Efficient phiC31 integrase-mediated site-specific germ-line transformation of *Anopheles gambiae*

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ABSTRACT

Current transgenic methodology developed for mosquitoes has not been applied widely to the major malaria vector *Anopheles gambiae*, which has proved more difficult to genetically manipulate than other mosquito species and dipteran insects. In this protocol, we describe phiC31-mediated site-specific integration of transgenes into the genome of *An. gambiae*. The phiC31 system has many advantages over "classical" transposon-mediated germ-line transformation systems because it allows integration of large transgenes at specific, characterised genomic locations. Starting from a general protocol, we have optimized steps from embryo collection to co-injection of transgene-containing plasmid and *in vitro* produced phiC31 integrase mRNA. We also provide hints for screening transgenic larvae. The outlined procedure provides robust transformation in *An. gambiae,* resulting in homozygous transgenic lines in approximately 2 to 3 months.

INTRODUCTION

Anopheles gambiae is among the most studied non-model organism for which molecular and genetic tools have been developed over the last two decades. This is due to its role as the main African vector of *Plasmodium falciparum*, the most deadly human malaria parasite.

Since the establishment of the *An. gambiae* genome sequence in 2002 an abundance of proteomic, transcriptomic and genome wide association data have been produced to try to understand the intimate relationships between the mosquito and *Plasmodium* parasites with the aim of identifying rational new means to control malaria transmission [1-5]. Other features of *Anopheles* biology relevant to disease transmission have also been intensively studied, including mating behaviour, sex determination, olfactory response and insecticide resistance [6-9].

An. gambiae has previously been perceived as intractable for functional genetic studies, largely because of reduced transformation efficiencies compared to other mosquitoes and the more complex husbandry required to maintain colonies. To surmount this, several researchers have used *Drosophila* or *Anopheles stephensi* as a recipient species to analyse the function of *An. gambiae* genes [10-14] as these species are more robust and amenable to genetic transformation, and in the case of *Drosophila*, possess a complex genetic toolset. However, these heterologous systems have their limitations, since presumed orthologous genes may behave differently in *An. stephensi*, or in *Drosophila*, which is a non-blood feeding diptera [10,15].

In parallel, transient RNAi was demonstrated as an alternative for functional analysis in *An. gambiae* [16]. This methodology has proved successful for silencing gene expression in mosquito tissues encountered by *Plasmodium* parasites during its development in the mosquito [16-20]. However, this method only permits loss-of-function studies, is labour intensive, and offers little selectivity over which tissues are targeted for knockdown.

Transposon mediated germ-line transformation of *An. gambiae*

Soon after the first report of successful transformation in *An. stephensi* [21] and the discovery that the *piggyBac* transposable element could mediate germ-line transformation in many

different insect species, Grossman *et al.*[22] succeeded in producing *An. gambiae* transgenic mosquitoes. However, in contrast to several insect species, including mosquitoes such as *Aedes aegypti*, *An. stephensi* and *Anopheles albimanus,* transformation efficiency was much lower in *An. gambiae* and more technically challenging [22,23]. Since then, several modifications to the protocol have improved the efficiency considerably, as outlined in Lobo *et al.*[24]. Technical details for producing transposon-mediated transgenic mosquitoes including *An. gambiae* have been published by several groups [24-27].

The major attributes of transposon-mediated transformation are the inherent randomness of the integration site and the post-integration mobility of transposons allowing these approaches to be used for mutagenesis screens, including 'classic' exon and enhancer trapping strategies, as reported in *An. stephensi* by O'Brochta *et al.* [28,29]. Transposons can be used to compare gene expression at multiple genomic sites [30]. Conversely, these characteristics can be severe drawbacks for other types of functional genetic analysis. Non-defined integration sites lead to position effect variegation and renders phenotypic comparison between transgenic lines difficult without the generation, characterization and maintenance of multiple independent homozygous lines [26,31]. This initial characterisation can be extremely labour intensive involving the generation of multiple isoparental lines. The randomness also means that apparent phenotypic differences may relate to mutagenesis at the insertion site rather than transgene function, which complicates analysis. Furthermore, the generation of lines with multiple sites of transgene integration within a genome is common, which again hinders phenotypic studies [26]. To overcome some of these problems, the phiC31 system has several characteristics that are advantageous for many functional genetic applications.

Overview of the phi C31 system in *An. gambiae*

The phiC31 system uses the properties of the site-specific integrase from the bacteriophage phiC31, which mediates recombination between an attachment site (attB) that is normally present in the genome of *Streptomyces lividans*, and an attachment site (attP) present in the circular genome of the bacteriophage phiC31. Recombination between the two att sites integrates the phage genome and forms two hybrid sites, attL and attR that are not targets for the phiC31 integrase, thus the integration event is irreversible[32].

To establish this system *de novo* in any organism, the first step is to integrate an attP site into the genome. In *An gambiae*, this was initially performed using attP tagged *piggyBac* transposons, that harbour a fluorescent marker gene under the control of the synthetic eye/nervous system-specific 3xP3 promoter, whose expression is easily visualised in mosquito larvae [33] (Figure 1 A). The resulting transgenic lines are called "docking strains". The docking strains need to be characterised for homozygous fitness, to ensure line robustness, as well as the presence of single chromosomally integrated attP sites, to enable the production of phase two mosquitoes, described below, with integrase-mediated insertions at these unique, identical sites.

The second step is to co-inject embryos from the docking strain with a transgene carried on a plasmid containing an attB site, and a source of the phiC31 integrase to promote site-specific recombination in the germ cells of the injected embryos (Figure 1B). In most studies, *in vitro* produced mRNA is used as a source of integrase [33,34], but plasmid templates have recently also proved suitable (A. L., unpublished ; S. Fuchs, T. Nolan & A.

Crisanti, unpublished) in mosquitoes. From this second step, also named phase two, establishment of homozygous lines is straightforward as virtually all transgenics will have an identical transgene at the same location and therefore mass mating can be performed. This is critical for *An. gambiae,* a mosquito species that mates in swarms. Increases in transformation efficiency may also be derived from only using G0 larvae that transiently express the marker gene to establish subsequent crosses, as our experience indicates that these are more likely to generate transgenic offspring (E. P. & N. P., unpublished).

Advantages of the phiC31-based transgenesis system in *An. gambiae*

The phiC31-based transgenesis system offers several advantages over other systems, which should be considered when adopting this technology. These include i) integration of transgenes at specific, characterised genomic locations, ii) insertion of large constructs (larger than 42 kb, the size of the phage phiC31 genome), iii) irreversible integration of the transgene, iv) the predominance of single gene insertions and vi) potential increases in transformation efficiency (see details in Fish *et al.*[35]). Furthermore, it provides a simplified mating procedure to identify transformants, which should all be identical. This is a considerable benefit for *An. gambiae* that is not easily amenable to single pair mating. Overall, the ability to mitigate position effects on transgene expression is likely to be of principal importance for most applications, allowing for more efficient comparison between transgenic lines during phenotypic characterisation[31].

In *D. melanogaster*, the efficiency of phiC31-mediated transgenesis is reported to be approximately 5-fold higher than that mediated by P element transposase[36]. However, in mosquitoes extensive comparisons have not been described [31,33,37]. The overall benefit of the development of the phiC31 system for producing transgenic *An. gambiae* has led to recent developments of novel tools in this mosquito including a synthetic homing endonuclease-based gene drive system [34] and the TALen system for targeted mutagenesis[38]**.**

Further advances could be obtained by generating a minimal set of docking strains that bear an attP site on each of the three chromosomes of *An. gambiae*. These would need to be selected from a large batch of *piggyBac*-derived attP carrying transgenics, to optimise fitness and efficiency of site-directed modification. These tools would aid the development of efficient conditional expression systems, including Gal4/UAS in *An. gambiae*. In addition to gain-of-function studies, such a system would also permit functional analysis of alleles by a combination of stable RNAi and *in vivo* complementation to alleviate the otherwise laborious process of allele-specific transient RNAi[18].

One of the limitations of *Anopheles* transgenesis in general is the lack of a method for convenient line preservation, such as cold or dry storage. This means that lines may have to be reared continuously. Although efforts need to be focused on producing a suitable line preservation system, the relative efficiency of phiC31 transformation combined with the ability to create lines with insertions in identical sites means that it is feasible to re-generate the lines *de novo* as required, rather than maintaining them for years.

Experimental design

Mosquito embryos Whatever the strategy chosen for producing transgenic *An. gambiae* mosquitoes, embryos will be exposed to stress at the time of injection from receiving exogenous nucleic acid material. Therefore, the quality of the embryos is paramount.

Beside specific care of the females from which embryos are collected, it is highly important to rear the larval juvenile stages to produce those mothers in optimal, healthy conditions. Particular care should be given to larval density and larval food access. Although not based on extensive testing, our experience leads us to highly recommend use of embryos produced from the first egg batch of young females.

Designing AttB containing plasmids The standard docking strains currently available harbour the ECFP marker gene. Therefore a second fluorescent marker gene, usually YFP or DsRed, is necessary for efficient differentiation and selection of transgenics harbouring the transgene of interest. To be able to screen transgenics at all post embryonic stages, the most convenient regulatory region to drive the selectable marker gene is currently the 3xP3 promoter, which yields expression in the eye and nervous system in mosquito larvae (Figure 2A). As the number of easily differentiated fluorescent proteins is not extensive the choice of markers needs to be carefully evaluated using published guides and based on the availability of suitable filter sets [39]. Several plasmids with an attB site associated with different fluorescent proteins under the control of the 3xP3 promoter have been already constructed for phiC31 integrase-mediated transformation of *Aedes* and *Anopheles* mosquitoes and are available from the respective laboratories [33,37,40,41]. Additional constructs developed for *Drosophila* might also be considered [42].

