period of ~4 d[16,36].

The SB system is particularly well suited for gene delivery because it does not preferentially insert transposons into active genes[37–39]. There are ~200 million potential sites in the mouse genome into which an SB transposon can insert, which means that tracking transposition activity by cataloging integration sites is essentially impossible. However, transposition can be quantified using an excision assay (Fig. 2) in which, regardless of where it integrates, the transposon leaves a plasmid with a definitive ‘footprint’ that can be quantified by PCR[36]. The excision assay is relatively simple and can be used to determine the activity of the transposon system quantitatively whenever there is a question about its activity, for example, either under novel conditions or with constructs that have not been validated in tissue-cultured cells.

The power of the SB system to confer long-term expression of transgenes has been demonstrated in mice for several models of human disease. SB-mediated long-term expression of clotting factors such as Factor VIII and Factor IX in the liver has cured hemophilias A and B, respectively[40–42]. The procedure has also been used by us and others to treat mice with genetic deficiencies for fumarylacetoacetate hydrolase deficiency[43–45] as well as by us for

β-glucuronidase and iduronidase deficiencies associated with mucopolysaccharidosis Type VII and Type I, respectively[16]. Hydrodynamic delivery to mouse liver has also been used with other nonviral vectors including the ϕC31 integrase system[46], ϕBT1[47,48] and a variety of plasmids encoding transgenic cassettes[14,29] The SB system has also been used to deliver genes to other organs of the mouse including skin to treat junctional epidermolysis bullosa[49], the brain to retard glioma xenographs[50,51] and to lung allografts to prevent fibrosis[52]. A bibliography of papers on the SB transposon system is available at http://beckmancenter.ahc.umn.edu/html/data.html.

Designing an SB vector for gene delivery

A list of some of the available SB transposon constructs, and their full sequences, can be found at http://www.cbs.umn.edu/labs/perry/plasmids/plasmid.html. There are four aspects that should be considered in designing SB transposon vectors for gene delivery.

The expression cassette—This consists of transcriptional regulators (hereafter for convenience referred to as the promoter) and a gene or sequence that is to be expressed. The desired biological effect determines the composition of the expression cassette. The choice of promoter is dependent on several variables, including promoter strength, duration of expression, stringency of expression in certain cell types, and overall length. For example, the CAGGS promoter[53], which is a fusion of transcriptional elements from the β-actin gene and the cytomegalovirus (CMV) early promoter is considered to be an exceptionally strong, fairly ubiquitous driver of transcription in many cell types[54,55]. In contrast to the early CMV promoter, which is often shut off in liver cells after a few days or weeks[56,57], the CAGGS promoter is active over months if not the lifetime of the cell in which it resides[58,59]. However, owing to the promiscuous expression of the CMV and CAGGS promoters, they also will drive expression in other cell types, including stem cells[60] and antigen-presenting cells that could exacerbate immunological responses to the transgene product that will abolish its overall presence in the animal[16]. Consequently, a combination of tissue-specific promoters, insulator or boundary elements[61], and *mir* motifs for regulation of translation by miRNAs[62] or addition of siRNA genes[63] have been used to limit expression to liver cells.

Transposase source and plasmid configuration—As noted earlier, the SB system is comprised of the transposon and the SB transposase enzyme. In most cases, the transposase source is an expression cassette on a plasmid and the transposon is carried on another plasmid (Fig. 3a, *trans*). However, the SB expression plasmid can be included on the transposon plasmid (Fig. 3a, *cis*). The advantage of the *cis* configuration is that only a single plasmid preparation, with appropriate quality control for elimination of bacterial endotoxins, is required and every cell is guaranteed a constant ratio of the SB transposase gene and the transposon with its gene/sequence of interest. The advantage of the *trans* configuration is that there is more flexibility for varying the ratio of the transposase gene and the transposon. For most initial studies, the *trans*-delivery is the preferred choice.

An alternative to *trans*-delivery of the SB system with an SB transposase-expressing plasmid is injection of SB transposase mRNA with the transposon plasmid. This method has not been widely tested, but it works, albeit at a lower rate of transposition than is generally achieved through *trans*- or *cis*-delivery of the SB gene[17,45]. This alternative has the advantage of limiting transposition to a 1- or 2-d period before the transposase mRNA and protein decay, but the inherent disadvantage is the exceptional instability of mRNA both in most solutions and in the vascular system.

Molecular stoichiometry of SB transposase to the transposon—Optimal transposition rates depend on having a ratio of four transposase enzymes per transposon—less

than four transposase molecules will not support transposition, and excessive levels of transposase will quench transposition by a process called *over-production inhibition* (Fig. 3b)[64–67]. The relative levels of transposase and transposons can be manipulated by the ratio of transposase genes to transposon and the relative strength of the promoter driving the SB transposase gene. Thus, delivery of the SB system in the *trans* configuration allows the transposase to transposon ratios to be finely adjusted by modifying the ratio of the plasmids; in the *cis* configuration, a promoter of appropriate strength in the target cell must be experimentally determined and then incorporated into the final *cis*-plasmid. The study by Mikkelsen *et al*. is the most complete report of the effects of a variety of promoters used for the SB gene in a *cis* configuration[66].

Transposon size—This aspect is poorly understood because most end-points for determining effectiveness of delivery involve a screen for gene expression or simple insertion of the gene into a chromo-some. However, both assays depend not only on transposition rate, but also on the efficiency of uptake of the plasmids into the nuclei of cells and stability of expression of the transgene in the transposon. The efficiency of gene expression, which is taken as a measure of efficiency of gene delivery and transposition, decreases with size of the transposon-plasmid. As shown in Figure 3c, an SB transposon of ~6 kb, which would accommodate an expression cassette of ~5.5 kb, has ~50% the efficiency of gene transfer as an optimally sized transposon of 2.0−2.5 kb. A size limitation has not been observed for the Tol2 transposon system in the single report of effects of size[44]. Accordingly, if size of the transposon or its plasmid is an issue, then *trans*-delivery is the preferred method.

Since its first appearance in 1997 (ref. 31), the SB system has been further developed to give higher transpositional activities. Improvements in the transposon inverted terminal repeats have been made[36,52,68,69] and the original SB10 transposase has also been improved[65,70,71]. More recently, efforts to construct SB transposases that can target specific integration sites have been deployed[72–74].

Hydrodynamic injection

The original conditions for hydrodynamic delivery were worked out in the labs of Jon Wolff and Dexi Liu[12,13]. In the mouse, hydrodynamic delivery of DNA requires injection of a large volume (10%, vol/wt) of the mouse body weight of DNA, in a saline solution that is isotonic with blood and suitable for intravenous administration, through the tail vein in <10 s (Fig. 4). Accordingly, depending on the experience of the person doing the injections, DNA can be delivered to the livers of one dozen mice or more in an hour if the materials are organized and the mice are healthy.

Control plasmids

Depending on the experiment, certain control injections may be necessary. These may include a ‘no transposase’ control, in which case the *trans*-configuration of delivery the plasmid with the SB transposase gene is replaced with a plasmid that lacks the SB gene expression cassette or has the inactive mutant ΔDDE gene[31]. In the *cis*-configuration of delivery, the SB gene is omitted from the vector. A control for delivery of any expression cassette that may be difficult to evaluate could include adding a small (e.g., 0.1−1 μg) amount of a CMV-luciferase or CAGGS-luciferase plasmid for bioluminescent imaging; this slight amount of luciferase plasmid should be sufficient to produce 10^9–10^{10} photons s^{-1} (Fig. 5).

In this protocol, we present a step-by-step procedure for hydrodynamic delivery of SB transposon-containing plasmids to mouse liver cells. We include methods for assaying the effectiveness of delivery and the effectiveness of transposition of expression cassettes, or other

genetic cargo, from the carrier plasmid to a recipient DNA molecule, which is generally a chromosome.

