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TITLE:

Site-directed $\phi C31$ -mediated integration and cassette exchange in *Anopheles* vectors of malaria

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KEYWORDS:

Anopheles, $\phi C31$, site-directed, docking, integration, cassette exchange, mosquitoes, transgenic.

SUMMARY:

The protocol describes how to achieve site-directed modifications in the genome of *Anopheles* malaria mosquitoes using the $\phi C31$ system. Modifications described include both the integration and the exchange of transgenic cassettes in the genome of *attP*-bearing docking lines.

ABSTRACT:

Functional genomic analysis and related strategies for genetic control of malaria rely on validated and reproducible methods to accurately modify the genome of *Anopheles* mosquitoes. Amongst these methods, the $\phi C31$ system allows precise and irreversible site-directed integration of transgenes, or the substitution of integrated transgenic cassettes via recombinase-mediated cassette exchange (RMCE). This method relies on the action of the *Streptomyces* $\phi C31$ bacteriophage integrase to catalyze recombination between two specific attachment sites designated *attP* (derived from the phage) and *attB* (derived from the host bacterium). The system uses one or two *attP* sites that have been integrated previously into the mosquito genome and *attB* site(s) in the donor template DNA. Here we illustrate how to stably modify the genome of *attP*-bearing *Anopheles* docking lines using two plasmids: an *attB*-tagged donor carrying the integration or exchange template and a helper plasmid encoding the $\phi C31$ integrase. We report two representative results of $\phi C31$ -mediated site-directed modification: the single integration of a transgenic cassette in *A. stephensi* and RMCE in *A. gambiae* mosquitoes. $\phi C31$ -mediated genome manipulation offers the advantage of reproducible transgene expression from validated, fitness neutral genomic sites, allowing comparative qualitative and quantitative analyses of phenotypes. The site-directed nature of the integration also substantially simplifies the validation

of the single insertion site and the mating scheme to obtain a stable transgenic line. These and other characteristics make the φ C31 system an essential component of the genetic toolkit for the transgenic manipulation of malaria mosquitoes and other insect vectors.

INTRODUCTION:

The ability to modify the genome of mosquito vectors of diseases reliably and reproducibly has bolstered *in vivo* functional validation of genes and opened the doors to realizable genetic vector control strategies, such as those targeting *Anopheles* mosquitoes that transmit malaria¹.

Early mosquito genome editing relied solely on transposable element (TE)-mediated transformation, with *piggyBac* being the most commonly used transposon in *Anopheles*²⁻⁴. However, the random nature of TE integration can lead to undesirable modifications such as gene knockouts (insertional mutagenesis) and significant position effects on transgene expression⁵⁻⁸. Multiple insertions are also a common occurrence when using *piggyBac*^{5,9}, which makes the validation and the isolation of transgenic lines with single insertions laborious. Other drawbacks include their potential remobilization, as observed in the germline of *Anopheles stephensi* when providing a source of *piggyBac* transposase¹⁰⁻¹², and their limited size of DNA cargo (10-15 kb in length) with transformation efficiency declining with increasing size of the donor plasmid^{13,14}.

Site-directed integration approaches were introduced to circumvent these issues. The most common site-directed genome modification in mosquitoes is that mediated by the φ C31 system (**Figure 1a**). This is driven by a viral integrase that catalyzes the recombination between two heterospecific attachment (*att*) sites occurring naturally in the genome of the bacteriophage φ C31 (*attP*) and in the *Streptomyces* bacterium host (*attB*)¹⁵. Recombination of the two sites is unidirectional and results in the formation of hybrid sites (*attL* and *attR*). The recombination of such hybrid sites (leading to DNA excision) would require not only the presence of an active viral integrase but also another phage-encoded recombination factor^{16,17}. A stable integration site is thus generated that relieves the issue of potential undesired remobilization¹⁵. Moreover, the system allows the integration of large cargoes (e.g., integration of >100 kb constructs was reported in *D. melanogaster*¹⁸), significantly increasing carrying capacities. Integration occurs in a single predefined genomic locus which greatly simplifies the validation of insertion and the mating scheme to obtain a stable transgenic line. Finally, the site-directed nature of the integration allows normalization of expression as alternative transgenes are located in the same locus and therefore are regulated within the same neighboring genomic context. Indeed, one of the main applications of the technique is the direct comparison of phenotypes conferred by different transgenes following insertion into an identical locus.

Achieving φ C31-mediated integration involves two phases: phase I is the creation of transgenic docking lines carrying *attP* site(s), and phase II is the site-directed integration of an *attB*-flanked cargo in the genome of the docking line¹⁹. The creation of phase I docking lines has relied on the TE-mediated random integration of *attP*-tagged constructs and thus involved an initial laborious process (including southern blot and inverse PCR analyses on single-female progeny) to isolate and validate transgenic lines carrying a single integration event in unique, transcriptionally active, and fitness neutral genomic locations. Nevertheless, several docking lines for φ C31-mediated

single integration have been developed and validated in *An. gambiae*^{19–22} and in *An. stephensi*^{23–25} (**Table 1**). Each of these lines varies in terms of the genomic location of the docking site and the strain-specific genetic background and from them a great variety of new transgenic lines can be created. The complex validation of TE-mediated integrations for producing docking lines can now be circumvented by the CRISPR/Cas9 technology²⁶; however this relies on the a priori knowledge of neutral loci to be targeted and their surrounding sequences.

ϕ C31-mediated integration has been applied extensively to insect genome editing from the model organism *D. melanogaster*²⁷, to the mosquitoes *Aedes aegypti*^{13,28}, *Ae. albopictus*²⁹, *An. gambiae*¹⁹, and *An. stephensi*²⁴, as well as other insects including *Ceratitis capitata*³⁰ and *Bombyx mori*³¹.

A limitation of ϕ C31-mediated integration, especially in view of potential field releases for vector control, is the integration in the mosquito genome of the entire *attB*-bearing donor plasmid, including undesirable sequences such as antibiotic-resistance gene markers and plasmid backbone components of bacterial origin. To address this, a modification of the standard system, recombinase-mediated cassette exchange (RMCE), was implemented that allows the precise replacement of a previously integrated transgenic cassette with a new donor DNA (**Figure 1b**). This is achieved by using two inverted *att* sites flanking the donor and recipient cassettes at each end, which drives two independent recombination events to take place simultaneously resulting in cassette exchange without integration of the plasmid backbone. This improved design circumvents the integration of undesired sequences and expands the application of ϕ C31 systems to include for example the integration of unmarked DNA cargos by screening for the loss of a previously integrated fluorescent marker³².

RMCE was achieved first with *D. melanogaster*³² and later applied successfully to non-model insects including *An. gambiae*^{9,26,33}, *Ae. aegypti*³⁴, *Plutella xylostella*³⁴, and *B. mori*³⁵. Several *An. gambiae* docking lines for RMCE have been developed and validated in *An. gambiae*^{5,9,26} (**Table 1**). To our knowledge, RMCE is yet to be explored in other *Anopheles* vectors species.

To date, the ϕ C31 system has been used widely in *Anopheles* mosquitoes to introduce and study a variety of molecules including antimalaria effectors^{19,24,36}, components of the GAL4/UAS system to overexpress and knockdown genes for insecticide resistance studies^{9,33}, regulatory elements, reporter genes^{5,21,37}, and gene-drive elements^{26,38}.