PhiC31 integrase source To establish the procedure for the phiC31 integrase-based transformation system in *Anopheles* as well as in *Aedes* mosquitoes [31,37,40], *Bombyx mori* [43] and *Drosophila* [35,36], *in vitro* transcribed mRNA was used as a source for phiC31 integrase. The use of mRNA is particularly suitable for non-model organisms, since it doesn't rely on specific promoters to drive expression of integrase in the germ-line. However, since integrase mRNA is prone to degradation if precautions are not adhered to, this can lead to variability in transformation efficiency [35]. An alternative way to provide the phiC31 integrase is to co-inject helper plasmid, which is inherently more stable than mRNA. The recent successful use of the pKC40 helper plasmid[44] in six independent experiments (A.L., unpublished) showed that this is efficient, although direct comparison of relative efficiency against mRNA has not been performed. The pKC40 plasmid carries the phiC31 integrase gene, with an additional carboxyl nuclear localization sequence, driven by the *Drosophila hsp70* promoter. The recent development of additional helper plasmids driving integrase expression under germ-line specific promoters such as *nanos* or *vasa* (S. Fuchs, T. Nolan & A. Crisanti, unpublished) may also contribute to overall increased convenience of the system.

Further sophistication of the *An. gambiae* system has been achieved [41] through the development of "self docking strains" that carry a genomic source of integrase similar to that described in *Drosophila* [42]. In these strains the phiC31 integrase is expressed specifically in the germ-line, therefore only the attB plasmid containing the transgene needs to be injected into the embryos, resulting in an increased survival rate up to 60%. This system has proved to be efficient [41], and although phase 2 integrations are stable, two options exist for the subsequent removal of integrase expression. Firstly, the integrase and fluorescent marker genes in the self-docking strains were designed to be excised using Cre-*loxP* following site-specific integration (J.M.M. & P.E., unpublished). Secondly, since the fluorescent marker is different in the self-docking strains (Figure 2B), these can be used as integrase 'driver' line, to provide integrase *in trans*, following a cross to an existing characterised differently marked standard docking strain. In the embryos derived from these crosses only the attB plasmid needs to be injected, and screening of a G0 outcross to

wild type will detect those transgenics only carrying the donor plasmid and not the integrase marker.

Here, we describe the step-by-step procedure for efficient site-specific transformation of *An. gambiae* using established docking strains. An outline is provided in Figure 3. The protocol includes the preparation of the embryos for injection, the *in vitro* synthesis of integrase encoding mRNA and the selection of transgenic mosquitoes up to the establishment of homozygous lines. We have emphasised the procedure for *in vitro* production of mRNA encoding integrase and the associated care as this procedure will likely be the entry point for phiC31-mediated transgenesis in other mosquito or insect species lacking refined molecular tools, such as a characterized promoter to drive efficient phiC31 integrase expression. In addition, we have included a video to document the delicate initial step of embryo alignment.

MATERIALS

REAGENTS:

- *Anopheles gambiae* attP docking strains or self-docking strains (see Table 1 for available strains)
- phiC31 integrase-containing plasmid (see REAGENT SETUP)
- Transgene-containing plasmid (see REAGENT SETUP)
- LB broth (cat. no.12780-052, Invitrogen)
- Ampicillin (cat. no. A9518-5G, Sigma Aldrich)
- RNAse away spray (cat. no. 7002, Molecular BioProducts)
- EndoFree Maxiprep purification kit (cat. no. 12362, Qiagen)
- Genomic DNA extraction kit (RedExtract-N-Amp™ Tissue PCR Kit, cat. no. X-NAT-1KT, Sigma)
- Ultrapure Agarose (cat. no. 16500-500 , Invitrogen)
- 50X TAE buffer (cat. no. 24710-030 , Invitrogen)
- Ethidium bromide (cat. no. GEPBET02-AF, EUROBIO) ! CAUTION Ethidium bromide is a mutagen. Wear a lab coat and protective gloves.
- 1Kb ladder (cat. no. N3232S, NEB)
- ssRNA ladder (cat. no. N0362S, NEB)
- *BamHI* Restriction enzyme (BamHI HF, cat. no. R3136S, NEB)
- Nuclease-free water (cat. no. 9932, Ambion)
- Ethanol 100% (absolute for analysis)
- 3M Sodium Acetate pH 5.5 (cat. no. 9740, Ambion)
- 0.5 M EDTA pH 8.0 (cat. no. 9269G, Ambion)

- *In vitro* RNA transcription kit (mMessage mMachine T7 ULTRA, cat. no. 1345, Ambion)
- RNA purification kit (MEGAclear kit, cat. no. 1908, Ambion)
- 0.22 µm-filtered 25 mM NaCl
- Halocarbon Oil 700 and 27 (cat.no H8898 & H8773, Sigma-Aldrich)
- microinjection buffer pH 7.2 (see REAGENT SETUP)
- Artificial sea salts (0.3 g/L, see MR4 Methods in *Anopheles* research, http://www.mr4.org/Publications/MethodsinAnophelesResearch.aspx)
- Tetramin baby fish food (Tetra)
- Deionized sterilized water for G0 embryo hatching
- 10% sucrose solution (any commercial food sugar supplier)

EQUIPMENT

- Laboratory gloves (eg. cat. no. XC-INT-M, Microflex)
- Double-sided sticky tape (cat. no. 415, 3M)
- 18 mm X 18 mm glass coverslips (eg. cat. no. BB018018A1, Thermo Scientific Menzel)
- Glass slides (eg. cat. no. AG00000112E01, Thermo Scientific Menzel)
- Fine forceps (N°5, Dumont)
- Fine paintbrush N°2/0 (1.3 mm) (eg. cat. no. 8400, Raphael)
- Whatman Filter papers for embryo alignment (cat. no. 1450-090, Whatman)
- Microinjection needles: Quartz micropipettes with filament (OD: 1.0 mm, ID: 0.70 mm) (cat. no. QF-107010, Sutter instrument) or Alumino-silicate glass capillaries with filament (OD 1.0mm, ID 0.64 mm) (cat. no. AF100-64-10, Intracel, Herts, UK) (see EQUIPMENT SETUP)
- Microloader pipette tips (cat. no. 5242956003, Eppendorf)
- Modeling clay
- Plastic Petri dish (diameter 90 mm ; for storing pulled needles and for G1 screening)
- Filter paper 100% cellulose for G1 screening (thickness 0,13 mm, filtration speed 50 sec, weight 64 g/m², retention 10-20 µm, any brand or Fiorioni- http://www.filtres-fioroni.com/)
- Transfert pipettes (cat. no. 225-1S, Samco Scientific)
- Microscope slides with wells (eg cat. no. 101432648EPOXY, Thermo Fisher)
- Natural Dry Cellulose Sponge N°4 (cat. no. 95030072, Spontex)
- 2L Popcorn boxes

- Hygienic Dental Dam Latex" (cat. no. H02146, Coltene/Whaledent)
- RNase free microfuge tubes 1.5 ml (cat. no. 12400, Ambion)
- 0.22 µm Microfilters (Ultrafree-MC sterile, cat. no. UFC30GVOS, Millipore)
- 50 ml Polypropylene Tubes (cat. no. 352098, Corning)
- Insectaries (26°C, 80% relative humidity, 12:12h light photoperiodicity)
- Black box or insectary with inverted day/night photoperiod
- Standard mosquito rearing material (see MR4 Methods in *Anopheles* research; http://www.mr4.org/Publications/MethodsinAnophelesResearch.aspx)
- Needle puller (Sutter P-2000, Sutter Instruments)
- Inverted microscope (eg. TS100 Eclipse, Nikon)
- Femtojet microinjector (Femtojet Express, Eppendorf)
- Joystick Micromanipulator (eg. Leica or MN-151 Narishige)
- Fluorescence stereomicroscope or macroscope (eg. AZ100, Nikon) with appropriate filter sets
- UV light to visualize gels (eg. Chemidoc, BioRad)
- Spectrophotometer (eg. ND-1000, Nanodrop)
- Oviposition chambers, custom-built (see EQUIPMENT SETUP)

REAGENT SETUP

Embryo harvesting Rear mosquitoes according to standard protocols with an adequate larval density:food ratio to obtain large and healthy mosquitoes that will emerge synchronously (MR4 standard protocol http://www.mr4.org/Publications/MethodsinAnophelesResearch.aspx). Approximately seven days before injection, prepare a large cage (30 x 30 x 30 cm) with freshly emerged adults (about 200 females and 100 males) and provide sugar (10% sucrose) *ad libidum*. Four days post emergence, ensure efficient blood feeding of females. Females will be able to lay suitable eggs on the 3rd and 4th days post-feeding. For embryo collection, place a small amount of deionized water in the cap of an oviposition chamber (see EQUIPMENT SETUP), place chamber on cap and introduce about 10 females through the dam (Figure 4). Place the chamber in the dark to allow females to lay eggs over a 20 minute period. Set up two oviposition chambers to ensure enough eggs for each injection series. Females are then removed and eggs left in the tube cap for 30 min in the insectary until they begin to melanize to light grey. At that stage they are suitable for alignment and injection [24,25] and are

transferred to the injection room conditioned at 18-20°C to slow down embryo development. Such embryos should be injected within 2 hours of laying to ensure optimal development of the embryos. After this time, chorion hardening may render the eggs difficult to inject. Under optimal rearing conditions, at least 250 eggs can be recovered from each chamber.
CRITICAL Females need to be inseminated for egg laying. If females do not lay eggs, control that females are inseminated by dissecting their spermathecae.