MATERIALS

REAGENTS

- Mice We routinely use 8−10 week-old C57BL/6 mice, available from the National Cancer Institute (NCI, Frederick, MD). We have successfully used other strains from other sources (Balb/c, FVB/N, NOD.129(B6)-*Prkdc*^{scid} *IDUA*$^{tm1\ Clk}$/J, and various congenic substrains) ! CAUTION Mice should be handled as per local Institutional Animal Care and Use Committee and Institutional Biosafety Committee approved protocols.
- Anesthetic cocktail: ketamine HCL (Phoenix Pharmaceutical Incorporated); acepromazine maleate (Phoenix Pharmaceutical Incorporated); and butorphanol tartrate (Fort Dodge Animal Health) (see REAGENT SETUP)
- Lactated Ringer's (LR) solution (Henry Schein Company, cat. no. 1533592)
- D-Luciferin firefly luciferase substrate, potassium salt (for *in vivo* imaging; Xenogen Corporation, cat. no. XR-1001). ▲ CRITICAL Store at −20 °C.
- 0.5% (vol/vol) Bleach solution [sodium hypochlorite (NaOCl)]
- Vector DNA containing gene for delivery (see REAGENT SETUP)—see http://www.cbs.umn.edu/labs/perry/plasmids/plasmid.html for details of vectors available from authors on request
- Primers (see Table 1)
- Invitrogen Optimized Buffer A Kit (Invitrogen; cat. no. K1220−02A)

EQUIPMENT

- Eye drops
- Mouse restrainer (Braintree Scientific, cat. no. TV-150) or equivalent
- Recovery cage(s) (*Extra*-clean; Allentown Micro-VENT Mouse cage) for the injected mice—often we inject more than one mouse in a single session and so the recovery cage is not the same as the cage containing the mice pre-injection.
- 45−50 °C waterbath and/or heat lamp
- Heating pad
- Scale for weighing mice (Ohaus compact electronic scale; Fisher Scientific, cat. no. 01−919−33)
- Bleach
- Alcohol pads
- Box of 1-ml TB syringes
- Box of 3-ml syringes
- Box of 18-gauge needles
- Box of 27-gauge butterfly needles
- Shaver to remove mouse hair

REAGENT SETUP

Anesthetic cocktail—In a 10-ml sterile vacutainer tubes (Becton Dickenson, cat. no. BD 2006−12), add 9 ml normal saline, 0.8 ml stock ketamine HCl (100 mg ml^{-1}), 0.1 ml butorphanol (1 mg ml^{-1}) and 0.1 ml acepromazine (10 mg ml^{-1}) to give a final anesthetic mixture of 8 mg ml^{-1} ketamine HCl, 0.1 mg ml^{-1} acepromazine maleate and 0.01 mg ml^{-1} butorphanol tartrate. This drug cocktail is premixed and is stable for 4 weeks at room temperature (20−25 °C). Protect anesthetic mixture from light.

Luciferin stock solution—Prepare a stock solution of luciferin (28.5 mg ml^{-1}) by dissolving 1 g substrate in 35 ml sterile Dulbecco's PBS (Cambrix Bio Science, cat. no. 17 −512F). Filter-sterilize solution through a 0.22-μm sterile filter. Store reconstituted substrate in 1 ml aliquots at −20 °C. Protect substrate from light. Shake well before use.

Preparation of vector DNA—Vector DNA can be prepared with an Endo-Free kit, for example, Maxi Kit (Qiagen, cat. no. 12362), or can be commercially prepared (e.g., Aldevron Inc.). We store the DNA at ~1−2 μg μl^{-1} in 10 mM Tris–HCl, pH 7.2, 0.1 mM EDTA at 4 ° C.

Preparation of samples for numerical evaluation of transgenes, genome copy number and EPs in liver (Step 23)—DNA is isolated from ~50 mg liver specimens using a kit for isolation of total cellular DNA from mammalian tissues, for example, the DNeasy Tissue Kit (Qiagen, cat. no. 69504) according to manufacturer's instructions.

Preparation of samples for EP standard curve (Step 24)—A standard curve for the plasmid EP that relates the copy number of excision plasmid product (EP) as a function of the PCR threshold cycle (C_t) number has to be obtained (Fig. 2). For this, a defined number of copies of the reference DNA sequence (*mockEP*, a plasmid that lacks the complete transposon and therefore mirrors the EP) in a defined number of genomes is prepared by diluting a known amount of plasmid into a known amount of genomic DNA and calculating the relative copy numbers using the known molecular weights of the plasmid and the genomic DNA (≈2.2×10^{12} g mol^{-1} for the mouse genome). Experimentally, the standard curve for EP is obtained through serial dilutions of known quantities of plasmid containing the mockEP in genomic DNA prepared from mouse liver. Alternatively, a standard EP curve can be obtained through serial dilutions of genomic DNA prepared from livers of mice 24 h after injection with 25 mg (the same amount as the transposon plasmid with GOI) of the mockEP (~7×10^{12} molecules in the case of a pT2/BH-based mockEP plasmid; see *Plasmid Info* at http://www.cbs.umn.edu/labs/perry/). Twenty-five microgram of transposon plasmids represents a delivery of ~20,000−40,000 DNA molecules (depending on transposon size) per hepatocyte. On the basis of quantitative PCR (qPCR) curves that detect down to 0.05 copy per cell at their limit, we have estimated that ~1,000 plasmids are associated with each liver cell (diploid genome) 1 d after hydrodynamic delivery[16]. The mockEP used in our studies was pT2/BH-based in which the transposon was deleted between two flanking *Bam*HI sites. The number of EP molecules can be standardized to the number of genomes by quantifying the number of genomic copies of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (Step 23A). In this case, a standard curve of GAPDH is obtained through serial dilution of the genomic liver DNA into TE buffer (10 mM Tris pH 7.6, 1 mM EDTA). Figure 2c shows the type of curve that can be obtained.

EQUIPMENT SETUP

General setup considerations—The necessary materials should be set up in a biosafety cabinet or appropriate hood, as shown in Figure 6. Place sufficient 1- and 3-ml syringes, 18-gauge needles, 27-gauge butterfly needles and alcohol pads on table for the entire procedure.

If using a water bath: wipe the water bath with a 0.5% (vol/vol) bleach solution. Turn heat control for water bath on *high* and fill 600-ml beaker with 0.5% bleach solution. Put beaker on top of heat block and read temperature using a thermometer. When solution temperature is ~35−40 °C, turn heat to *low*. Residual heat will raise temperature to 45−50 °C. If using a heat lamp: Turn on heat lamp and place first cage under it to warm mice

Recovery cage—Turn on heating pad to *medium* for several minutes and then turn down to *low*. Place half of recovery cage on the warmed (40−50 °C) pad to provide a temperature gradient in the cage, which allows the animal to find its preferred warmth.

PROCEDURE

Prepare mice for injection • TIMING 1 min

1| Anesthetize 25−30 g mice by administering 50 μl of anesthetic cocktail i.p., prepared as indicated in REAGENT SETUP. We inject 25−30 μl of anesthetic cocktail to mice weighing <20 g to avoid rendering them unconscious.

▲ CRITICAL STEP The mouse should NOT be unconscious but moving slower (as if it were 'drunk'). The hydrodynamic procedure will increase the effect of the anesthetic. If the mouse is unconscious, there is a significant chance of animal death if injected hydrodynamically.

? TROUBLESHOOTING

2| Weigh mouse. Weighing is easier and thereby more accurate when the mouse is 'drowsy' than when the mouse is fully active.

Prepare DNA sample for injection • TIMING 3 min

3| Before entering the mouse room (i.e., in the lab), transfer the desired amount of stock DNA solution into a 1.5-ml Eppendorf tube. Then, while the anesthetic takes effect, calculate the volume required for hydrodynamic injection based on the weight of the mouse [10% (vol/wt)]. For example: a 20 g mouse will be injected with 2 ml of a DNA solution that may range from 0.05 to 30 μg ml^{-1}; we customarily use ~10−12.5 μg ml^{-1} initially with modifications to the dose in subsequent experiments that depend on the level of gene expression attained compared to that desired.