This protocol describes how to perform 1) site-directed integration of an *attB*-flanked cargo and 2) RMCE of a construct flanked by inverted *attB* sites into the genome of *Anopheles* docking lines. This is achieved by using two plasmids: a donor *attB*-tagged plasmid carrying the transgene of interest, and a helper plasmid expressing the ϕ C31 integrase. The major malaria vectors *An. gambiae* and *An. stephensi* are used as specific examples, however these protocols are applicable to other *Anopheles* species.

[Place Figure 1 here]

133 PROTOCOL:

134
135 NOTE: A schematic workflow of the illustrated protocol is shown in **Figure 2**.

136 137 1. Design of ϕ C31 *attB*-tagged plasmids (Figure 3)

138 139 1.1. Create *attB* donor plasmids carrying the following essential components:

140 141 1.1.1. Dominant fluorescent marker

142
143 1.1.1.1. Choose a promoter to drive the expression of the fluorescent marker.

144
145 NOTE: For *Anopheles* transgenesis, fluorescent markers are usually under the regulation of the
146 *3xP3* promoter³⁹, which drives expression in the eyes and nerve cord. Alternatively, the *PUBc*
147 promoter⁵ can be used when expression in multiple tissues is desired. Donor plasmids and
148 docking lines used as examples in this protocol are marked using the *3xP3* promoter.

149
150 1.1.1.2. Choose a fluorescent protein (FP) that is compatible with that of the receiving docking
151 line so that they are readily distinguishable.

152
153 NOTE: Do not use the same marker that is already present in the docking line and avoid the
154 simultaneous use of GFP (green)/YFP (yellow) and GFP (green)/CFP (cyan) as they are very
155 difficult to reliably differentiate. Donor plasmids used as examples in this protocol are marked
156 with either DsRed or YFP as they are to be integrated in a docking line marked with CFP.

157 158 1.1.2. *attB* recombination site(s)

159
160 1.1.2.1. Use a **single *attB*** site for integration of a transgenic cassette (single-*attB* design) (**Figure**
161 **3A**).

162
163 1.1.2.2. Use **two inverted *attB*** sites for RMCE (double-*attB* design) where the sites lay inverted
164 in respect to one another and enclose the donor DNA template (**Figure 3B**).

165
166 NOTE: The orientation of the *attB* site(s) must be compatible with that of the *attP* site(s) present
167 in the docking line.

168 169 1.1.3. Desired transgene cargo

170
171 1.1.3.1. Use any other desired features to be integrated in the mosquito genome based on the
172 specific purpose of the experiment. Here, we describe the integration of an antimalarial effector
173 molecule into the genome of *An. stephensi* and the integration of the components of the
174 GAL4/UAS system into *An. gambiae* mosquitoes.

175 176 1.1.4. Plasmid backbone components

1.1.4.1. Include, amongst other essential components for plasmid replication in bacteria, a marker for plasmid selection in vitro (i.e., an antibiotic resistance gene).

NOTE: The plasmid backbone will be integrated in the mosquito genome in the single-*attB* design for integration (**Figure 3A**), while it will not be inserted in the double-*attB* design for RMCE (**Figure 3B**).

2. Preparation of plasmids for the microinjection mix

NOTE: The protocol illustrated here involves the use of two plasmids: an *attB*-tagged donor plasmid carrying the transgene of interest, and a helper plasmid that expresses the $\phi C31$ integrase under the regulation of the *Drosophila Hsp70* promoter⁴⁰.

2.1. Purify donor and helper plasmids using an endotoxin-free plasmid purification kit.

NOTE: Sequence the final plasmid preparation used for injection to verify the integrity of all components.

2.2. Combine appropriate amounts of the two plasmids to obtain a mix with a final concentration of 350 ng/ μ L of the donor plasmid and 150 ng/ μ L of the helper plasmid when resuspended in injection buffer.

NOTE: When calculating the necessary volume of mix, consider that 10-15 μ L are sufficient for each day of planned injections and DNA can be prepared in advance and stored at -20 °C. Integrase helper plasmid concentrations of 60-500 ng/ μ L and donor plasmid concentrations of 85-200 ng/ μ L have also been reported^{21,22,26,41}.

2.3. Precipitate the DNA by adding 0.1 volumes of 3 M sodium acetate (pH 5.2) and 2.5 volumes of ice-cold 100% EtOH and vortex. A white precipitate should be immediately visible. Having highly concentrated initial plasmid preparations (i.e., ~1 μ g/ μ L) improves precipitation efficiency.

NOTE: Stopping point - The precipitate can be stored at -20 °C overnight.

2.4. Centrifuge at 15,000 x *g* for 20 min at 4 °C, discard the supernatant, and wash the pellet with 1 mL of ice-cold 70% EtOH.

2.5. Wash the pellet with 1 mL of ice-cold 70% EtOH and centrifuge at 15,000 x *g* for 5 min at room temperature.

2.6. Discard the supernatant without disturbing the pellet and air dry.

2.7. Resuspend the pellet in 1x injection buffer (0.1 mM Na₃PO₄, 5 mM KCl, pH 7.2, 0.22 μ m filter sterilized) to reach a total final concentration of 500 ng/ μ L.

NOTE: Assume that some DNA will be lost during the precipitation process; therefore, add a smaller volume of injection buffer first, check the concentration on a spectrophotometer (e.g., Nanodrop), and then add an appropriate remaining volume to reach 500 ng/ μ L.

2.8. Ensure that the DNA is thoroughly resuspended, prepare aliquots of 10-15 μ L each and store them at -20 °C.

2.9. On the day of injection, thaw one aliquot and centrifuge at 15,000 x *g* for 5 min to remove any particulate residues.

NOTE: An alternative method for particulate removal is to filter the solution through a 0.22 μ m filter. Avoid the presence of particulate residues in the injection mix as they lead to needle blockage during embryo microinjection.

3. Microinjection of embryos from an *Anopheles* docking line

3.1. Blood feed 4-7-day-old mosquitoes from the desired docking line 72 h prior to microinjection (i.e., for injection on Monday and Tuesday feed females on the previous Friday; for injection on Thursday and Friday feed females on Monday of the same week).

3.2. Blood feed wild-type (WT) mosquitoes (i.e., mosquitoes with the same genomic background of the docking line) on the same day; these will be needed for outcrossing.

NOTE: The size and quality of the blood meal affect egg quality, so it is recommended to always use fresh blood (i.e., blood drawn within the previous 7 days). Arm feeding or feeding on mice may increase the quality and quantity of eggs, however these methods are not encouraged. Specific approved protocols will be necessary for human and animal use.

3.3. Perform embryo microinjections

3.3.1. Perform *An. gambiae* embryo microinjections in 25 mM NaCl⁴² by targeting the posterior pole of the embryo at a 45-degree angle. For a detailed protocol for embryo collection, alignment, and microinjection refer to Pondeville et al.⁴³ and Lobo et al.⁴⁴.

3.3.2. Perform *An. stephensi* embryo microinjections in halocarbon oil 700:27 (2:1) by targeting the posterior pole of the embryo at a 30-degree angle. A detailed protocol for embryo collection, alignment, and microinjection can be found in Terenius et al.⁴⁵ and Lobo et al.⁴⁴.