PhiC31 integrase-containing plasmid For *in vitro* transcription of mRNAs encoding phiC31 integrase, a plasmid containing the phiC31 integrase gene coupled with a RNA polymerase promoter is required. We use the original pET11phiC31poly(A) [35,36] provided by Pr Calos. Other transcription plasmids containing mutated and insect codon-optimized phiC31 integrase have been designed with the aim to increase transformation efficiency but such improvements have not been observed in *A. aegypti* or *An. stephensi* [14,31].

Transgene-containing plasmid The transgene has to be cloned into a recipient plasmid containing an attB attachment site plus a screening marker distinguishable from the one present in the docking strain. Several plasmids with an attB site associated with different fluorescent proteins under control of the 3xP3 promoter have been already constructed for phiC31 transformation of *Aedes* and *Anopheles* mosquitoes and are available from the respective laboratories [37,40,41]. Information on the phiC31 phage sequence can be found at the accession number AJ006589 and information on the 3xP3s synthetic promoter at the GeneBanK accession number AB779767.1 and at http://flybase.org/reports/FBtp0017474.html.

1X microinjection buffer pH 7.2 Add 0.1 ml of sodium phosphate buffer 0.1 M pH 7.2 and 1 ml of KCl 0.5 M to deionised water. Adjust volume to 100 ml. Filter-sterilize (0.22 μm), aliquot in small volumes and store at 4°C or -20°C.

Water-saturated Halocarbon oil Mix 9 ml Halocarbon Oil 700, 1 ml Halocarbon Oil 27 and 10 ml molecular biology grade water gently to avoid forming an emulsion. Leave to stand at room temperature (20°C-22°C) overnight to allow the Halocarbon oil to become water saturated.

EQUIPMENT SETUP

Oviposition Chambers Cut the bottom of 50 ml polypropylene tubes and cover this opening with a piece of dental dam latex. Make a small cut in the membrane to introduce mosquitoes.

A piece of lab latex glove is an alternative. Add deionized water to the cap of the polypropylene tube to serve as the oviposition site (Figure 4).

Pulling Needles for Microinjection We have used both quartz and alumino-silicate glass needles. Pull quartz needles using the following parameters on a Sutter P-2000: HEAT 750, FIL 4, VEL 40, DEL 150, PUL 165, which are similar to those described in Lobo *et al.*[24]. Pull alumino-silicate needles using the following parameters: HEAT 430, FIL 120, VEL 50, DEL 210, PUL 250. Parameters may need to be adjusted slightly depending on the accuracy of the alignment of individual P-2000 pullers and variation in needle batches. It is useful to pull a large number of needles to replace ones that break or get clogged during injections (usually about 5 needles per 150 embryos). Pulled needles are stored on modeling clay in a closed Petri dish at room temperature (RT: 20-22°C) until use. The tip of the needles is sealed during pulling but will usually open sufficiently when attempting to inject the first embryo.

! CRITICAL Although quartz micropipettes are more expensive than alumino-silicate glass needles and require the higher temperature of a laser puller, they perform better. Some researchers have also been successful in using commercial Femtotips[27]. Quartz needles are more resistant than other glass needles to damage often caused during the penetration of the hard embryo chorion. The presence of filaments increases flow, which aids the filling of the injection needle and solution flow while injecting.

! CRITICAL Wear gloves when pulling needles to avoid degradation of mRNA whilst injecting embryos if using mRNA as a source of integrase.

! CRITICAL If the needle shape after pulling is not reproducible; check first the quality of the needle batch. If the puller is not reproducible, refer to the puller set up instructions. It is critical to use and maintain the needle puller correctly to obtain pulled needles of reproducible quality.

Filling needles Back fill needles using 3-5 µl of plasmid or plasmid/mRNA mix using Microloader pipette tips (Eppendorf).

Injection slides and embryo alignment coverslips Tape two coverslips on a glass slide 1 cm apart. Place on the edge of each coverslip a piece of double-sided sticky tape, which will later receive the coverslip with aligned embryos. Prepare coverslips for collecting aligned embryos by placing one piece of double-sided sticky tape on each. Prepare an excess of small squares of filter paper with sharp scissors to minimize fibers (see Supplementary video 1). Injection slides and coverslips can be prepared in advance and stored in a dust-free environment.

Screening methods and fluorescence microscopy Screening of fluorescent larvae can be done with most fluorescence microscopes with appropriate filter sets to distinguish between

the different fluorescent markers [39,45]. For ECFP, a 421/456nm activation - 461/504nm emission filter set and for dsRed a 538/588nm activation - 600/652nm emission filter set can be used. We favour the use of a stereomicroscope or AZ100 Nikon macroscope, due to the convenience of handling specimens. There are multiple methods available to screen G1 larvae on mass, which all rely on reducing mobility of the larvae whilst keeping them moist, either by chilling or filtering, so that they can be viewed under a stereoscope and fluorescent transformants easily recovered. One simple method that can be used involves filtering on cellulose discs and preservation on damp sponges during manipulation. For this method, prepare 90 cm disks of 100% cellulose filter papers to fit into Petri dishes. Draw lines 0.5 cm apart to screen larvae by row (Figure 5). This will facilitate orientation under the microscope and enable the complete screening of large batches of larvae.

PROCEDURE

! CRITICAL **Steps 1-31 & 38-39** (i.e. up to embryo injection) have to be carried in an RNAse free environment when injecting standard docking strains with mRNA encoding the phiC31 integrase, to avoid RNA degradation. Clean the bench and pipettes with an RNAse away spray. Always wear gloves during preparation of mRNA, from establishing the culture to the final mix preparation. Use RNAse free microfuge tubes.

CRITICAL It is best to make fresh (less than 10 days) preparations of DNA and mRNA. We used to make the injection mix the week before injections. For long-term storage, store nucleic acid preparations in RNAse free 70% ethanol.

CRITICAL We recommend verifying the quality and identity of the plasmid to be injected at the relevant steps, as the whole process to obtain transgenic is 2 to 3 months.

Preparation of plasmid DNA carrying the transgene TIMING 3 days

1. Once the DNA of interest is cloned in an attB-recipient vector and its sequence verified, make a fresh transformation of the plasmid using standard protocols.

   ! CRITICAL STEP To obtain a good yield, it is necessary to make a culture from a freshly transformed clone.

! CRITICAL STEP We recommend using SURE2 cells (Stratagene) to avoid recombination during bacterial growth.

CRITICAL STEP If using mRNA as a source of integrase, **step 9** can be carried out in parallel to step 1-4.

2. The next day, grow a single colony in 1 ml of LB supplemented with 100 µg/ml ampicillin for 8 hours at 220 rpm 37°C with aeration.
3. Transfer 100 µl to a 1 liter flask containing 100 ml LB and 100 µg/ml ampicillin and grow overnight at 240 rpm 37°C with aeration.
4. Extract plasmid DNA with an endotoxin free purification kit according to the manufacturer's instructions. Elute DNA with 500 µl of pre-warmed nuclease-free water. Measure DNA concentration (250 to 500 µg should be recovered). Confirm identity of the plasmid by restriction enzyme digest.
5. Precipitate plasmid DNA with 0.1 volumes 3M sodium acetate and 2.5 volumes of 100% ethanol, 30 min at -20°C.

   CRITICAL STEP It is possible to aliquot DNA into smaller quantities before ethanol precipitation to avoid potential DNA degradation upon repetitive freezing and thawing.
6. Centrifuge at 15,000g for 15 min at 4°C.
7. Wash the DNA with 1 ml of 70% ethanol (100% ethanol diluted in nuclease-free water).

PAUSE POINT Plasmid DNA can be stored in 70% ethanol at -20°C until use.

8. **(OPTIONAL)** If using self-docking strains follow Option A, if using helper integrase plasmid follow Option B. Otherwise, proceed directly to **step 9** to prepare integrase mRNA.

   Option A: Self-docking strains

   i. Centrifuge attB plasmid DNA from **step 7** at 15,000g for 5 min, 4°C.
   ii. Air-dry the DNA pellet for 5 min and resuspend at about 300 ng/µl in 1X injection buffer.
   iii. Measure the DNA concentration to ensure no DNA has been lost during the precipitation. Check also the DNA purity by measuring OD 260/280, which should not be less than 1.8.
   iv. Spin again at 15,000g for 5 min to remove any particulates. The DNA is now ready for injection at **step 39**.

   Option B: Helper plasmid.

   i. Centrifuge attB plasmid DNA from **step 7** at 15,000g for 5 min, 4°C. Air-dry the DNA pellet for 5 min and resuspend at about 1 µg/µl in nuclease-free water.
   ii. Mix plasmids at a ratio of 7:3 attB plasmid:helper plasmid.

iii. Precipitate DNA as described in **steps 5-7**.

iv. Centrifuge DNA mix at 15,000g for 5 min, 4°C.

v. Air-dry the DNA pellet for 5 min and resuspend the DNA mix in 1X injection buffer at a final concentration of 500 ng/µl.

vi. Measure the DNA concentration to ensure no DNA has been lost during the precipitation. Check also the DNA purity by measuring OD 260/280, which should not be less than 1.8.

vii. Spin again at 15,000g for 5 min to remove any particulates. The DNA is now ready for injection at **step 39**.

mRNA phiC31 integrase synthesis TIMING **4-6 days**

9. Transform pET11-PhiC31polyA integrase-containing plasmid, establish cultures, extract plasmid DNA and confirm plasmid identity as described in **steps 1-4**.

PAUSE POINT The DNA can be stored at -20°C until required.