▲ CRITICAL STEP We find that if an animal weighs >25 g and >2.5 ml is injected, the expression of the reporter genes does *not* increase (E. Aronovich, J. Frandsen & R.S. McIvor, unpublished data). Accordingly, we have set an upper limit for the injection volume at 2.5 ml.

4| Place an 18-gauge needle on 3-ml syringe and draw up the amount of LR calculated in Step 3 plus an additional 200 μl to take into account fluid that will remain in the 27-gauge butterfly needle line after the injection is complete.

5| Inject ~1 ml of the LR from Step 4 into the tube and mix the DNA by withdrawing and re-injecting LR 'up and down' in the tube two to three times. At the end, after all of the solution in the Eppendorf tube has been recovered into the syringe, make sure that the DNA is thoroughly mixed by rocking the syringe several times using the air bubble to mix. As an example, for injection of a 20-g mouse with 25 μg DNA we would transfer 12.5 μl of stock DNA(at 2 μg μl^{-1}) into the Eppendorf tube and mix it into 2.2 ml LR solution (2 ml + 200 μl) from Step 4; we generally store our plasmids at concentrations of 1−2 μg μl^{-1}.

6| Remove the 18-gauge needle and replace it with a 27-gauge butterfly needle. Remove the air from the syringe by tapping it and then press plunger slowly to fill the butterfly line. Set

syringe aside until injection. The DNA solution should be stable at room temperature for the duration of the injection time if properly stored in a buffer that lacks free divalent cations such as Mg^{2+}.

Hydrodynamic injection • TIMING 1 min

7| Place the tail of the mouse under a heat lamp or in a 45−50 °C water bath for 10−20 s. This will increase the vascular volume in the tail and make the tail vein more easily visualized.

▲ CRITICAL STEP If using water bath: ensure that the temperature does not exceed 50 °C or the tail will be burned.

▲ CRITICAL STEP When using a heat lamp, avoid overheating the mice; they die quickly when heated too much or for too long. Attach the heat lamp on a ring stand rod ~45−50 cm high. Place the cage under the lamp. The length of time that the mouse can stay under the lamp depends on how close the lamp is to the cage; we find that at a height of 45 cm the mice can rest up to 15 min, which is sufficient time to inject five mice. When a mouse is being overheated, it becomes sluggish and usually will dig into the bedding at the corners of the cage. If you notice this, either move the cage farther away from the heat lamp or turn off the lamp for 2−5 min until the mouse regains normal activity.

? TROUBLESHOOTING

8| Place the mouse in a restrainer tube (Fig. 7a) and wipe the tail with an alcohol pad.

? TROUBLESHOOTING

9| Place the tail with the lateral side up between the thumb and forefingers. Inject the needle into the vein ~2−3 cm from the tip of the tail, making sure that the bevel of the needle is up (Fig. 7b). Once in the vein, you may see a little backflow of blood into the tubing of the butterfly needle. In white or nude mice, you can actually see the needle inside the vein.

▲ CRITICAL STEP As there are several variations for mouse tail-vein injections, an experienced person should use his/her normal injection procedure. A butterfly needle is not necessary, but we find it is easier to control the tip of the needle during the injection.

10| Press on plunger of the syringe with even force. The plunger will move smoothly with ease. Do not force it. If the plunger will not move easily, remove the needle and reinsert it anterior to the previous injection site about midway between the first injection site and the body of the mouse. If a third attempt is made, it can be done using the other vein on the opposite side of the tail.

▲ CRITICAL STEP If the plunger stops moving, you have blown the vein. Usually, the mouse is removed from the experiment. You can retry repositioning the needle. However, we have found that often delivery is compromised and not as efficient in these animals.

11| Inject the entirety of the DNA sample into the tail vein within a period of 4−7 s.

▲ CRITICAL STEP This short period of time, from start to finish, is critical. An optimal duration of the injection is ~4−7 s. Injections lasting 8−10 s will have moderate success and injections taking > 10 s are considered unsuccessful and are not included in test groups.

? TROUBLESHOOTING

12| When finished, remove needle and, if there is any bleeding or blood, apply light fingertip pressure to the injection site until the bleeding stops.

Recovery phase • TIMING 60 min for full recovery

13| After injection, remove the mouse from the restrainer as quickly as possible and place eye drop in its eyes to prevent them from drying out owing to anesthesia. We hold the mouse until its respiration rate returns to approximately the normal rate.

14| Return the mouse to the recovery cage, half of which should be on a heating pad set at low heat (see EQUIPMENT SETUP). The mouse is considered to have recovered when it is up and back to normal activity. The mice may be sufficiently stable for the first blood drawing or other tests, such as *in vivo* bioluminescence, as soon as 30 min after hydrodynamic injection.

▲ CRITICAL STEP Monitor breathing to verify that the mouse survived the injection. The mouse's breathing may slow to one breath every 1 or 2 s. After 5−15 s the breathing rate should increase to near normal, 94−163 breaths per min. If the mouse stops breathing or gasps, chest massage may be required to promote breathing and recovery.

? TROUBLESHOOTING

15| Repeat the entire procedure with the next mouse. In general, a cohort of several mice is injected one after the other, with occasional inspection of the recovering mice to ensure they are not in distress. The only limitation on the number of mice injected over a single period is the investigator's ability to avoid confusing various DNA solutions that are being injected, keep accurate records that include injection times and gross physiological responses to the injection procedure and all the while monitor the health of the recovering mice. We have injected up to 50 mice in a day but generally inject only ~20 in a single session. When a large number of animals are injected, a team of two investigators is best to ensure all the tasks can be accomplished while maintaining vigilance over mouse activities.

Evaluation of the efficiency of the hydrodynamic injection by *in vivo* bioluminescent imaging • TIMING 10 min or more per image set (depending on exposure of images)

16| The effectiveness of nucleic acid delivery can be evaluated as soon as 30 min after hydrodynamic injection through bioluminescent imaging[59]. Weigh mice and use Table 2 to determine the appropriate dose of anesthesia.

▲ CRITICAL STEP If imaging equipment is not available, blood can be drawn from mice using standard methods and assayed for activity of the GOI.

17| Inject anesthetic i.p. Wait 2−5 min, until the mouse is fully anesthetized. Note that the level of anesthesia is higher than that used in Step 1 because for this procedure the mouse must be completely anesthesized for compliance with animal care regulations.

▲ CRITICAL STEP Do not overdose the mouse with anesthetic—if the mouse goes into respiratory distress it will probably die. Owing to the variation in responses to anesthetic by various mouse strains and mutant lines, there is no simple method to determine the anesthetic dose other than by experimentation. Check with appropriate animal care and veterinary staff for any guidelines they can provide for particular lines of mice.

? TROUBLESHOOTING

18| If necessary, shave the ventral area over the liver on the underside of the mouse. Generally, only dark-coated mice must be shaved for acceptable imaging.

19| Inject 100 μl luciferin i.p. and wait at least 5 min before imaging; or inject 50 μl i.v. and image immediately. For detection of firefly luciferase activity, either i.p. or i.v. delivery of luciferin can be employed. For detection of Renilla luciferase, substrate should be delivered i.v.

▲ CRITICAL STEP For the i.p. luciferin injections, several mice can be injected for imaging after hydrodynamic DNA delivery. Imaging should be carried out between 5 and 25 min after administration of the luciferin; after 30 min the level of luciferase activity drops significantly and the assay will be compromised.

▲ CRITICAL STEP Sometimes, imaging multiple animals at one time results in one animal apparently lacking expression in terms of the color-coding. This could be an artifact owing to strong signals from one animal interfering with detection of the image from another animal that has a lesser signal. Accordingly, in this circumstance, mice that do not show a signal should be imaged separately to ensure that their signals are appropriately measured.