3.3.3. Transfer eggs immediately after injection in a Petri dish filled with sterile distilled water (pH 7.2) and return them to insectary conditions.

3.4. Upon hatching, transfer G₀ larvae into a tray with salted distilled water (0.1% tonic salt) daily and rear to pupae.

3.5. Record hatching rate (i.e., number of larvae hatched/number of embryos injected).

NOTE: Embryo movement aids hatching, so gentle swirling is desirable. Hatching should start ~48 h after injection. Since injection may cause a slight developmental delay it is advisable to keep monitoring for late-hatching larvae for 3-4 days.

4. Crossing and screening of transformed individuals

4.1. [OPTIONAL STEP] Screen G_0 (injected) 1st or 2nd instar larvae (L_1 - L_2) for transient expression of the fluorescent marker.

4.1.1. Use a fine-tip glass pipette to transfer G_0 L_1 - L_2 larvae to a microscope slides with wells. Place one larva in each well.

4.1.2. Use a fluorescence stereoscope with the appropriate filter to screen for the presence of transient expression of the fluorescent marker.

NOTE: The pattern of transient expression is dictated by the promoter used. When using the *3xP3* promoter, transient expression of the fluorescent marker is visible in the anal papillae (see Figure 6 in Pondeville et al.⁴³)

4.1.3. Rear G_0 positive individuals separately.

4.2. Sort G_0 pupae by sex under a stereoscope (Poulton et al., this collection).

4.3. Let males emerge in separate cages in groups of 3-5 (founder families) and add a 10-fold excess of age-matched WT females.

NOTE: Since males mate multiple times, it is important to provide an excess of WT females to maximize the mating chances of each male.

4.4. Let females emerge in separate cages in groups of 10-15 (founder families) and add an equal number of age-matched WT males.

NOTE: If there is limited space in the insectary, females can emerge all together in a single cage. The female to male ratio can be as low as 1 male to 3 females.

4.5. Allow adults to mate for 4-5 days and provide females with a blood meal.

NOTE: Blood feed and collect eggs from G_0 females multiple times to maximize the chances of getting transformants from multiple gonotrophic cycles.

4.6. Blood feed WT individuals at the same time for outcrossing.

4.7. Collect eggs and rear emerging next generation G_1 s.

4.8. Screen G_1 L₃-L₄ larvae for appropriate fluorescence to identify transformants.

4.8.1. Collect larvae in a Petri dish lined with filter paper or on a microscope slide and screen using a fluorescent stereoscope with appropriate filters for the presence of the marker introduced with the *attB*-tagged cargo.

NOTE: Fluorescence driven by the *3xP3* promoter is visible in all postembryonic stages and the screening may be performed on younger larvae, however these are more fragile and must be handled relatively carefully. Pupae can also be screened.

4.8.1.1. For single-*attB* designs for integration screen for the presence of the new and pre-existing marker; they should both be present since the new cassette is inserted next to the original one (Figures 3A, 4).

NOTE: Screening exception for single *attB* designs: When using marker-less docking lines²², screen for the presence of the new marker only. When using docking lines where integration results in the inactivation of the pre-existing marker²¹, screen for the presence of the new marker and the loss of the pre-existing one.

4.8.1.2. For double-*attB* designs for RMCE, screen for the presence of the new marker and the loss of the pre-existing one, only the newly introduced marker should be present since the new cassette replaces the original one (Figures 3B, 5).

NOTE: Occasional integration events can be recovered in RMCE experiments where only a single *attP* recombined and thus both markers will be present. The screening of G_1 individuals can be carried out also at the pupa stage following the same procedure (Poulton et al., this collection).

4.9. Transfer transformed G_1 individuals into a larval tray and rear to pupae. Discard non-fluorescent individuals and individuals with an unexpected marker expression pattern.

4.10. Sort transformed G_1 pupae by sex and cross them *en masse* with opposite-sex age-matched WT individuals.

4.11. Allow adults to mate for 4-5 days, provide a blood meal, collect the eggs, and rear the next generation G_2 progeny.

4.11.1. For single integration experiments, collect eggs directly from the *en masse* cross as the integration site is identical in all individuals.

4.11.2. For RMCE experiments, collect eggs from single females and maintain progeny separate until molecular assessment is complete due to the potential presence of two alternative cassette

orientations (Figure 3B).

4.12. Screen the G₂ progeny (at either the larva or pupa stage) for the presence of the fluorescent marker (50% of the individuals are expected to be positive), discard non-fluorescent progeny.

4.13. Set aside a subset of G₂ positive individuals for molecular analysis, rear the rest to adulthood.

NOTE: If all G₂ individuals must be kept alive, molecular analysis can be conducted on single adult's legs⁴⁶ or pupal case DNA extractions (L. Grigoraki personal communication). Alternatively, molecular analysis can be performed after all the G₂ individuals have oviposited and eggs have hatched.

4.14. Allow adult males and females to intercross in the same cage to establish the new transgenic line.

NOTE: For RMCE experiments, adult intercross must occur between siblings deriving from a single female until orientation of insertion is determined via molecular analysis.

5. Molecular validation of the insertion site by DNA amplification (PCR)

5.1. Prepare a map of the predicted insertion site in the genome of the docking line after transformation.

5.1.1. Single integration: Ensure that the predicted insertion site carries the original docking construct plus the whole sequence of the donor plasmid between the two hybrid sites *attL* and *attR* (Figure 3A).

5.1.2. RMCE: Ensure that the predicted insertion site is identical to that of the docking line where hybrid inverted *attL* sites replace the original inverted *attP* sites and the exchange template replaces the cassette originally present between them (Figure 3B).

5.2. Design oligonucleotide primers to amplify the insertional junction at either side of the integration locus.

5.2.1. Single integration: Design oligonucleotide primer pairs that span across the *attR* and/or *attL* sites. One primer must bind to the previously integrated docking construct and the other to the newly integrated transgene (Figure 3A).

5.2.2. RMCE: Cassette replacement can occur in **two different orientations** with respect to the chromosome (designated A and B). Design alternative combinations of 4 oligonucleotide primers to give a discrete product in only one of the orientations, with one pair being diagnostic for orientation A, and the other for orientation B (Figure 3B, 6).

5.3. Extract genomic DNA from G₂ positive individuals and perform the diagnostic PCR and gel electrophoresis to visualize the presence of expected diagnostic amplicons from the predicted integration site maps.

NOTE: DNA may alternatively be extracted from single adult's legs⁴⁶ or pupal cases (L. Grigoraki personal communication).

5.4. Sequence PCR products to confirm expected sequences.

[Place Figure 2 and 3 here]

REPRESENTATIVE RESULTS:

The protocol illustrated here enables to generate a stable *Anopheles* transgenic line in ~10 weeks (assuming a 21-day mosquito life cycle).

Post-injection larval hatching rates in *An. gambiae* are expected to be generally lower than *An. stephensi*, however hatching rates between 10-50% have been reported^{9,20,24,26,33,43,47}. Given appropriate injection technique, hatching rates of ≥20% are generally sufficient to yield transformants. DNA uptake by the embryos can be assessed by screening young larvae for transient expression of the fluorescent marker. In successful RMCE experiments in *An. gambiae* using the 3xP3 promoter up to 50% of the surviving G₀ larvae showed episomal expression of the marker in the anal papillae⁴⁸.