10. Linearize 40 µg of pET11-PhiC31polyA plasmid with BamHI HF at 37°C overnight as recommended by the manufacturer but using RNASE-free water as a diluent.
11. Next morning, check for digestion completion by running an aliquot (500 ng) on a 1% agarose gel in 1X TAE buffer; a unique band at 8kb is expected.
12. Stop the digestion and precipitate the digested plasmid by adding 1/20 volume 0.5 M EDTA, 1/10 volume 3M Sodium Acetate and 2 volumes of 100% ethanol. Leave 30 min at -20°C.
13. Centrifuge at 15,000g 15 min, 4°C.
14. Rinse the pellet with 1 ml of 70% ethanol (100% diluted in RNAse-free water).
15. Centrifuge at 15,000g, 5 min, 4°C.
16. Remove the supernatant and air-dry the DNA pellet before addition of 40 µl of RNAse-free water. Verify that DNA concentration is around 1 µg/µl using a NanoDrop and run an aliquot of the purified linear template on an agarose gel for quality control.

PAUSE POINT The linear DNA can be stored at -20°C until required.

17. Perform *in vitro* phiC31 integrase mRNA synthesis using the T7 ULTRA mMessage mMachine kit following the manufacturer's instructions. To inject approximately 2000 embryos, about 250 µg of mRNA is required. This amount can be obtained from 4 reaction tubes, each using 1 µg of the digested T7 phiC31 integrase DNA template from **step 16**. Incubate at 37°C for at least 4 hours (can safely be left overnight).

18. **(OPTIONAL)** Remove the DNA template using TURBO DNAse (included in the *in vitro* mRNA synthesis kit) and add a poly(A) tail to the mRNA according to the manufacturer's instructions. Although inclusion of this step adds to the overall time, it results in higher purity and stability of the mRNA and might increase the number of site-specific integration events.

CRITICAL STEP Remove 2.5 µl per RNA sample before adding the poly(A) polymerase and keep on ice; this control will be run on a gel next to the tailed mRNA after mRNA purification at **step 20**.

CRITICAL STEP Always keep the mRNA on ice and proceed directly to purification (**step 19**).

19. Purify the mRNA using the MEGAclear kit following the manufacturer's instructions. Use one column per two mRNA reactions. Elute each column with 2x50 µl of supplied elution solution prewarmed at 95°C. Pool the 200 µl eluted mRNA and keep on ice.
20. Measure mRNA concentration (200 to 260 µg should be recovered). Verify mRNA quality on an agarose gel run at 50V; before loading, denature 2.5 µl of mRNA (untailed mRNA from **step 18** and tailed one) and 3 µl of ssRNA ladder in ssRNA buffer following manufacturer's instructions. The untailed phiC31 integrase mRNA should be 1840 bp while the tailed one, if **step 18** was performed, should be over 2000 bp.
21. Precipitate the mRNA with 5M ammonium acetate supplied in the MEGAclear kit following manufacturer's instructions.

PAUSE POINT mRNA can be stored when in 70% ethanol (100% ethanol diluted in nuclease-free water) at -20°C. After this step, there is no other pause point until storage of the injection mix at **step 31**.

22. Recover the precipitated mRNA by centrifugation, 15,000g for 15 min, 4°C, rinse with RNAse-free 70% ethanol. Resuspend the air-dried pellet in 200 µl of nuclease-free water prewarmed at 65°C and keep the tube on ice.

    ! CRITICAL STEP Do not air dry the pellet too much as it will be difficult to resuspend.
23. Determine mRNA concentration to verify full recovery of mRNA after precipitation. Check the mRNA purity by measuring the OD 260/280, which should be about 2.0. Proceed directly to injection mix preparation.

Preparation of DNA-mRNA mix for injection TIMING 1 to 2 days

24. Centrifuge plasmid DNA from **step 7** at 15,000g for 5 min, 4°C.
25. Air-dry the DNA pellet for 5 min and resuspend at about 1 µg/µl in nuclease-free water.
26. Measure the DNA concentration to ensure no DNA has been lost during the precipitation. Check also the DNA purity by measuring OD 260/280, which should not be less than 1.8.
27. According to the concentration of the purified plasmid DNA (from **step 26**), add an adequate volume of plasmid DNA to the purified mRNA to recover the recommended concentration after co-precipitation in 1X injection buffer. Plasmid DNA is injected at 250 ng/µl (if plasmids are under 12 kb) and mRNA encoding phiC31 integrase at 800 to 1000 ng/µl.

! CRITICAL STEP When using larger plasmids, we observed a decrease in transformation efficiency. To increase efficiency, increase DNA concentration up to 350 ng/µl. However, high concentrations can clog needles. This can be limited by a larger needle tip; however, a larger tip will result in a decrease in embryo survival [24].

28. Precipitate the DNA-mRNA mix with 5M Ammonium Acetate (supplied in the MEGAclear kit) following manufacturer's instructions. After the 70% ethanol wash and centrifugation, resuspend the air dried pellet in 1X injection buffer prewarmed at 65°C and keep the tube on ice.

! CRITICAL STEP Do not air dry the pellet too much as it would be difficult to resuspend.

29. Filter the mix with a 0.22 µm microfilter at 13,000 g for 5 min at 4°C.
30. Verify injection mix quality on an 1% agarose gel run at 50V, running side by side: 3 µl of denatured filtered injection mix, 500 ng of plasmid DNA preparation from **step 26** and 3 µl of ssRNA ladder in ssRNA ladder buffer. The plasmid DNA should present 2 bands corresponding to different types of circular DNA structure and the mRNA should be over 2 kb.
31. Aliquot 15 µl samples of the injection mix and store on ice. Small aliquots are recommended as mRNA production is expensive and mRNA sensitive to degradation.

PAUSE POINT Store aliquots at -80°C until embryo injections. The mix should not be refrozen once thawed.

Embryo alignment for microinjection TIMING 10-20min/ batch of 50 embryos

CRITICAL: Methods for aligning *Anopheles* eggs have been well described [24-27,46]. Our procedure is very similar to those described in [24,26]. As the method is intricate, we provide a video (See Supplementary video 1).

CRITICAL For alignment and injections, condition the room at 18-20°C to slow down embryo development.

32. Place a small square of filter paper on a glass slide and add a drop of 25 mM NaCl to moisten the paper, leaving a fine line of buffer around the edges. Ensure no air bubbles are trapped between the paper and the glass slide.
33. Deposit a drop of 25 mM NaCl on the glass slide near to the edge of the filter paper.
34. With a fine paintbrush, transfer about 50 embryos into the drop of NaCl. Align embryos against the filter paper- which should be wet enough to ensure the embryos are in contact with the solution, but not so wet that it attracts them. Align 30 to 50 eggs with the anterior pole (the larger one) against the paper and with the dorsal side (flattened one) facing up.

! CRITICAL STEP Do not let the embryos become dry. If needed, add drops of 25 mM NaCl to the paper to maintain moisture as required.

35. Once embryos are aligned, absorb the solution from the wet filter gently but quickly with fresh filter paper. This will allow the embryos to partially desiccate.
36. Remove the alignment filter paper gently and apply the coverslip to glue the embryos to the tape. Turn over the coverslip and immediately cover embryos with a drop of 25 mM NaCl or Halocarbon oil.

CRITICAL STEP Both 25 mM NaCl or Halocarbon oil have been used successfully for *An. gambiae* transformation [33,47]. Use of Halocarbon oil has the advantage of improved visibility of both the embryos and needle tip, and increases the delimitation of the periplasm space and yolk in the embryos. However, its use requires an additional step of oil removal to ensure high survival rate of the injected embryos.

37. Position the coverslip on the injection slide (see EQUIPMENT SETUP), and transfer it to the microscope stage for injection. To inject a large number of eggs, it is convenient to work in team of two persons, one aligning and one injecting. This way, it is possible to inject continuously.

CRITICAL STEP The quality of eggs is of great importance for transformation success. We select embryos on several criteria as described by Lobo *et al.* [24] to maximize survival after injection. White or yellowish eggs, eggs that are too dark, small or thin eggs, and those with visible defects in the chorion or in their morphology, are discarded. Moreover, we try to align

eggs that are at a similar melanization stage, which facilitates injecting as they will have similar chorion hardness. The level of desiccation is also critical. Under-desiccated embryos will expel yolk after the injection needle is removed and are likely to die ; over-desiccated embryos will be soft and difficult to inject and are more likely to sustain serious damage. A set of aligned un-injected embryos can be used as a quality control for hatching rate, which should be of 95-100% leading to adult emergence rate of about 80% in optimal conditions.

Embryo microinjection TIMING **4 to 8 days**

CRITICAL Injections are carried out at a 200X magnification as previously described [24,25].

38. Thaw an aliquot of the injection mix on ice. If injecting a self-docking strain, use the injection mix from **step 8A**. If using a helper plasmid as the source of integrase, use the injection mix from **step 8B**. If using mRNA as an integrase source, use the injection mix from **step 31**.
39. Fill a pulled needle (see EQUIPMENT SETUP) with 3 to 5 µl of injection mix and mount it on the micromanipulator.
40. Focus on the embryo floats and gently position the needle until it is visible in the same optical field. The angle of the needle from the horizontal coverslip plane should be between 15 and 30 degrees.
41. Set up the parameters of the Femtojet Express (constant pressure Pc and injection pressure Pi). They should be adjusted depending on the capillary size, the embryo batch and the state of maturation and desiccation. Generally, Pc is around 1000 HPa and Pi varies between 2000-3000 HPa. If injecting under Halocarbon oil, the droplet size can be monitored between injections by injecting into the oil and adjusting the FemtoJet parameters to give the required droplet size. If the needle is very thin and properly broken, injections can be made by using the CLEAN function, and no resistance should be felt during chorion penetration.
42. Inject embryos on their ventral side, which faces up, at the posterior pole (the thinner one), between the posterior of the floats and the posterior pole of the embryo. Inject as much mix as the embryo can support without inflicting damage. Inject as many eggs as required.