20| Place the animal on its back onto black construction paper (Fig. 8). If the animal cannot be positioned stably on its back with its underside fully exposed, tape down its legs.

21| Image the mice according to manufacturer's instructions. We use the Living Image program for which the basic guidelines are provided in Box 1.

▲ CRITICAL STEP Exposures should not exceed 5 min—good delivery of a transgenic cassette with a promoter that provides robust expression in the liver usually requires ~1 s exposure for saturation (Fig. 8).

? TROUBLESHOOTING

22| Return mice to their cage (see EQUIPMENT SETUP) to recover until they are up and moving around.

▲ CRITICAL STEP Avoid hypothermia. Use heating pad set on low (warm) to keep mouse from shivering. The heating pad will help keep the mouse's internal temperature from dropping too low and causing hypothermia.

Excision assay for transposition efficiency • TIMING 2−3 d

23| After bioluminescent imaging, the animals may be killed and the excision assay performed. The excision assay[16,36] is the most convenient and comprehensive assay for transposition of SB transposons in a multicellular organ like the liver. Isolate ~0.1 mg total DNA from ~50 mg liver specimens using a kit for isolation of total cellular DNA from mammalian tissues, for example, the DNeasy Tissue Kit according to manufacturer's instructions.

24| Quantify DNA copy number by real-time qPCR (option A) or approximate copy number by conventional PCR (option B).

▲ CRITICAL STEP All PCR experiments require appropriate controls. Suitable negative controls should include samples that lack any DNA and samples that specifically lack the target DNA sequence. A positive control should be included when possible, but a plasmid source that might lead to contamination of the experimental sample should not be used. Internal PCR controls, for example, β-glucuronidase in Figure 2b, serve to validate the reaction mix and conditions of amplification.

(A) Determining DNA copy number by real-time qPCR

i. Set up a 25 μl reaction mixture for each sample, as tabulated below. All qPCRs should be run in triplicate and include appropriate controls and standard curve samples (see REAGENT SETUP). We use mouse single-copy gene sequences, such as GAPDH, as an internal control of genomic DNA content. A standard curve of GAPDH is obtained through serial dilution of genomic liver DNA from the hydrodynamically injected mice into TE buffer (10 mM Tris pH 7.6, 1 mM EDTA) and measuring the C_t numbers using the GAPDH-F and GAPDH-R primers (Table 1)[16].

Component	Amount	Final concentration
Liver DNA	500 ng	20 ng μl^{-1} (≈ 0.01 pM GAPDH gene)
GAPDH-F and -R (Table 1)	5 pmol each	200 nM each
2× IQ SYBR green supermix	12.5 μl	1×

BIOLUMINESCENT IMAGE ACQUISITION

We have experience only with Xenogen's IVIS Imaging System with Living Image program.

1. Initialize the system according to manufacturer's instructions.
2. Set stage at A or B (depending on number of mice imaged simultaneously). Use stage A for one to three mice imaged at the same time (depending on size of mice). Use stage B for three to five mice imaged at the same time (depending on size of mice).
3. Set imaging parameters as follows: Exposure time: 0.5 s to 5 min (duration depends on expression cassette and the time after injection). Binning—medium (we use most commonly 4 × 4 pixels setting—binning can be decreased if the image saturates). *F*-stop—1 (increasing the *F*-stop reduces saturation of the image).
4. Take a photo to verify position.
5. Select *lumination* overlay key.
6. Acquire image.

i. Carry out thermocycling. Typical conditions are tabulated below. We use an iCycler instrument (Bio-Rad).

Cycle number	Denature	Anneal	Extend
1	95 °C 2:00 min	—	—
2–41	95 °C 0:40 min	58 °C 0:40 min	72 °C 1:00 min
42	—	—	72 °C 5:00 min

ii. The DNA content (GAPDH gene number, *y*-axis) for each dilution is plotted as a function C_t value (*x*-axis) to obtain a standard curve for genomic DNA copy number. Figure 2c shows the type of curve that can be obtained.

(B) Conventional PCR for qualitative assessment of EP formation

i. Excision analysis using conventional PCR can be used to back up, at lower resolution, the qPCR results. Conventional PCR can also be used to compare transposition rates qualitatively in various reactions where the absolute numbers of events is not required.

Our conventional PCR protocol requires two rounds of amplification. We use a dNTP mix, PCR buffer and DMSO from the Invitrogen Optimized Buffer A Kit. For the first round, set up a 50 μl reaction mixture for each sample (including controls), as tabulated below.

Component	Amount	Final concentration
Liver DNA	100 ng	2 ng μl^{-1} (≈ 30,000 haploid genomes)
EP-F1 and EP-R1 primers	10 pmol each	200 nM
dNTPs (A, G, C, T)	10 nmol each	200 μM
5× Optimized Buffer A	10 μl	1× Optimized Buffer A
DMSO	2.5 μl 100% stock	5% (vol/vol)
Glycerol	2.5 μl 100% stock	5% (vol/vol)
Taq DNA polymerase	5 U	0.1 U μl^{-1}

ii. For the first amplification, we use the following conditions.

Cycle number	Denature	Anneal	Extend
1	95 °C 5:00 min	—	—
2−41	95 °C 0:40 min	58 °C 0:40 min	72 °C 1:00 min
42	—	—	72 °C 5:00 min

iii. Set up the second, nested round of amplification reactions as tabulated below.

Component	Amount	Final concentration
PCR mixture from reaction 1	5 μl	Not applicable
EP-F2 and EP-R2 primers	10 pmol each	200 nM
dNTPs (A, G, C, T)	10 nmol each	200 μM
5× Optimized Buffer A	10 μl	1× Optimized Buffer A
DMSO	2.5 μl 100% stock	5% (vol/vol)
Glycerol	2.5 μl 100% stock	5% (vol/vol)
Taq DNA polymerase	5 U	0.1 U μl^{-1}

iv. Use the following conditions for second round amplification.

Cycle number	Denature	Anneal	Extend
1	95 °C 5:00 min	—	—
2−31	95 °C 0:40 min	58 °C 0:40 min	72 °C 1:00 min
32	—	—	72 °C 7:00 min

v. We run 8 μl PCR product on an 1% (wt/vol) agarose gel. An EP band of ~460 bp is expected on the gel (Fig. 2b). In this example, the murine β-glucuronidase (GUSB) housekeeping gene served as an internal control that was amplified in the same PCR using primers GUSB-F and GUSB-R (Table 1). PCR amplification yields a GUSB PCR product of 358 bp.

• TIMING

It takes ~6−10 min per mouse from anesthesia to completion of the hydrodynamic injection although a group of 10−15 mice can be performed in <1.5 h as mice can be injected while others recover Steps 1−6, preparing mice and DNA solutions for injection, 4 min (per mouse

—cohorts may take less time since the preparation of the DNA solution is a single event for all mice in a given group)

Steps 7−12, hydrodynamic injection, 1 min (per mouse)

Steps 13−15, recovery phase, 60 min for full recovery; 30 min for sufficient recovery for bioluminescent imaging

Steps 16−22, evaluation of the efficiency of the hydrodynamic injection: *in vivo* bioluminescent imaging, 10 min or more per image set (depending on exposure of images)

Steps 23−24, excision assay for evaluation of transposition efficiency, 2−3 d after harvesting of liver

? TROUBLESHOOTING Troubleshooting advice can be found in Table 3.

ANTICIPATED RESULTS

A natural question is the reliability and extent of gene delivery using the hydrodynamic procedure. In our experiments, we inject ~25 mg of DNA, which depending on the size of the plasmid, corresponds to ~$2-4 \times 10^{12}$ plasmids per injection. Biodistribution studies have shown that, based on a CMV promoter that transiently expresses well in many cell types, >95% of the expressing plasmids are in liver, and 0.1−1% in other organs[12,75]. Assuming that immediately after injection ~1% of the injected DNA remains in the liver, which contains ~10^8 hepatocytes as well as Kupffer and sinusoidal epithelial cells in an adult mouse[76], there would be on the order of 1,000 transposon-containing plasmids per cell.