Generalized estimates of transformation efficiency are difficult to evaluate among laboratories and even among experiments as transformation depends on a complex interplay of variables including purity, concentration, size, and potential toxicity of the injected DNA, quality of eggs, pre- and post-injection handling of eggs, mosquito rearing, and most importantly the experience of the operator. Transformation rates up to 7% have been obtained for RMCE in *An. gambiae* (calculated as the number of independent transformation events in the total G₀ individuals)^{9,26,33}, and up to 2.2% transformation rate for integration in *An. stephensi*. We suggest injecting at least 500 embryos, which should lead to the hatching of at least 100 G₀ larvae and to 2-7 G₀ adult founders from which stably transformed progeny can be obtained. If screening for transient expression in G₀ larvae, up to 40 positive larvae can be expected.

Examples of phenotypic validation of transformation via the screening of fluorescent markers regulated by the 3xP3 promoter are reported in **Figures 4-5**. **Figure 4** shows a new *An. stephensi* line obtained by insertion of a DsRed-marked cassette into a docking line marked with CFP (80.9, **Table 1**), resulting in G₁ progeny expressing both markers as indicated by the red and blue fluorescence detected in the eyes.

RMCE designs are instead expected to result in the replacement of the marker originally inserted into the docking line with that of the donor plasmid. **Figures 5A-B** illustrate this marker exchange in an *An. gambiae* docking line marked with CFP (A11, **Table 1**) where after successful RMCE the CFP marker is lost and the YFP marker is acquired resulting in yellow (but not blue) eye and nerve

cord fluorescence³³. Occasionally, RMCE can result in a single integration event instead of the exchange of the desired transgenic cassette as illustrated in **Figure 5C**, where a larva marked with both the original CFP and the new YFP markers is shown. It is reported that up to 50% of the total number of transformation events are single integrations^{9, 33}.

When screening for the presence of a fluorescent marker it is crucial to distinguish its signal from possible background autofluorescence. This is particularly important when using CFP as *Anopheles* larvae display natural blue autofluorescence (**Figure 6A**). Increasing the magnification and focusing on the tissues and organs where fluorescence is expected to be driven by the promoter is necessary to identify true CFP-positive individuals as illustrated in **Figure 6B** using the 3xP3-CFP marker.

Individual transformants are finally assessed molecularly via PCR to confirm the expected insertion site. **Figure 7** reports the PCR validation in individuals from an exchange *An. gambiae* line showing the two potential orientations of insertion in the mosquito genome³³.

[Place Figures 4-7 here]

FIGURE LEGENDS:

Figure 1. Site-directed genome modifications, single integration (A) and recombinase-mediated cassette exchange (RMCE) (B), using the ϕ C31 system. The ϕ C31 integrase (INT, grey double arrow) catalyzes the recombination between the *attB* site(s) (purple striped) present in a donor plasmid and the *attP* site(s) (blue striped) present in a receiving docking line, which results in the formation of hybrid sites *attL* and *attR*. A) Integration is achieved when single *attB* and *attP* sites recombine and results in the presence of two integrated markers (blue and red). B) RMCE occurs when two *attB/P* sites recombine simultaneously and results in the replacement of the cassette between the *att* sites of the docking line (blue marker) with that carried by the donor plasmid (red marker). C) Partial nucleotide sequences of *attP* (purple) and *attB* (blue) and the hybrid sites *attL/R*. Recombination occurs between the 'TT' core sequences highlighted in bold black.

Figure 2. Workflow diagram for site-directed ϕ C31 genome modification in *Anopheles* mosquitoes.

Figure 3. Molecular basis of ϕ C31-mediated single integration (A) and RMCE (B). A) Schematic maps of the genomic insertion in an *An. stephensi* docking line (80.9, Table 1) carrying a single *attP* site and marked with CFP (top), a single-*attB* design donor plasmid marked with DsRed (middle), and the expected insertion site resulting after successful integration (bottom). B) Schematic maps of the genomic insertion in an *An. gambiae* docking line (A11, Table 1) carrying two inverted *attP* sites and marked with CFP (top), a double-*attB* design donor plasmid marked with YFP (middle), and the expected insertion site resulting after successful RMCE (bottom). Wavy line: mosquito genome; Striped arrows: *piggyBac* transposon arms; 3xP3: promoter of the fluorescent marker; SV40: viral terminator; Ori: origin of replication; AmpR: ampicillin resistance gene. Crossing lines represent the site(s) of recombination between *attP* and *attB* sites.

Numbered black arrows represent primer binding sites for the molecular validation of the insertion locus (step 5 of the protocol). Fully annotated single and double *attB*-tagged plasmids are available from the authors upon request.

Figure 4. Validation of ϕ C31 single integration in *An. stephensi* larvae (dorsal view). A) The docking line (80.9, Table 1) expresses CFP in the eyes under the regulation of the *3xP3* promoter. B) Successful integration results in the expression of the newly acquired DsRed as well as the original CFP marker in the eyes.

Figure 5. Validation of ϕ C31 RMCE in *An. gambiae* larvae (ventral view). A) The docking line (A11, Table 1) expresses CFP under the regulation of the *3xP3* promoter in the eyes (e) and the nerve cord (nc)⁵. B) Successful RMCE results in the swap of fluorescent marker from CFP to YFP³³. C) Single integration event occurred during RMCE experiment where the transformant larva expresses both the CFP and YFP markers. This larva carries GAL4/UAS components that cause a broad expression pattern of YFP, particularly strong in the abdominal muscles (am).

Figure 6. CFP autofluorescence in *An. gambiae* larvae (dorsal view). A) Side-by-side image of a positive (CFP+) and a negative (CFP-) L₄ larva using the CFP filter. B) Close-up image of the larval eyes that reveals a CFP+ vs CFP- individual.

Figure 7. Molecular validation of the orientation of cassette insertion in representative transgenic *An. gambiae* created by ϕ C31 RMCE. The transgenic cassette can be inserted in one of two alternative orientations (A or B) in respect to the insertion site. Each PCR reaction (I - IV) uses a combination of primers (5-8)³³ designed to give a diagnostic amplification fragment for each orientation as indicated in the schematic plasmid maps. T1: representative transgenic individual carrying orientation of insertion A; T2: representative transgenic individual carrying orientation of insertion B; WT: wild type; DL: docking line; -: reaction negative control where water was used as template. This figure has been modified from Adolfi et al. (2019)³³.