! CRITICAL STEP Do not inject in the terminal end of the posterior pole as it is harder and more difficult to penetrate with a needle. A useful drawing can be found in Lobo *et al.* [24]. Depending on manipulator preference, injection can be performed either by moving the

microscope plate or by moving the needle using the joy stick of the micromanipulator. The tip of the needle should open on first contact with an embryo.

CRITICAL STEP To prevent clogging and to check that the needle isn't clogged, use the CLEAN function between each injected embryo. Change needles that cause excessive embryo damage. An embryo that continues to lose yolk material from the wound site will not survive.

TROUBLESHOOTING

43. Submerge the coverslip supporting injected embryos in a dish with about 1 cm of deionized-sterilized water supplemented with artificial sea salts. If injecting under Halocarbon oil, position the coverslip vertically to allow the oil to flow away from the eggs.

! CRITICAL STEP Do not add food until larvae hatch to avoid microbial development that might impair embryo recovery from injection.

44. Discard the remaining thawed injection mix at the end of the injection day. Transfer the vessels containing the injected eggs to insectary conditions until larvae hatching (48h to 72h post-injection).

    CRITICAL STEP Using this protocol, a hatching rate of 25-50% can be obtained using the standard docking strains and up to 60% with the self-docking strains, in which less amount of nucleic acid are injected.

Rearing injected embryos to 3rd or 4th instar larvae TIMING 8 to 9 days

45. Collect newly hatched larvae daily and transfer them to fresh rearing trays with clean water supplemented with salts. Ensure larvae of the same developmental stage are placed in the trays.

TROUBLESHOOTING

46. Carefully control larval density and food as larval health has a strong impact on adult size and fecundity (MR4, chapter 2). Feed larvae with Tetramin baby fish food from the 1st to 2nd instar. Then, rear larvae as recommended in MR4 standard protocols (MR4, chapter 2.4.4) up to the 4th instar. In optimal conditions, no or very few larvae will die between the 1st and the 4th instar.

Screening transient fluorescence in generation 0 (G0) larvae TIMING 1 day

CRITICAL A good indicator for successful transformation is the presence of G0 larvae with transient fluorescence due to episomal presence of injected DNA plasmid. Transient fluorescence can be observed in the posterior region of larvae (Figure 6). From recent experiments we observed that more transformants are recovered from progeny of G0 larvae expressing transient fluorescence than from progeny of G0 larvae that do not (E.P.& N.P., unpublished). Therefore breeding only from G0 larvae expressing transient fluorescence can save time and space for G1 production, rearing and screening.

47. Screen transient fluorescent G0 larvae by placing individual larvae in wells of an 8 well glass slide, and observe fluorescence under the stereomicroscope. Minimize the amount of water in each well without drying the larvae, to facilitate rapid screening. Following this protocol, transient fluorescence can be observed in about 30 to 50% of larvae depending on injection series.

TROUBLESHOOTING

48. Transfer larvae expressing transient fluorescence to the rearing tray with their previous rearing water and rear them until pupation which will occur over the two following days.

Crosses of G0 adults and rearing progeny (generation 1, G1) TIMING 1 month

49. When G0 adults emerge, collect males and females daily and place in two separate cages. Collecting newly emerged mosquitoes daily ensures that females remain virgin. Alternatively, sexing and separation can be performed at the pupal stage. In parallel, collect virgin males and females from the docking strain of the same age as the G0 adults.

50. Set up backcrosses of G0 males to virgin females from the docking strain 3 to 5 days after emergence, when males are sexually mature. Make pools of 10 G0 males plus 50 virgin females from the docking strain to optimize mating opportunities of each male. Allow at least 4 days for mating and then combine all G0 males and their corresponding mated females in a large cage.

    CRITICAL STEP To minimize space needed for cages, it can be convenient to use disposable 2 liter popcorn containers. The wild-type strain, rather than the docking strain, can be used for outcrossing if required to increase genetic diversity.

CRITICAL STEP As *An. gambiae* mosquitoes need large amounts of space to mate, use cages or boxes with a minimum volume of 2 liters for crosses as this will ensure a minimum of 75% mated females in 4 days.

51. Collect G0 females over a week and place them all together in a large cage (30 x 30 x 30 cm) and backcross to a 5-fold excess of mature virgin males (older than 3 days) from the docking strain.

CRITICAL STEP As *An. gambiae* mosquitoes need large amounts of space to mate, use cages or boxes with a minimum volume of 2 liters for crosses as this will ensure a minimum of 75% mated females in 4 days.

52. Blood-feed females from each cross (G0 males x docking females; G0 females x docking males) 4 to 5 days after having established crosses.
53. Harvest the first batch of eggs and provide a second and even a third blood-meal and collect eggs after each blood-meal to obtain additional progenies. In our experience, we have not observed transgenics in the 3rd batch, if none were present in the first two batches. However, under normal conditions, where transformants are observed in the first two batches, a 3rd batch allows more G1 positive larvae to be collected, helping with transgenic line establishment.
54. Control the G1 larval density and food quantities carefully to maximize larval survival as described in **step 46**.

Screening G1 transgenic larvae TIMING **4 to 6 days**

55. Collect 200 to 300 larvae, preferably L3-L4 stages, and place them on an 8 cm diameter circular cellulose paper on top of a moist sponge (Figure 5). Let the sponge absorb enough water that larvae do not move whilst keeping enough moisture to preserve the larvae.

    CRITICAL STEP Remove food from the water before pipetting larvae otherwise it will result in autofluorescence background during the screen.

56. Transfer the filter paper containing the larvae to a Petri dish under the fluorescence of a stereomicroscope to select transgenic larvae.

! CRITICAL STEP We frequently observe G1 larvae with fluorescence restricted to one eye or both eyes and that showed no expression in the anal papillae as expected from expression driven by the 3xP3 promoter (Figure 7). They are presumably false positive transgenic G1, as no stable transgenic progeny have been obtained from these larvae (E. P. & N. P., unpublished). Nevertheless, we noticed that presence of such larvae is correlated with finding genuine transgenic larvae in the same G1 batch. In addition, we observed this type of larvae only in G0 female progeny (E. P. & N. P., unpublished), suggesting that the false positive

signal might be generated from the injected DNA plasmid maintained as an episome from the injected embryo over one generation.

TROUBLESHOOTING

57. Carefully transfer transgenic G1 larvae with a fine paintbrush back to small trays filled with their previous rearing water. Add a small amount of food and rear normally until adult emergence.

Establishment of homozygous transgenic lines TIMING 2 months

58. At G1 adult emergence, separate males and females into two different cages and backcross them when sexually mature utilizing a 20-fold excess of the opposite sex from the docking strain.
59. Blood-feed the cages two or three times to maximize the numbers of transgenic mosquitoes obtained. In the G2 generation, select only fluorescent larvae, which should represent 50% of total larvae.
60. From a subset of fluorescent larvae extract genomic DNA using standard methods[47] and confirm by PCR the presence of the canonical phiC31 recombination sequences attL and attR (see Table S1 for primers and PCR parameters) and the presence of transgene (using transgene-specific primers). At this stage, fluorescent larvae are heterozygous and should be positive for attP, attL and attR. It is advisable to verify the integrity of the transgene by PCR cloning and sequencing.
61. Intercross G2 positive heterozygous mosquitoes and blood-feed them.
62. Select G3 homozygous larvae on the basis of higher fluorescence intensity than that of heterozygous larvae. At this stage, about 25% of larvae should be homozygous for the integrated transgene if it has no harmful effect.

! CRITICAL STEP Keep an amplified back-up of the heterozygous transgenic mosquitoes until establishment of the homozygous line. Moreover, try to keep at least two independent pools derived from the transgenic G1 mosquitoes (progeny from G0 males and G0 females for example). PhiC31 integrase is a site-specific transformation system and in our experience with *An. gambiae*, we have not yet observed non-specific integration. However, insertions into pseudo-attP sites cannot be excluded as they are documented in mammalian and *Drosophila* cells as well as in the mosquito *A. aegypti* [36,40,48]. Maintaining two independent heterozygous lines should ensure that one of the lines contains the insert at the expected site.

63. Extract DNA from the leg of each G3 mosquito to be used for intercrossing using the RedExtract-N-Amp tissue genomic DNA extraction kit according to the manufacturer's instructions- except that volumes are 5-fold reduced. Perform PCR on

the extracted DNA as described in **step 60** ; homozygous mosquitoes should be attL and attR positive but attP negative.

64. Set up inter-crosses with G3 homozygous adult mosquitoes and blood-feed to obtain a homozygous line in G4.

65. Check that the line is homozygous in subsequent generations by fluorescence and by PCR.

TROUBLESHOOTING

Troubleshooting advice is provided in Table 2.