Figure 2 shows examples of expected results for assay of the first step of transposition—the excision of the transposon from its carrier plasmid. The excision assay can quantify EPs down to the level of one excised transposon plasmid per 10,000 cells[36].

Figure 5 shows the reproducibility of hydrodynamic delivery to the liver and an example of the distribution of cells that take up transgenic DNA for expression in the liver. The efficiency of delivery to liver varies about tenfold from mouse to mouse as measured by expression of luciferase from a transposon-containing plasmid 24 h after injection (Fig. 5a). The duration of transposition following hydrodynamic delivery to the liver appears to be relatively short, ~3−4 d if a short-duration promoter like the CMV promoter is used to direct transcription of transposase[77]. Within the first day, as many as 40%, but more often 10−20% of hepatocytes (Fig. 5b and **c**)takeup ~100 transposon-containing plasmids each[16] but only express those which are able to enter the nucleus for transcription. In ~1−5% of cells, a transposition event occurs that moves the transposon into a mouse chromosome[16,40,43]. After ~2−4 weeks, Southern blots indicate that there is <1 copy of transgenic DNA per liver cell[18]. Thus, of the 10^{12} injected transposons, we estimate that ~10^6 (10^{-4}%) actually integrate into liver cells, which in mice can be sufficient to treat diseases effectively such as hemophilia and lessen the effects of other diseases such as lysosomal storage disorders. The retention of plasmids may be dependent on their CpG contents and the strain of mouse.

Figure 8 shows examples of two mice imaged 1 d after hydrodynamic injection. A hybrid CMV/β-actin promoter was used to regulate expression of the firefly luciferase gene. The bioluminescence imaging allows estimation of luciferase gene delivery *in vivo*.

Figure 9 shows the first reported example of hydrodynamic delivery of the SB system into mice[40]. In this example, the human Factor IX gene (hFIX) behind an EF1α promoter was delivered with either a functional SB10 transposase or the inactive mutant form in *cis*.

Expression of hFIX at therapeutic levels was achieved over the lifetime of the mice when functional transposase was co-delivered but not when the mutant transposase was delivered.

ACKNOWLEDGMENTS

We thank the Arnold and Mabel Beckman Foundation for support of our work and all members of the Beckman Center for Transposon Research for a long history of contributions of ideas and results. We appreciate the help of Mr. Joel Frandsen for teaching us the intricacies of hydrodynamic injection and our veterinarian technician, Brenda Koniar. The authors were partially supported by National Institutes of Health grant 1PO1 HD32652-07.

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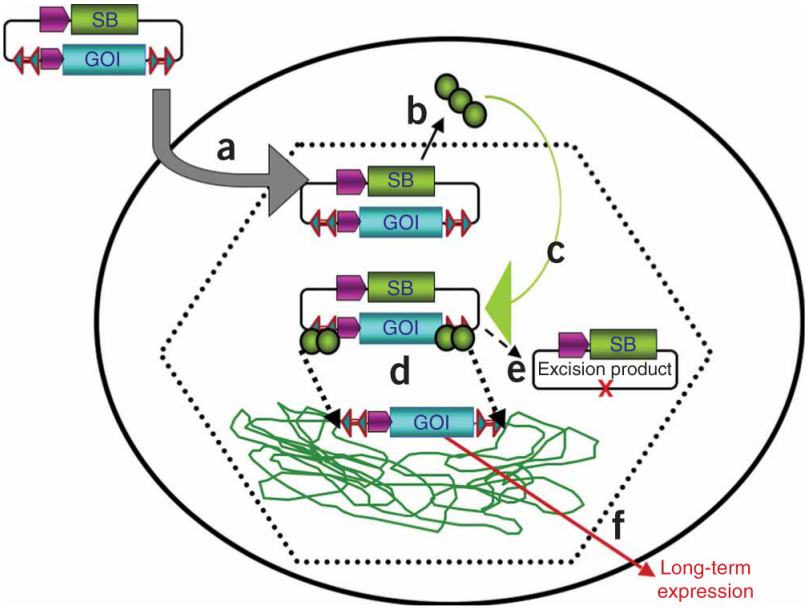

Figure 1.

Schematic of *Sleeping Beauty* (SB) transposition. DNA transposition consists of a cut-and-paste reaction in which a transposon containing a gene of interest (GOI, shown in blue, with its promoter shown in magenta) is cut out of a plasmid and inserted into another DNA molecule, in this case a mouse chromosome. The cleavage reaction occurs at the ends of the inverted terminal repeats (inverted set of double arrowheads in the diagram) of the SB transposon[31]. SB transposons integrate only into TA-dinucleotide basepairs (~200 million in mammalian genomes). The inverted terminal repeats are the only DNA sequences required by the transposase enzyme for transposition. The transposase gene can be on the same plasmid (*cis* configuration as shown) or delivered on a separate plasmid (*trans* configuration, see Fig. 3) to permit greater flexibility in experimental design. (**a**) The plasmid carrying the SB transposase gene and transposon enters a cell (large back oval) as a result of hydrodynamic delivery and proceeds through the nuclear membrane (dotted hexagon) by a poorly understood process. (**b**) The SB transposase gene (SB, green) is transcribed from a promoter (magenta arrowhead) and the mRNA translated in the cytoplasm to give an appropriate level of enzyme (green circles). (**c**) The SB transposase molecules enter the nucleus and bind to the transposon, two at each end, to bring the transposon ends together (shown in detail in Fig. 3b). (**d**) Four transposase enzymes work in concert to cleave the plasmid at the termini of the transposon and paste it (dotted lines) into chromosomal DNA (green tangled lines). (**e**) A plasmid excision product is left behind in this reaction (the site whence the transposon left is marked by an X). (**f**) Integration into a chromosome can confer long-term expression of the GOI that is contained within the transposon.

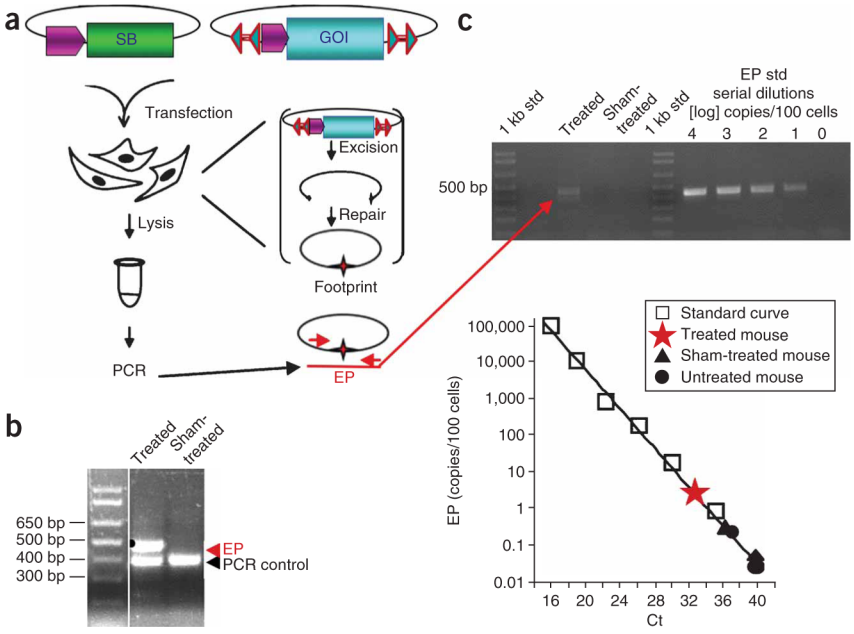

Figure 2.