DISCUSSION:

The accurate design of *attB*-tagged plasmids that are compatible with the docking line of choice is paramount for the success of the experiment. Careful consideration must be given to the choice of the marker used for the screening of transformants, including the fluorescence color and its pattern of expression, which will be subject to the pattern already present in the docking line. It is necessary to use fluorescent markers that are easily distinguishable: good marker combinations include RFP (red)/CFP (cyan), RFP (red)/GFP (green), RFP (red)/YFP (yellow), and YFP (yellow)/CFP (cyan), while combinations to avoid are YFP (yellow)/GFP (green) and CFP (cyan)/GFP (green). The *3xP3* promoter³⁹, specific to the eyes and nerve cord, is the most frequently used to drive the expression of fluorescent markers for mosquito transgenesis. Indeed, all the *Anopheles* docking lines currently available utilize this promoter. Alternative regulatory regions are that of the *An. gambiae* polyubiquitin gene (PUBc)⁵ or the viral promoter IE1²⁰, which drive expression in multiple tissues. When used along with *3xP3*, these promoters would expand the possible color combinations and even the use of the same fluorophore. The indicated promoters are active throughout the mosquito life cycle allowing screening and

fluorescence monitoring at all life stages. An additional consideration during plasmid design is the size of the cargo to be integrated or exchanged. While the $\phi C31$ system has remarkable carrying capacities¹⁸, it should be considered that the size of the donor plasmid generally correlates negatively with transformation efficiency²².

In the described protocol the source of integrase is a helper plasmid expressing the enzyme ubiquitously⁴⁰. The ubiquitous presence of the integrase may lead to the transformation of somatic cells if microinjections are not precisely directed to the area where the germline forms. While such transformation events will be lost as they are not heritable, somatic effects can decrease the fitness of injected individuals. To avoid this and increase transformation efficiency, integrase expression can be restricted to the germline, for example by using the *vasa* promoter^{22, 26}. Other protocols describe the use of *in vitro* transcribed messenger RNA (mRNA) as source of $\phi C31$ integrase^{19,24,43}. However, this involves the laborious preparation of mRNA and requires careful handling of the injection mix and the use of RNase free reagents to avoid degradation. Plasmid sources of integrase have been demonstrated in both *An. gambiae*^{9,21,22,26,33,37} and *An. stephensi* (A.A. personal communication) to be reliable and lead to efficient transformation, and are thus our preferred option. A further option for integrase delivery is its *in vivo* production in self-docking helper lines. Such lines were created in *An. gambiae* that express the $\phi C31$ integrase under the regulation of the germline-specific promoter *nanos* and were found to lead to an improved survival and transformation efficiency²⁰. However, potential fitness loads imposed by the *in vivo* production of the integrase enzyme on the helper line must be considered.

As with other transgenic techniques, special care must be reserved to the rearing and crossing of individuals deriving from injected embryos to maximize the chances to recover transformants. Individuals that have stably inherited the transgene can be firstly recovered at the G₁ progeny. However, early signs of potential transformation can be evaluated by the presence of transient episomal expression of the fluorescent marker in the anal papillae and/or nerve cord of G₀ first and second instar larvae when using the 3xP3 promoter⁴³. While the presence of transient fluorescence suggests successful plasmid delivery, it does not guarantee heritable germline transformation. Similarly, the lack of transient expression does not exclude successful transformation. Nevertheless, it has been observed that transiently positive individuals are more likely to yield transgenic progeny compared to transiently negative ones^{43,48}. In expert hands, rearing and crossing of only positive individuals may be an option to reduce mosquito numbers. However, given the importance and fragility of small G₀ larvae, the least amount of manipulation is still advisable and the rearing of all G₀ individuals is always recommended.

The mating scheme reported in this protocol is designed to maximize the chance of mating and to isolate independent transformation events. However, if insectary space or personnel availability is an issue, G₀ adults can be pooled by sex in single cages if enough opposite-sex individuals are provided. Such a setup will not allow discrimination between multiple transformation events occurring in individuals from the same cage. Depending on the experimental setup, the presence of a double (single integration) or single (RMCE) marker is expected during the screening process. In single integration experiments it is important to verify the presence of the original marker from the docking line, while in RMCE is important to verify

the loss of the previously integrated marker. Indeed, it is not uncommon in RMCE designs to recover transformants in which single integration instead of exchange occurred due to the recombination of a single *attP* site^{9,33}. In such individuals both fluorescent markers are present as well as the whole donor plasmid backbone highlighting the importance of conducting a thorough screening for both fluorescent markers.

While the presence of expected fluorescence patterns indicates successful transformation, molecular characterization of the insertion site must be undertaken. To do so, the preparation of accurate maps of the predicted insertion locus, including the flanking genomic regions of the docking line, is crucial for the design of adequate diagnostic oligonucleotide primers for gene amplification analyses. Single integration events result in the formation of *attR* and *attL* hybrid sites at the junction between the newly integrated DNA and the previously inserted cassette. These sites can be targeted for insertion site validation. In RMCE designs, the insertion of the donor cassette can occur in two alternative orientations in respect to the genomic locus, thus four primers can be used in alternative PCR combinations to detect which orientation the line carries. As the orientation of cassette insertion may affect transgene expression, in comparative gene expression analysis it is important to use lines carrying the same orientation of insertion.

When working with low numbers of transformants it may not be desirable to sacrifice whole individuals for molecular analysis. An option to this is conducting molecular analysis on DNA extracted from single adult's legs⁴⁶ as leg loss does not affect an adult female ability to mate and oviposit⁴⁹. However, there is a risk of damaging the individual in the process of leg removal. Success has been obtained using discarded pupal cases (L. Grigoraki personal communication), however the safest approach is to perform molecular analysis on G₂ parents after obtaining viable G₃ progeny.

In recent years, CRISPR/Cas9 has revolutionized the way of performing site-specific genome editing^{26, 41, 50, 51}. Unlike site-directed RMCE, CRISPR/Cas9-mediated gene integrations (knock-ins) are independent of the presence of pre-inserted recombination sites with only a one-step transformation event needed. Nevertheless, the CRISPR/Cas9 system relies on the presence of large known genomic sequences flanking the desired insertion site for successful homology directed repair as well as on the efficient site recognition mediated by guide RNAs. These conditions cannot always be met or may be laborious to troubleshoot and, given the availability of multiple docking lines in *An. gambiae* and *An. stephensi* and lines derived from them, the ϕ C31 system remains a very valuable tool to perform direct phenotypic comparisons between transgenes at the same genomic locations.

ACKNOWLEDGMENTS:

We are grateful to Kiona Parker (UCI) for providing images of transgenic *An. stephensi* larvae, and to Fraser Colman (LSTM) and Beth Poulton (LSTM) for providing transgenic *An. gambiae* larvae. Beth Poulton (LSTM) also provided precious assistance during the imaging of *An. gambiae* larvae. This work was funded by the Tata Institute for Genetics and Society (TIGS) and the LSTM's Director Catalyst Fund awarded to A.A. (DCF2014AA). A.A.J. is a Donald Bren Professor at the University of California, Irvine.

DISCLOSURES:

The authors have nothing to disclose.