Table 2. Troubleshooting

Step	Problem	Possible reason	Solution
Step 42	The embryos loose too much yolk	Embryos are not desiccated enough	Dry well the paper against the eggs after alignment before adding NaCl or halocarbon oil
		Too much mix is being injected	Decrease Pi or Ti
		The needle tip is too large	Replace the needle
		The eggs are too young	Wait the eggs become grey before aligning
	The embryos are difficult to penetrate because they are soft or "rubbery" in texture	Embryos have been over desiccated	Rehydrate more quickly the eggs after desiccation
		Embryos are too old	Inject a new batch of eggs
	Needles clog continuously	The mix may contain particulates.	Centrifuge the mix at top speed 5 min before pipetting
		Embryos may need more desiccation	Dry well the paper against the eggs after alignment before adding NaCl or halocarbon oil

Step 45	Hatching G0 larvae stuck in the egg case	Too much pressure is applied when transferring the aligned embryos onto the coverslip	Apply less pressure when transferring the aligned embryos onto the coverslip
Step 47	Few transient fluorescent G0 larvae are found	The injection mix is not enough concentrated	Ensure the injection mix is correct
		The mix does not enter in embryo cells	Attempt injecting into the yolk, rather than into the periplasmic space
	Weak transient fluorescence in G0 larvae	The injection mix is not concentrated enough	Increase the concentration of the injected plasmid DNA
Step 56	No transformants when the G0 transient fluorescence was weak	Not enough nucleic acids are delivered to embryos	Control the quality of the mix. Increase nucleic acid concentration of the injection mix
	No transformants when the G0 transient fluorescence was good	The rearing conditions are not optimal	Control your rearing conditions to maximize mosquito survival and fecundity
		The transgene may be toxic	Attempt injecting a neutral transgene

TIMING

Steps 1-7, Preparation of plasmid DNA carrying the transgene: 3 d
Step 8, Preparation of DNA injection mix if using self-docking strains or helper plasmid: Optional
Steps 9-23, mRNA phiC31 integrase synthesis: 4-6 d
Steps 24-31, Preparation of DNA-mRNA mix for injection: 1-2 d
Step 32-44, Embryo alignment and microinjection: 4-8 d
Steps 45-48, Rearing injected embryos and screening transient fluorescence: 10 d
Step 49-54, Rearing, crosses of G0 adults and progeny: 1 m
Step 55-57, Screening G1 transgenic larvae: 4-6 d
Step 58-65, Establishment of homozygous transgenic lines : 2 m

ANTICIPATED RESULTS

Using this protocol with the standard docking strain E (see Table 1), injection of roughly 2000 embryos should be sufficient to produce a transgenic line (assuming no toxicity of the expressed transgene), with a mean embryo survival rate of approximately 25%. This can be

achieved in 2-4 days of injections, depending on the experience of the injector and the help of a second person to align the eggs. With the self-docking strains, injection of 1000 to 1500 embryos is usually sufficient for obtaining a transgenic line with an embryo survival rate around 55 to 60%. Overall, the production of a single transgenic line will take roughly two to three months. However, injecting two different transgenic constructs in parallel over one-two weeks is manageable. This will allow two transgenic lines to be obtained almost simultaneously two-three months later.

AUTHORS CONTRIBUTION STATEMENTS

EP, NP, JM, AL, GL performed the research, CB, GL, PE designed and supervised the research, KV supervised the research, EP, AL, GL, PE and CB wrote the manuscript.

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The authors declare they have no competing financial interests.

FIGURE LEGENDS

Figure 1. Schematic representation of phiC31 site-specific transformation in *An. gambiae*. (A) Description of the initial phase to create standard docking strains containing an attP site in their genome. In *An. gambiae*, these strains have been produced by co-injecting the *piggyBac* transposase helper plasmid and a *piggyBac* transposon containing an attP site and a ECFP fluorescent marker gene under the control of the eye /nervous system-specific 3xP3 promoter. (B) The second phase is injecting into attP-containing embryos a plasmid containing an attB site, a fluorescent marker gene different from the one in the docking strain and the transgene along with a source for phiC31 integrase to mediate site-specific

recombination at the attP site. After recombination, attL and attR sites, insensitive to the integrase, are formed. As outlined in the main text, the source of integrase can be *in vitro* transcribed mRNA[33] or a helper plasmid (A. L., unpublished). In recently developed self-docking strains[41] the integrase is produced in the attP-containing embryo germ-line, and only the attB plasmid is injected into the embryos.

Figure 2. Fluorescent profile of larvae from standard docking strain and self-docking strain. (A) 4th instar larva of a standard docking strain associated with ECFP under control of the 3xP3 promoter[33]. (B) Larva of a self-docking strain associated with dsRed2 fluorescent marker under control of the constitutive promoter IE1[41].

Figure 3. Timeline and flow diagram for site-specific phiC31 transformation in *An. gambiae* mosquitoes. Timing and steps for the procedure are outlined whether using a standard docking strain or a self-docking strain.

Figure 4. Oviposition chamber. The bottom of a 50 ml polypropylene tube has been cut and covered by a dental dam with a slot to introduce mosquitoes. The inverted orange cap is half-filled with deionized water to allow females to oviposit.

Figure 5. G1 screening system. About two hundred 3rd to 4th instar larvae are transferred on a cellulose paper on top of a wet sponge. The paper is then transferred to a Petri dish for fluorescence screening.

Figure 6. G0 larvae expressing transient fluorescence. Embryos of the standard docking strain E (see Table 1) were injected with attB plasmids encoding (A) EYFP, or (B) DsRed2nls, each under control of the 3xP3 promoter. As the nucleic acids are injected into the posterior end of embryos, only the posterior cells where 3xP3 expression occurs are fluorescent in hatching larvae.

Figure 7. False positive G1 larvae. G0 embryos of an ECFP docking strain were injected with an attB plasmid containing EYFP under the control of the 3xP3 promoter. Some G1 larvae from G0 females express the EYFP screening marker in one eye (A) or two eyes (C), while the ECFP marker of the docking strain, also under the control of the 3xP3 promoter, is well expressed from eyes to anal papillae (B, D).

Table 1. *Anopheles gambiae* phiC31 docking strains and self-docking strains. This table provides the list of docking strains that are available for phase 2 integration. The name of the lines, chromosomal and genomic location (on the reference of PEST scaffolds) of the attP

insertion, associated promoter and fluorescent marker are shown (see references [33,41] for details).

Docking strain	PhiC31 source	Chromosome	Chromosomal location	Associated marker	Tested for phase 2
E	Injected	III	3R 31B 15801959	3P3-ECFP	yes
G	Injected	II	2L 22E 18922078	3P3-ECFP	no
H	Injected	II	2L 20A 1316139	3P3-ECFP	no
F	Germ-line	II	2L 23C 23661650	IE1-DsRed2	yes
Q	Germ-line	III	3L41A 15200903	IE1-DsRed2	yes

Table S1. Primers and PCR parameters to detect transgene site-specific integration.

Supplementary Video 1. ***Anopheles gambiae*** **embryo alignment for microinjection.**