Excision assay. (**a**) Theory of the excision assay. The first step of transposition is precise cleavage on both sides of the transposon (*excision* step, step *d* in Fig. 1). The gap in the donor-plasmid left by the transposon is repaired (step *e* in Fig. 1) to reform a circular plasmid lacking the transposon. The re-ligation step often leaves a *footprint* of an extra 5 bp (either TCTGA or TCAGA) at the site of the original transposon[36]. As a result of ligation of the plasmid, primers (red arrows in the bottom right corner of panel **a**) complementary to plasmid sequences located on either side of the site whence the transposon originated can bind to the repaired plasmid. PCR-amplification from the primers leads to a DNA sequence of predictable length and sequence (except for the footprint), which we refer to as the excision product (*EP*) (red line at the bottom right of panel **a**). The power of the assay is that regardless of where the transposon integrates, the EP from each event is the same thereby allowing quantification of the totality of transposition events. Thus, whereas identification of integration at any of 2×10^8 TA sites can only be determined through analysis of genomic DNA, the intensity of the PCR product from the repaired plasmid can be used to access the overall extent of transposition. The red line represents the EP from PCR and the red arrow indicates its location on the gel in panel **c**. Primer sites are chosen to be 100−200 bp outside of the inverted repeats of the transposon to ensure that the PCR product is efficiently synthesized, that the EP is distinguishable from the primers (and 'primer dimers' sometimes seen in gels of PCRs), and that the EP is distinguishable from the PCR amplification across the transposon from unexcised plasmids. (**b**) The level of transposition can be estimated through conventional PCR and analysis by agarose-gel electrophoresis. The gel shows a single band amplified from a genomic sequence (e.g., β-glucuronidase) in a liver sample from a sham-treated control mouse that received an injection of lactated Ringer's solution without DNA and an additional band representing the amplified EP (red EP) from a mouse injected with a transposon-containing plasmid. (**c**) The amount of EP can be quantitatively determined by real-time PCR using any of several instruments (the data here were generated using an iCycler (Bio-Rad Laboratories) with their IQ SYBR green supermix according to their guidelines). The data show the copy numbers of excision plasmid product (EP) as a function of the PCR threshold cycle (C_t) number when SYBR Green fluorescence is first detectable. The standard curve indicates that the signal is proportional to the input level of repaired plasmids after excision (open boxes). Triplicate samples were assayed and overlapped for all samples except that from the sham-treated mouse

(two triangles; the bottom triangle represents two results) and the untreated mouse (black circles with two of the samples overlapping at the bottom). Greater variation between samples is sometimes seen with the least concentrated samples because the slightest contamination can lead to a measurable signal. The gel at the top shows the final products from real-time PCRs that were resolved by electrophoresis using 1% agarose gel. Panel a is adapted from Figure 2 of Liu *et al*. (2004)[36] and panels **b** and **c** are from Figure 6 of Aronovich *et al*. (2007)[16]. GOI, gene of interest; SB, *Sleeping Beauty*.

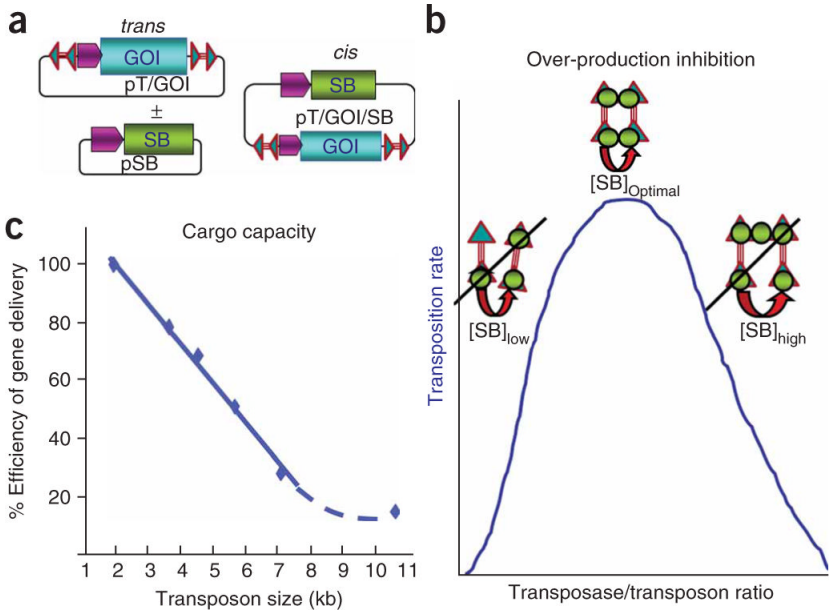

Figure 3.

Design of *Sleeping Beauty* (SB) transposons. (**a**) The *cis* and *trans* configurations for delivery of the SB system are shown with transcriptional regulatory sequences represented by the magenta arrowheads as described in Figure 1. In the *trans*-delivery mode, the transposase gene can be omitted as a negative control. An alternative *trans*-delivery is possible wherein the SB mRNA is delivered rather than its gene. (**b**) The phenomenon of overproduction inhibition is illustrated. The rate of transposition depends on the binding of four transposase enzymes to the transposon $[SB]_{optimal}$. If there are fewer than four transposase molecules $[SB]_{low}$, transposition will not occur and if there are more, then the additional transposase molecules will quench the reaction by competing with bound enzymes to prevent the bringing of the transposon ends together $[SB]_{high}$. (**c**) The effects of cargo (expression cassette) size on gene delivery as determined by long-term gene expression in dividing cells. The cause for the apparent decrease in transposition as a function of size is poorly understood because the length of the plasmid carrier sets the linear distance between the termini of the transposon once the expression cassette is longer than ~2 kb. Factors that may influence the efficiency of gene delivery include propagation through the plasma and nuclear membranes, where effects of DNA length have not been carefully evaluated. The diagrams in panels **b** and **c** are adapted, with permission, from Figure 3 of Geurts *et al.* (2003)[65] and Figure 2 of Hackett (2007)[67],respectively.

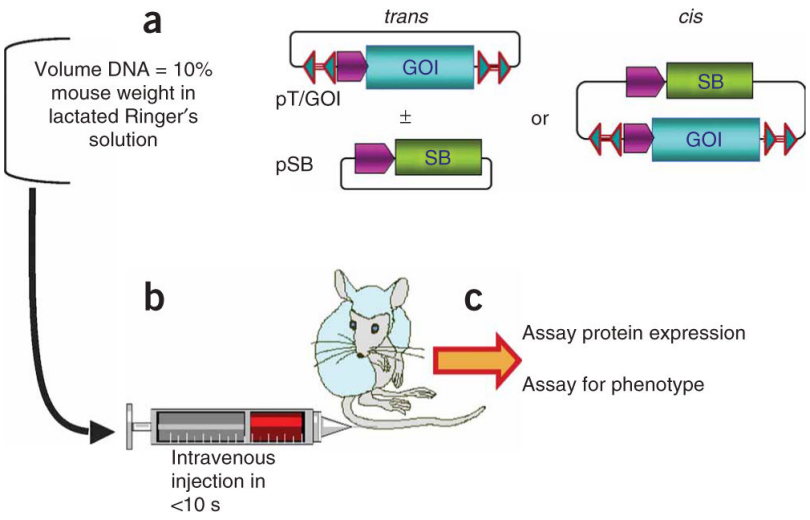

Figure 4.

Hydrodynamic injection procedure of a transposon containing a gene of interest and a source of *Sleeping Beauty* (SB) transposase encoded by an SB gene. The components of the SB system are the same as in Figures 1 and 3. *Trans*- and *cis*-deliveries are performed in the same way. (**a**) The desired amount of DNA (generally between 0.1 and 50 μg, although higher and lower amounts can be injected) is diluted into a volume of lactated Ringer's solution that is equivalent to 10% the mouse weight. (**b**) The DNA solution is injected into the tail vein of the mouse; injections that take 4–7 s are optimal. (**c**) Results from hydrodynamic delivery can be obtained as soon as 30 min after injection, depending on the gene of interest (GOI) and the assay.