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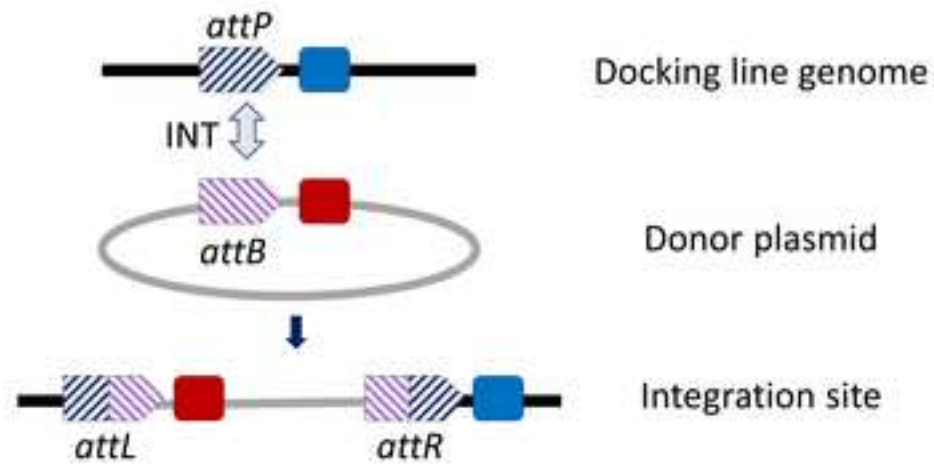
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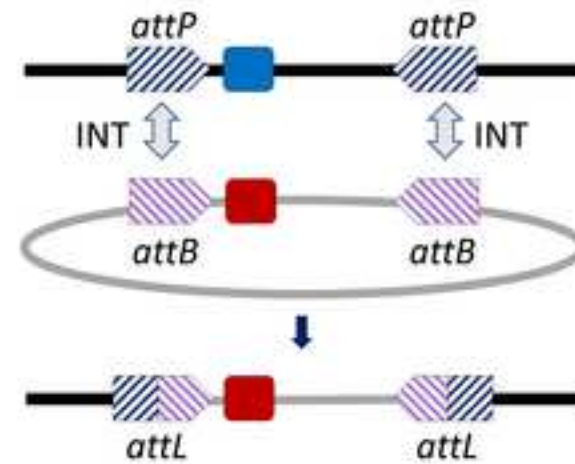
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A. ϕ C31 single integration



B. ϕ C31 RMCE



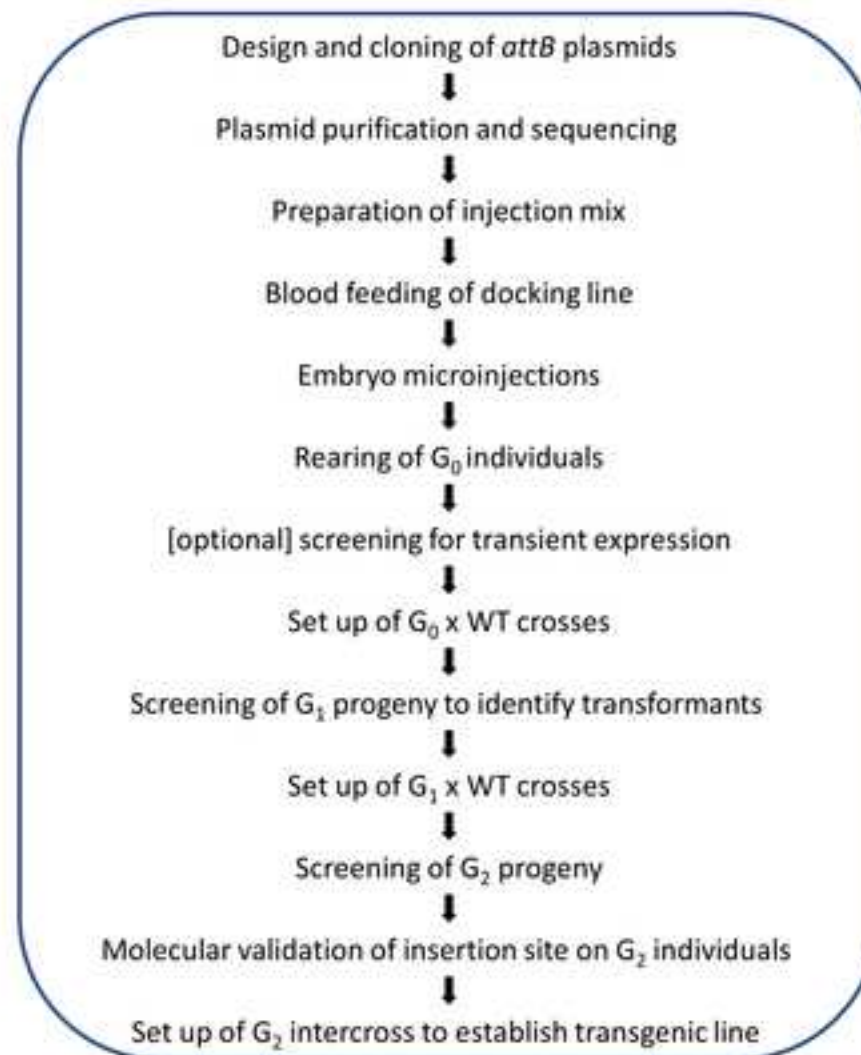
C.