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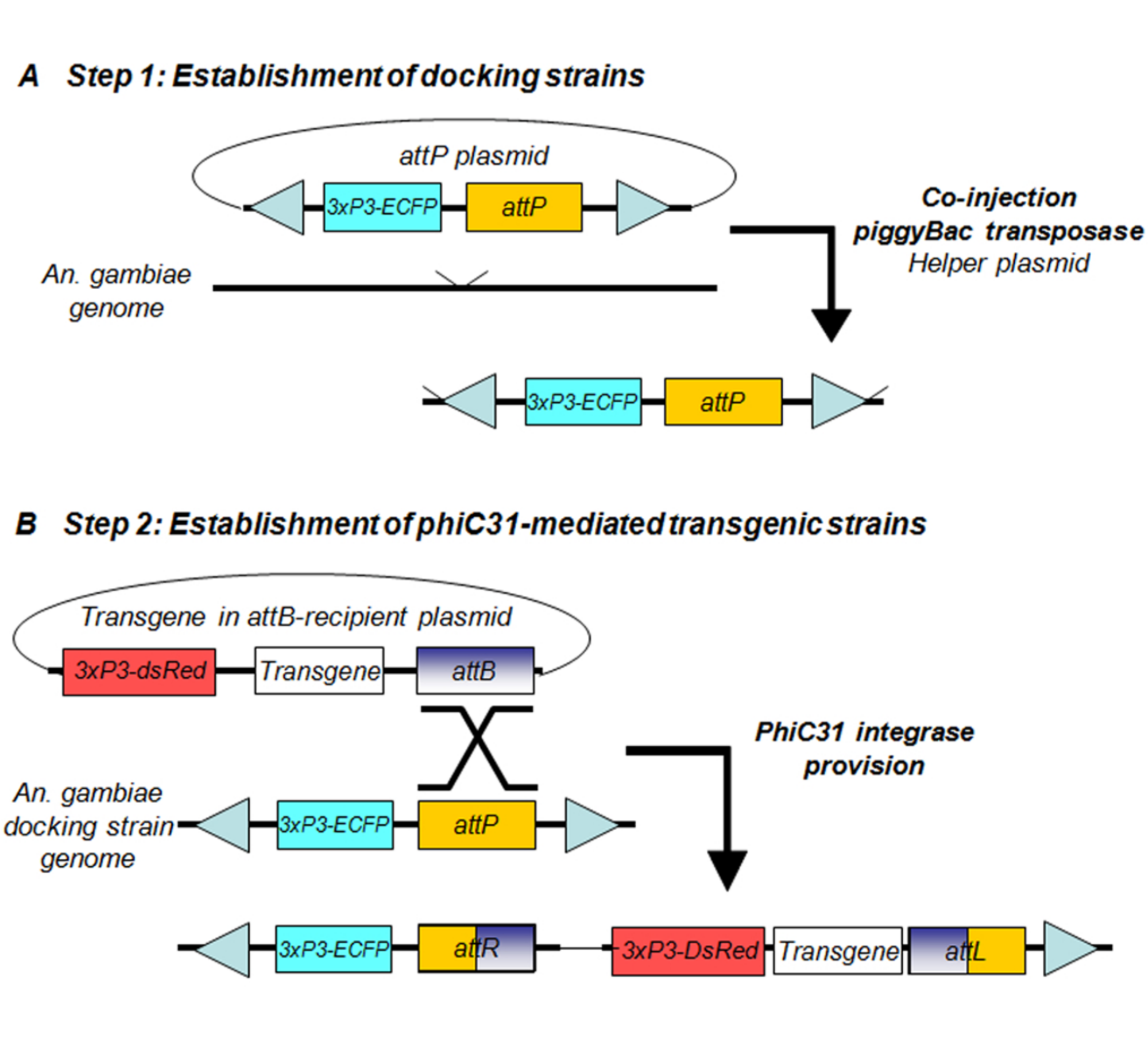
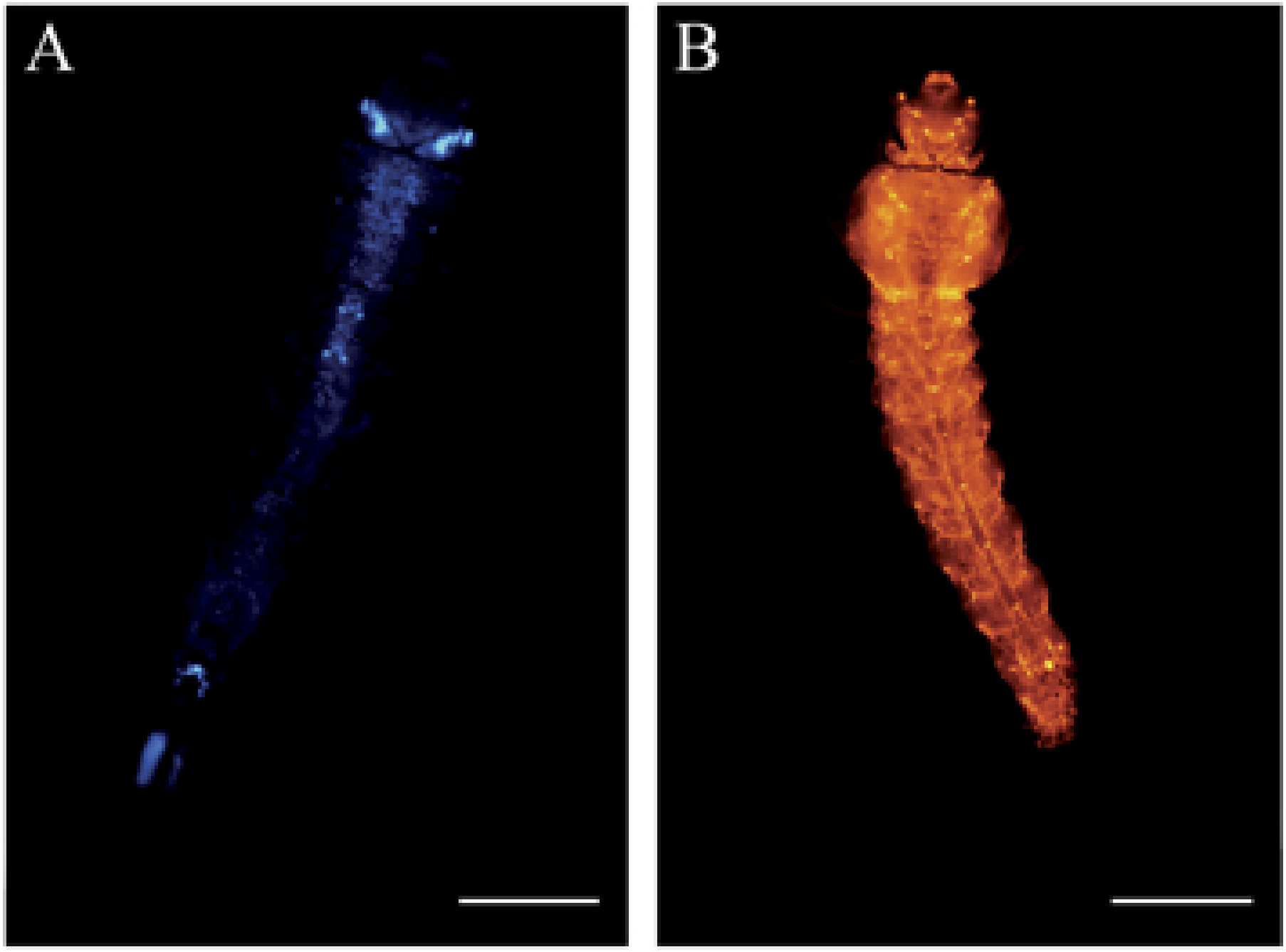

A Step 1: Establishment of docking strains
attP plasmid
3xP3-ECFP
attP
Co-injection
piggyBac transposase
Helper plasmid
An. gambiae
genome
3xP3-ECFP
attP
B Step 2: Establishment of phiC31-mediated transgenic strains
Transgene in attB-recipient plasmid
3xP3-dsRed
Transgene
attB
PhiC31 integrase
provision
An. gambiae
docking strain
genome
3xP3-ECFP
attP
3xP3-ECFP
attR
3xP3-DsRed
Transgene
attL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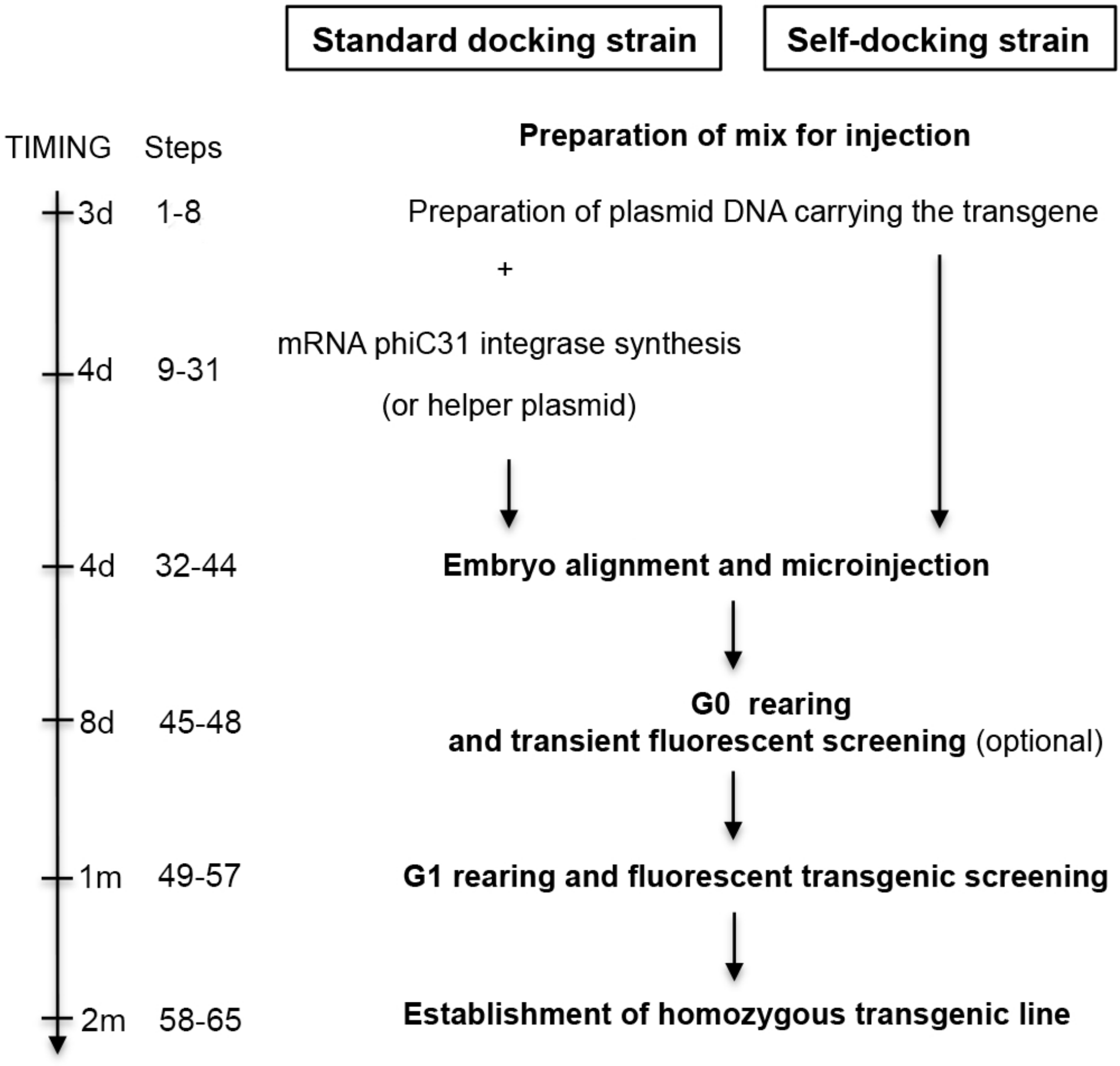
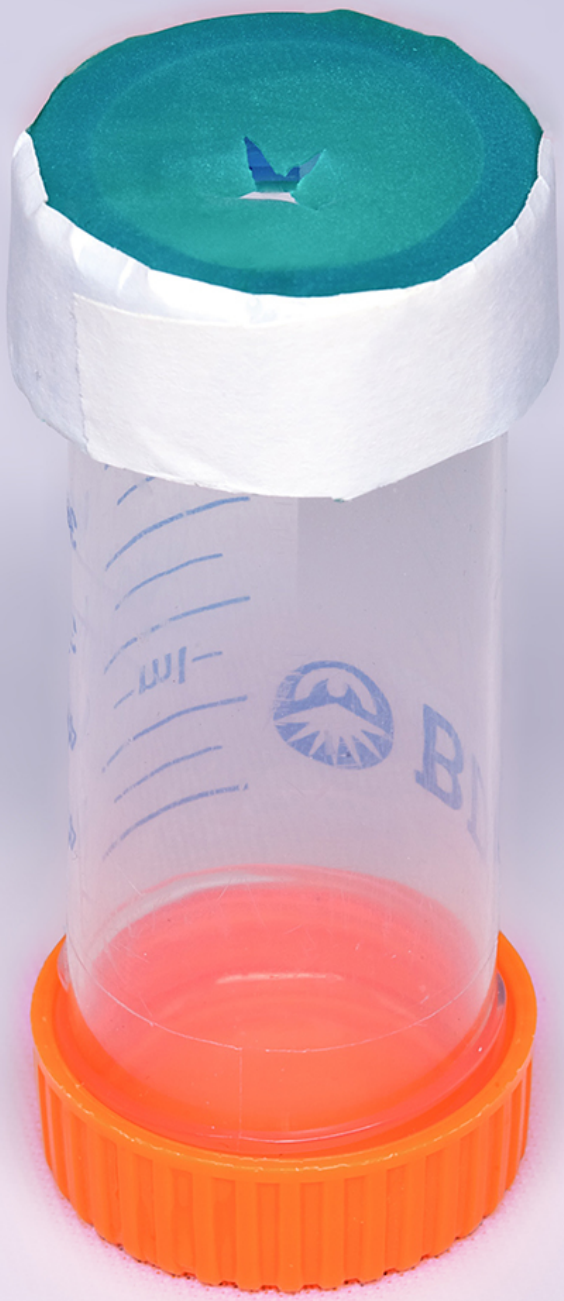
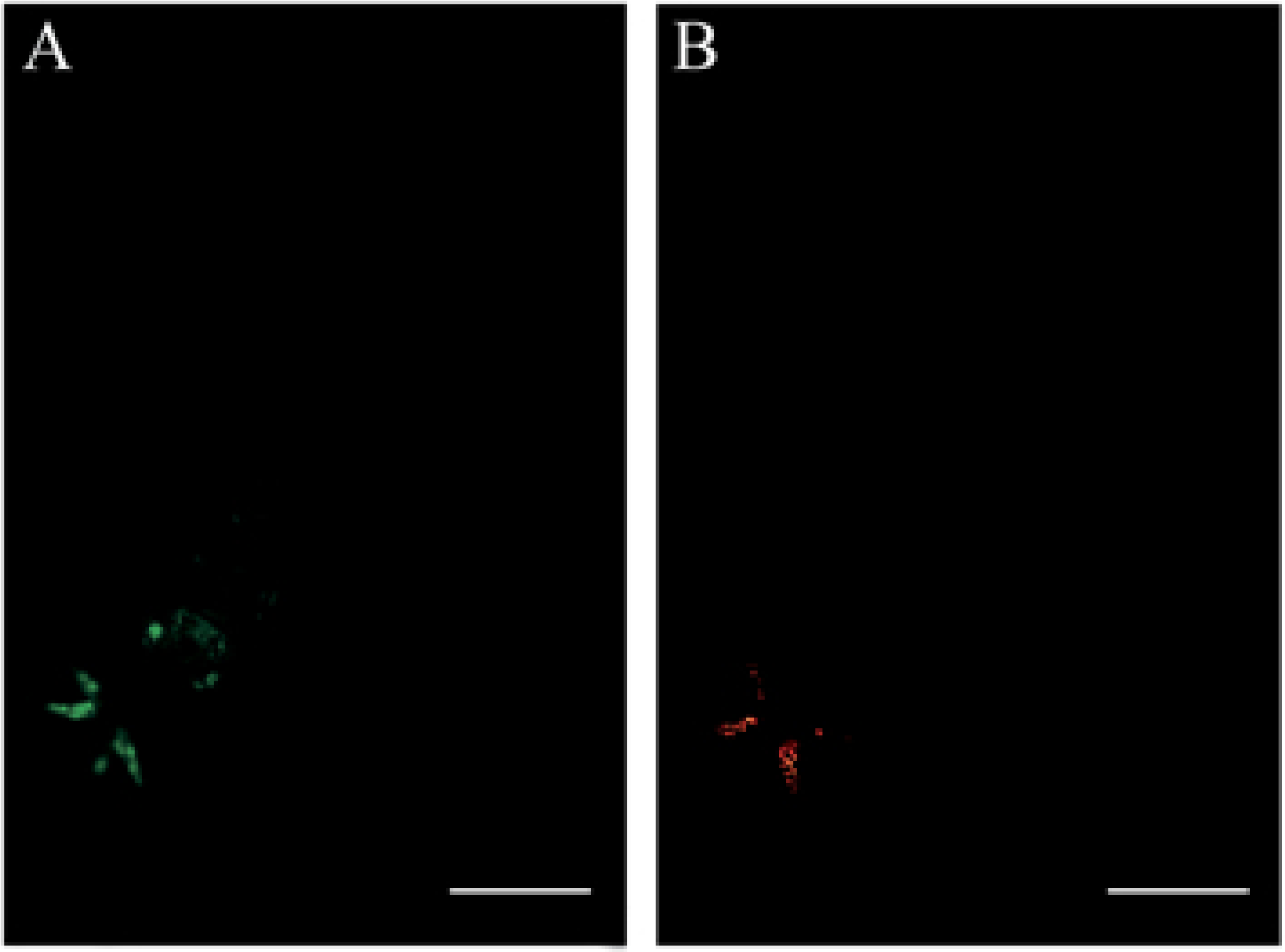
B