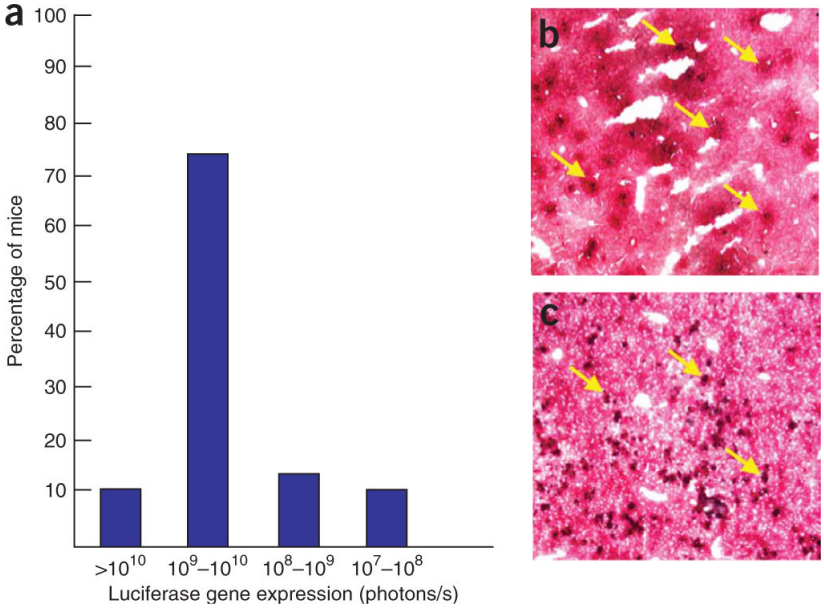

Figure 5.

Hydrodynamic delivery to liver. (**a**) The graph shows the reproducibility of delivery of a luciferase expression cassette. Seventy-five percent of the C57BL/6 mice express a luciferase transgene within a tenfold range (2×10^9 to 2×10^{10} relative light units). (**b**) The image shows the cellular distribution of expression of human β-glucuronidase (dark purple cells) in liver sections from a C57BL/6 mouse 24 h after hydrodynamic injection of transposons carrying the gene controlled by a hybrid cytomegalovirus (CMV)/β-actin promoter (yellow arrows show some of the cells expressing β-glucuronidase). (**c**) The image shows the cellular distribution of expression of human β-glucuronidase (dark purple cells) in liver sections from a β-glucuronidase-deficient C57BL/6 mouse 24 h after injection following hydrodynamic injection of the same transposon vector as in panel b. All of the cells are stained because those that do not express the enzyme take up enzyme produced by those cells that are expressing the transgene, a process called ‘cross-correction’. Panel **c** is adapted from Figure 1 of Aronovich *et al*. (2007)[16].

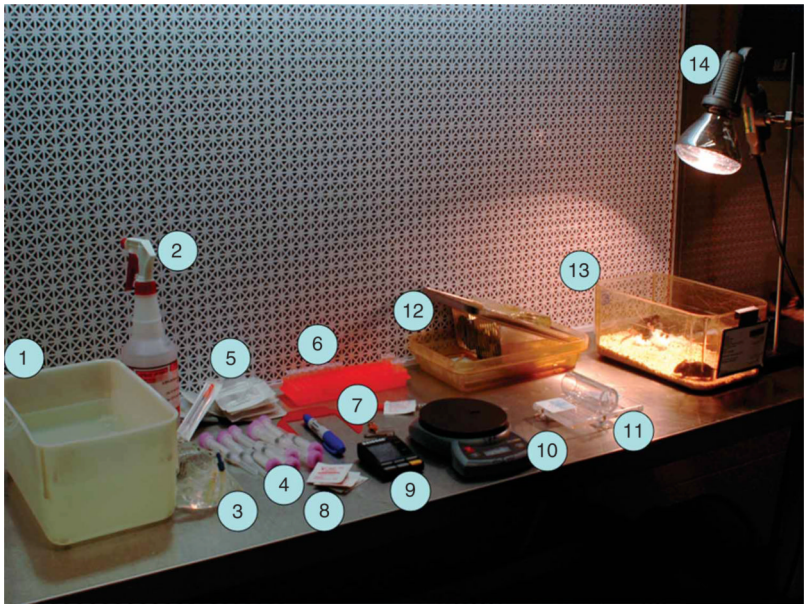

Figure 6.

Layout of equipment for hydrodynamic injection. The items, except for a marker pen in middle are numbered for easy identification: 1) Bleach bucket, for washing items and hands that are used in the hood in accordance with specific-pathogen free (SPF) procedure. 2) Bleach Spray bottle to facilitate washing in accordance with SPF procedure. 3) Lactated Ringer's solution for dilution and injection of transgenic DNA. 4) Three milliliter syringes for hydrodynamic injections. 5) Butterfly needles for hydrodynamic injections; the needle attaches to the 3-ml syringe. 6) Rack with samples that contain the DNA solutions for injection. 7) One-milliliter syringe with anesthetic drug already drawn up; it is covered because the anesthetic is sensitive to visible light. 8) Alcohol pads to sanitize tails. 9) Timer to measure duration of each injection. 10) Scale for weighing mice. 11) Mouse restrainer to contain mouse and reduce its activity during the injection. 12) Top of mouse cage set at an angle for better viewing. 13) Bottom of mouse cage for animal housing. 14) Heat lamp and ring stand to dilate veins in mice and keep them warm. The vertical rod in the stand is ~50 cm tall.

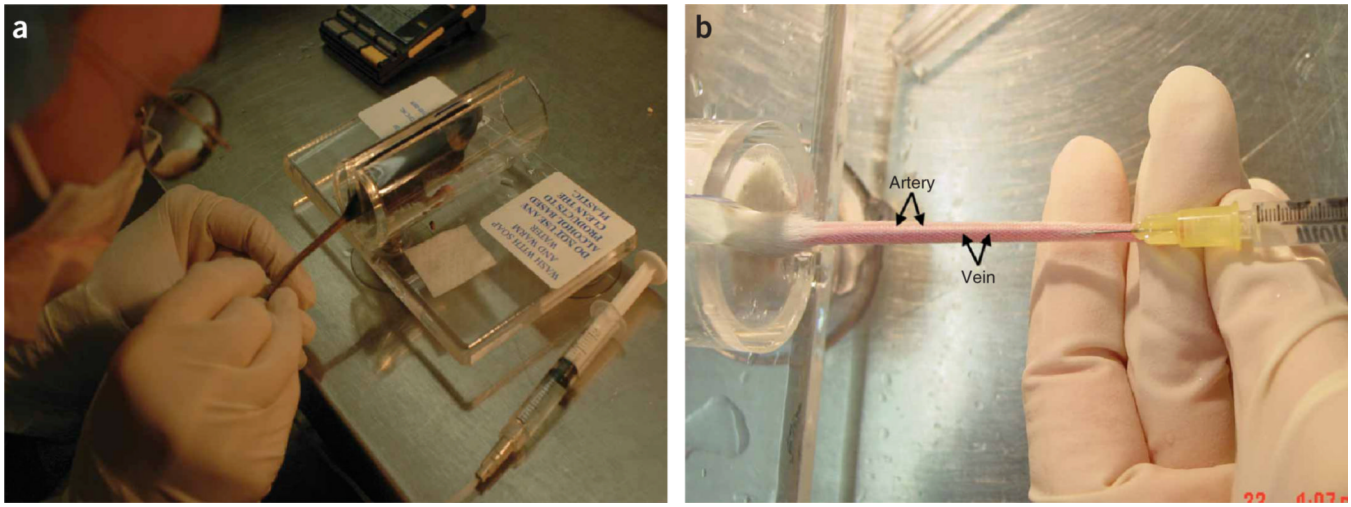

Figure 7.