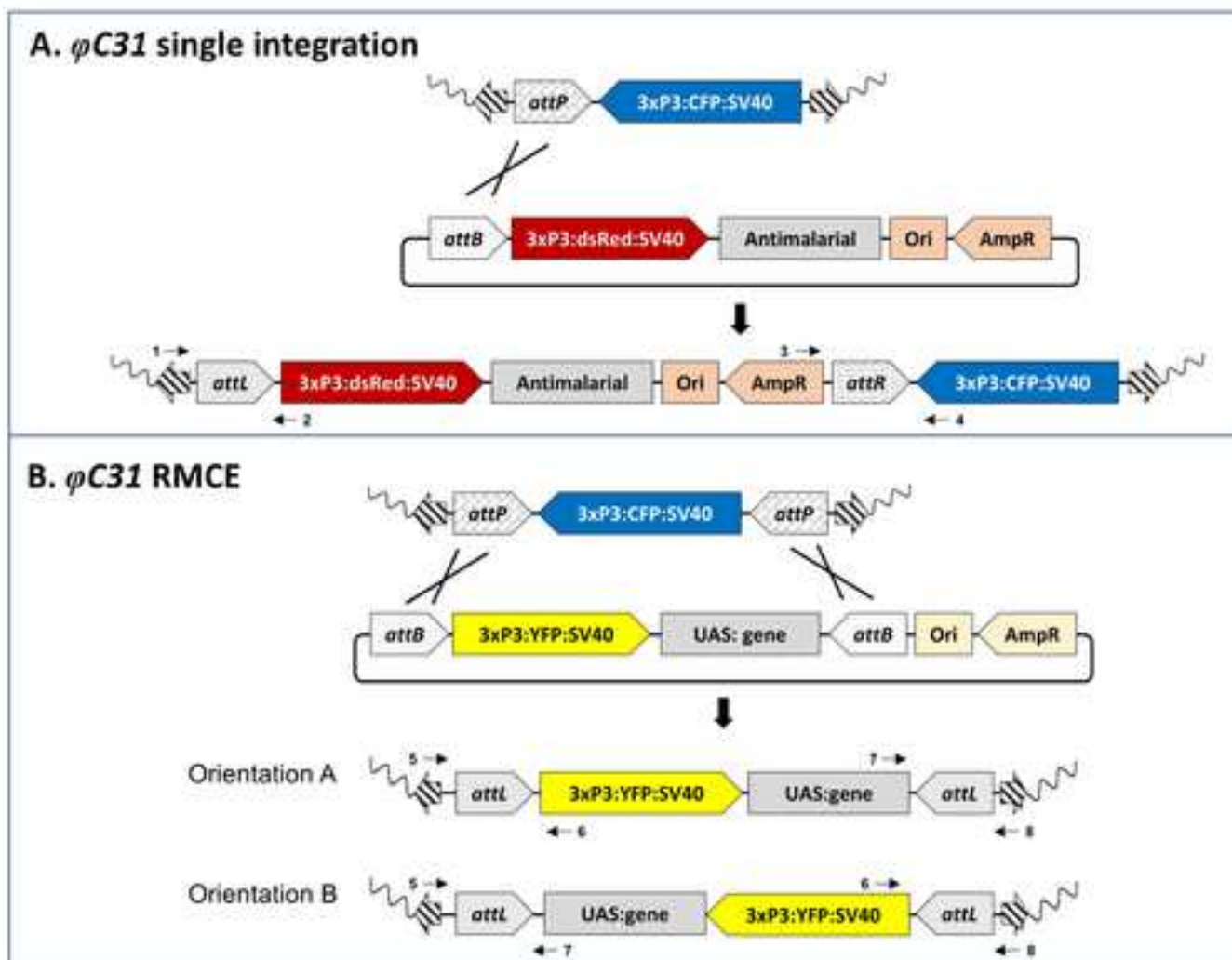
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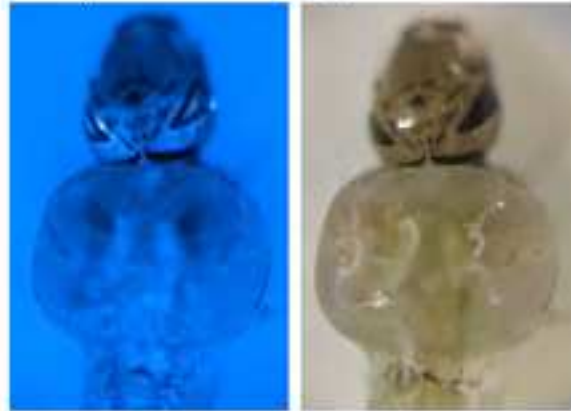
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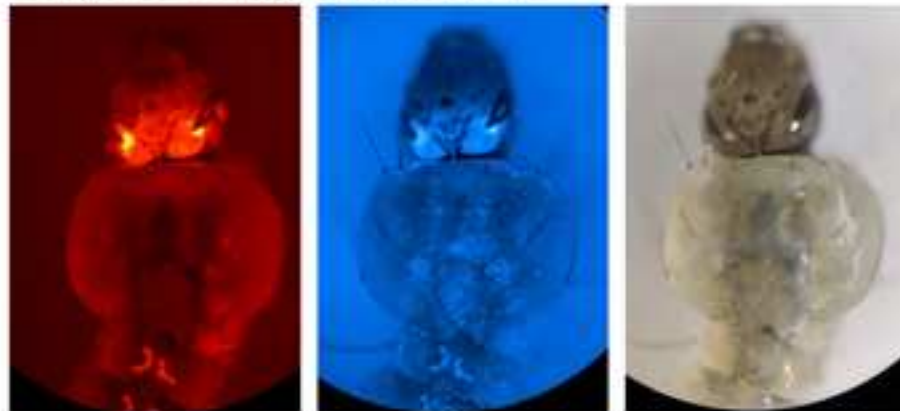


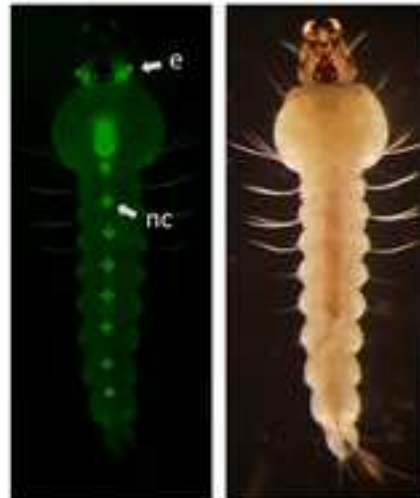
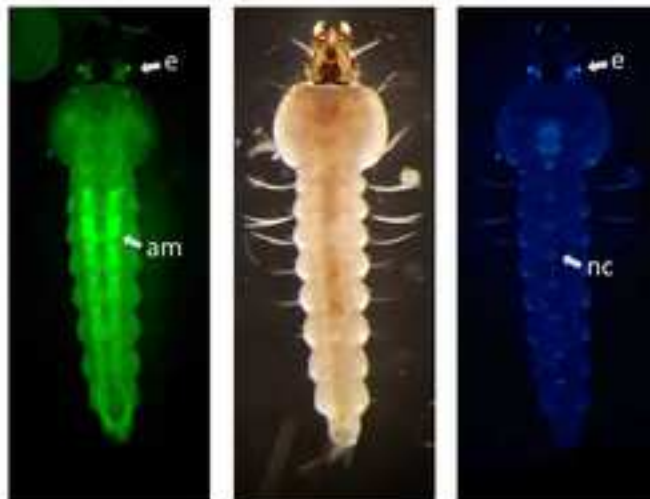