Standard docking strain
Self-docking strain
TIMING
Steps
Preparation of mix for injection
3d
1-8
Preparation of plasmid DNA carrying the transgene
+
4d
9-31
mRNA phiC31 integrase synthesis
(or helper plasmid)
4d
32-44
Embryo alignment and microinjection
8d
45-48
G0 rearing
and transient fluorescent screening (optional)
1m
49-57
G1 rearing and fluorescent transgenic screening
2m
58-65
Establishment of homozygous transgenic line

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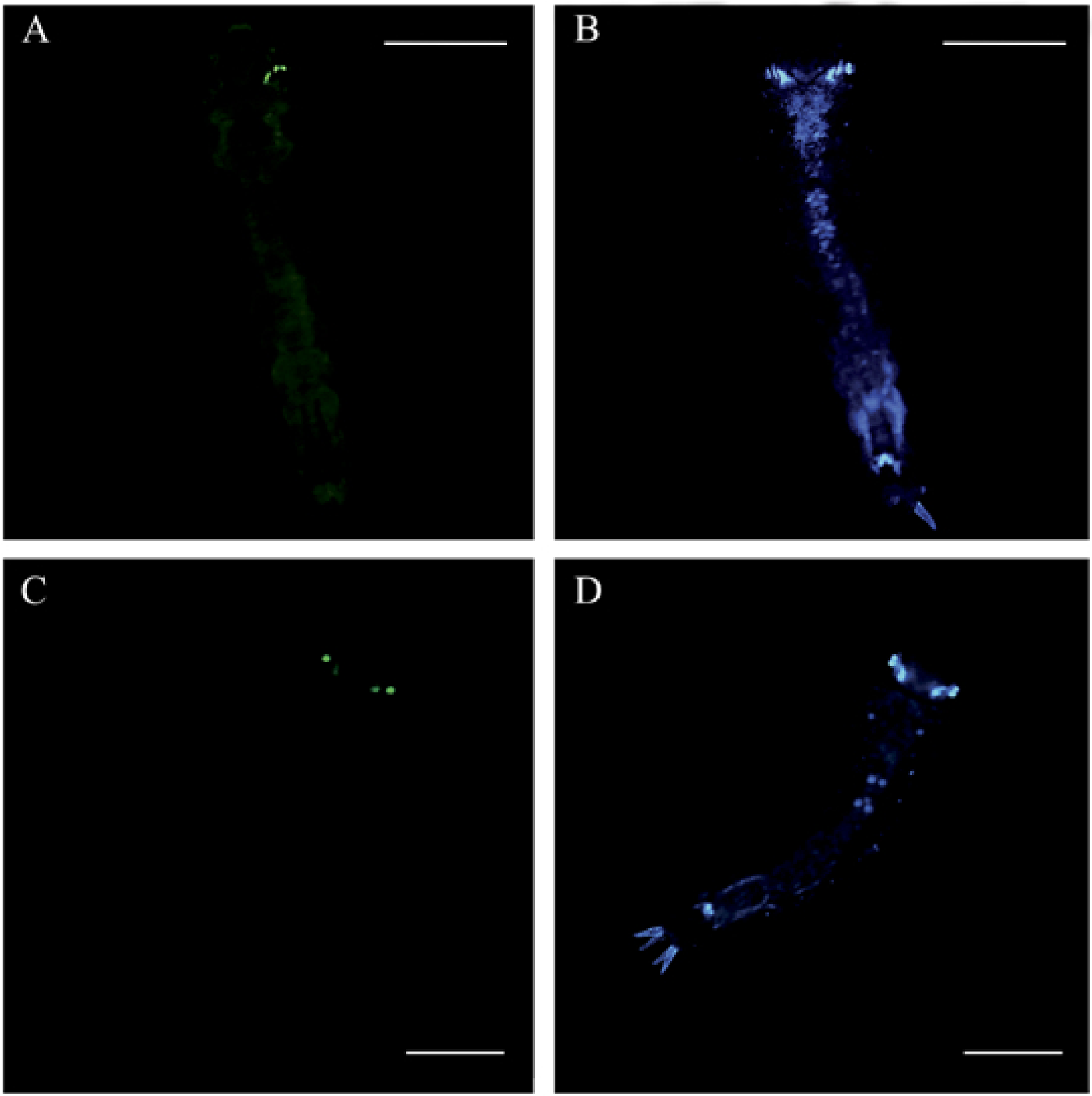
A
B
C
D