Hydrodynamic injection with mouse in restrainer. (**a**) The mouse is placed in a restrainer and the tail is rotated until the lateral vein faces up. In this example, the needle is in the right hand and is being placed into the tail vein for injection. (**b**) Detail of insertion of the needle into a tail vein. Two tail arteries run along the top and the bottom of the tail and two tail veins run on either side, midway between the arteries. The needle is inserted ~2−3 cm from the tip of the tail (which is under the injector's thumb in this image). The needle is almost completely inserted into the vein for best results. The tip of the tail behind the injection site may be bent downward by some injectors. The tail is pulled taut, but excessive force may result in the tail coming off in young mice. If the injection fails, a new site, roughly in the middle of the tail can be tried. If this fails, one can try the other tail vein on the other side. When the needle is removed, some blood will flow from the puncture; bleeding is stopped by pressing lightly (not squeezing tightly) the wound with a finger and thumb.

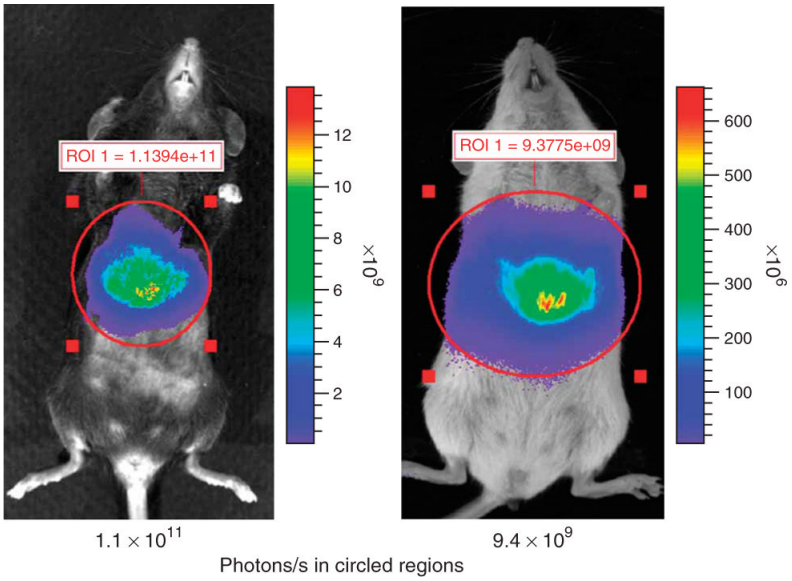

Figure 8.

Typical results from *in vivo* bioluminescence imaging of mice 24 h after hydrodynamic injection. Both mice are on black construction paper, which may appear differently according to background illumination. Color-coded luminescence scales for the circled regions are shown on the right. Exposures are 1 s. The imaging identifies the liver as the primary site of expression of a luciferase expression cassette. The mouse on the left is a C57BL/6 mouse (Imaged for 0.5 s) and the mouse on the right is a NOD.129(B6)-*Prkdc*^{scid}*Idua*^{tm1Clk} (imaged for 1 s). ROI, region of interest.

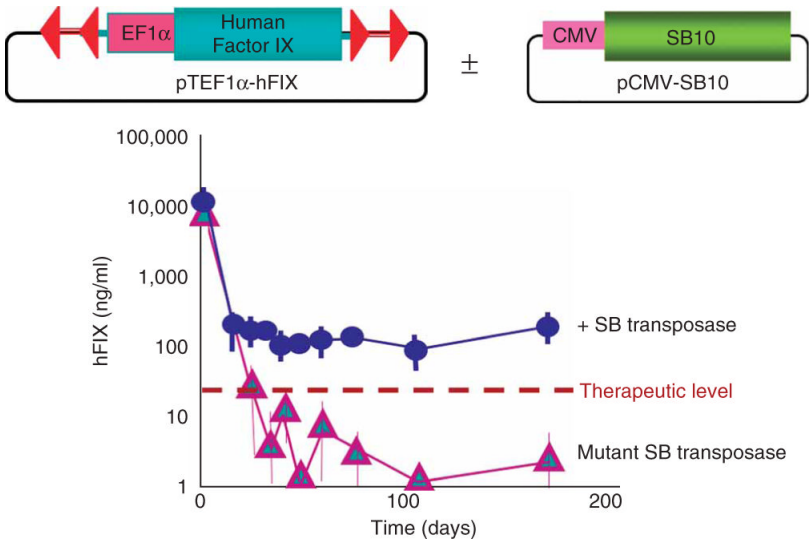

Figure 9.

Example of long-term gene expression following hydrodynamic delivery into mice. Results of delivery of the human clotting Factor IX (hFIX) gene in a *Sleeping Beauty* (SB) transposon into FIX-deficient mice. Long-term expression depended on the presence of a functional transposase (SB10 regulated by a cytomegalovirus (CMV) promoter). The top portion of the figure shows the constructs delivered in cis. The graphs show the levels of hFIX in the mice as a function of time after hydrodynamic injection relative to normal enzyme amounts (therapeutic level). This figure is adapted from Figure 4 of Yant *et al*. (2000)[40].

TABLE 1

Primers used in this protocol.

Oligo name	Sequence (5′ to 3′)	Comments
EP-F1	TGACGTTGGAGTCCACGTTC	Forward primer for detection of excision product (EP; Step 24)
EP-R1	GGCTCGTATGTTGTGTGG	Reverse primer for detection of EP for plasmids lacking an SB gene (Step 24)
EP-F2	CTGGAACAACACTCAACCCT	Forward primer for detection of EP (Step 24)
EP-R2	CCCAAGGTTTGAACTAGCTC (*cis*) CACACAGGAAACAGCTATGA (*trans*)	Reverse primers for detection of EPs for vectors in either the *cis*, or *trans* configured SB gene (Step 24)
GAPDH-F	TGTCTCCTGCGACTTCAACAGC	Forward primer for detection of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene used as an internal control (Step 24)
GAPDH-R	TGTAGGCCATGAGGTCCACCAC	Reverse primer for detection of GAPDH gene used as an internal control (Step 24)
GUSB-F	CTTCGACTGGCCGCTGCT	Forward primer for detection of GUSB gene
GUSB-R	GGCCACAGACCACATCAC	Reverse primer for detection of GUSB gene

TABLE 2

Doses for anesthetizing mice for imaging.

Animal weight (g)	Dose (ketamine HCl:acepromazine malieate: butorphanol tartrate)
<15	150 μl Anesthetic cocktail (1.2 mg:15 μg:1.5 μg)
15−20	200 μl Anesthetic cocktail (1.6 mg:20 μg:2.0 μg)
20−25	250 μl Anesthetic cocktail (2.0 mg:25 μg:2.5 μg)
>25	300 μl Anesthetic cocktail (1.4 mg:30 μg:3.0 μg)

TABLE 3

Troubleshooting table.

Step	Problem	Possible reason	Proposed solution
1: Anesthetic dosing	Mouse appears fully anesthesized	Overdose of anesthetic	Reduce dosing to account for variety in mouse strains
7: Heating mice	Vein not visible	Underheating	Heat tail longer
	Tails fall off or the mouse dies	Overheating	Watch mouse closely; mouse should slowly move around cage
8: Loading mouse into restrainer	Mouse not breathing	Mouse wedged into restrainer	Do not cover nose of mouse
11: Hydrodynamic injection	Injection takes longer than 10 s	Injection too slow	Omit mouse from test group
	Blown tail vein	Poor placement of needle; injection too rapid	Omit mouse from test group
	Mouse suffers cardiac arrest or develops fluid in its lungs	Unknown	Massage chest to assist labored blood flow and/or help clear lungs
14: Recovery	Death	Hypothermia	Place subsequent mice on heating pad set on low heat until they are moving
	Troubled breathing	Unknown	Massage chest
17: Imaging	Mouse dies	Overdose of anesthetic	Reduce dose in subsequent mice to account for strain differences
	Mouse is too active for imaging	Underdose of anesthetic	Increase anesthetic. The mouse may need to be taped down
21: Imaging	Signals not distinct	Overexposure	Reduce exposure time Image only one mouse at a time