A. ϕ C31 docking line



B. ϕ C31 single integration



A. ϕ C31 docking line**B. ϕ C31 RMCE****C. ϕ C31 integration during RMCE**

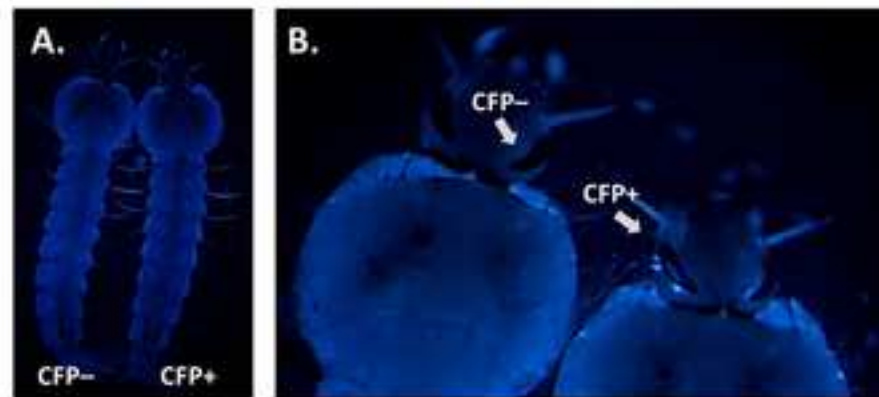


Figure 7

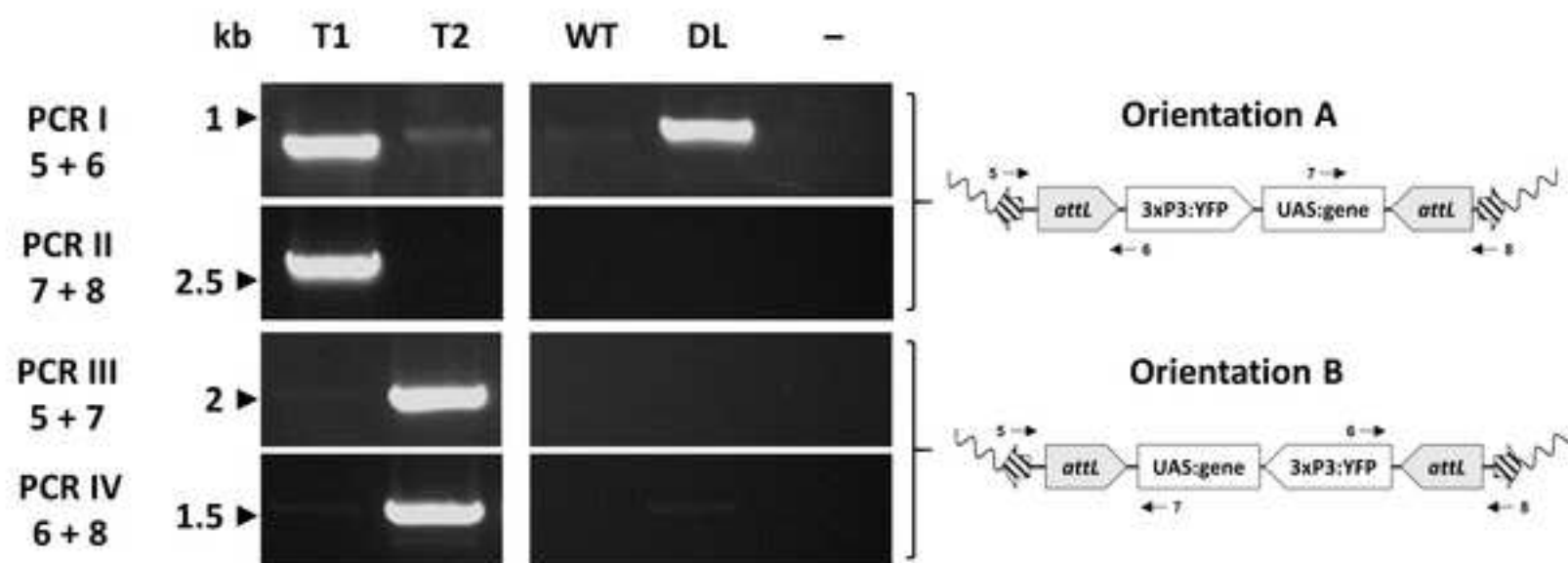


Table 1. *Anopheles attP* docking lines

Species	Strain	Name	<i>attP(s)</i>	Chromosome	Promoter-marker	Institution of origin	Reference
<i>An. stephensi</i>	Indian ^a	26.10 ^b	Single	2R	3xP3-eCFP	Univ. of California Irvine	25
<i>An. stephensi</i>	Indian ^a	44C ^b	Single	X	3xP3-eCFP	Univ. of California Irvine	23, 24
<i>An. stephensi</i>	Indian ^a	80.9 ^b	Single	2L	3xP3-eCFP	Univ. of California Irvine	This study
<i>An. gambiae</i>	G3	113	Single	2R	3xP3-eCFP	Univ. of California Irvine	This study
<i>An. gambiae</i>	KIL	E ^c	Single	3R	3xP3-eCFP	Keele Univ.	19, 43
<i>An. gambiae</i>	G3	X1	Single	2L	No marker	Univ. of Strasbourg	22
<i>An. gambiae</i>	G3	YAttP	Single	Y	3xP3-RFP	Imperial College London	21
<i>An. gambiae</i>	G3	A10 ^b	Double	2R	3xP3-eCFP	Liverpool School Trop. Med.	5
<i>An. gambiae</i>	G3	A11 ^b	Double	2R	3xP3-eCFP	Liverpool School Trop. Med.	9

a. Strain from Johns Hopkins University (gift of M. Jacobs-Lorena) and in culture at the Univ. of California Irvine for >20 years.
b. These lines are available from the authors upon reasonable request.
c. This line is available at the BEI repository www.beiresources.org as MRA-1163.

Name of Material/ Equipment	Company	Catalog Number
1.5 mL eppendorf tubes		
8-well microslides	VWR	MARI1216690
DNeasy Blood & Tissue Kit	Qiagen	69504
EndoFree Plasmid Maxi Kit (10)	Qiagen	12362
Ethanol, Absolute, Molecular Biology Grade		
Filter set CFP for Leica MZ FLIII Excitation 436/20 nm, extinction 480/40 nm	Leica	10446363
Filter set dsRED for Leica MZ FLIII Excitation 545/30 nm, extinction 620/60 nm	Leica	10447079
Filter set YFP customised for Leica MZ FLIII	Omega Optical	500QM25, 500QM35
Halocarbon oil 27	Sigma	H8773
Halocarbon oil 700	Sigma	H8898
Petri dishes		
Potassium chloride		
Sodium Chloride		
Sodium phosphate dibasic		
Sodium Acetate Solution (3 M), pH 5.2	Thermo Fisher Scientific (Life Technolog	R1181
Stable brush Size 0		

Comments/Description

JoVE62146R1**Site-directed ϕ C31-mediated integration and cassette exchange in *Anopheles* vectors of malaria**

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The authors would like to thank the editor and the 3 reviewers for their constructive comments. Please find authors' point by point responses to comments below in blue.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

Manuscript was proofread by all authors.

2. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Imperative tense was used wherever possible throughout the protocol. Please see track changes.

3. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

4. In the protocol, please work with a specific example to illustrate how this method is to be used. This will help with the filming. For example, 1.1.3: mention as an example here which cargo you will work with for the video.

Specific examples were included where applicable. Please see track changes.

5. Please highlight only up to 3 pages of protocol text for inclusion in the protocol section of the video.

3-page worth of the protocol has been highlighted.

6. Wherever possible, please provide volume and issue numbers in the reference list, and do not abbreviate journal names.

Reference list has been updated to conform with JoVE's referencing style.

7. Please sort the Materials Table alphabetically by the name of the material.

Modified as requested.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This manuscript from Adolfi et al overviews the main steps and procedures for the transformation of *Anopheles* mosquitoes via ϕ C31-mediated integration. The manual and the associated video that will follow will be a valuable resource, particularly for the training of new researchers with this peculiar experimental procedure. As expected from the experienced researchers involved, the work is of high standard and I recommend its publication. Minor comments/suggestions are included below.

Major Concerns:

None

Minor Concerns:

36 "two specific attachment (att) sites designated attP" - check writing
'att' was removed from text (line 36).

39 "This stepwise protocol" - suggest rewriting (e.g. Here we illustrate...), it sound linked/referred to previous sentence otherwise
modified as suggested using 'Here we illustrate' (line 39).

47 "the mating scheme to obtain a stable transgenic line" I would specify that this method simplifies the characterisation of single insertions (e.g. compared to transposase methods)

This comment was addressed in multiple parts of the manuscript.

The word 'single' was added in the abstract (line 47).

The sentences were included in the introduction to highlight the improved ease in the molecular validation and characterisation of the single insertion locus:

Line 60: 'Multiple insertions are also a common occurrence when using piggyBac^{5,9}, which makes the validation and the isolation of transgenic lines with single insertions laborious'.

Line 76: 'Integration occurs in a single predefined genomic locus which greatly simplifies the validation of insertion and the mating scheme to obtain a stable transgenic line'.

59 "position effects on transgene expression." Reference/s may be useful here (e.g.

<https://www.pnas.org/content/110/18/7176> and

<https://www.nature.com/articles/ncomms4977?origin=ppub>)

Suggested references and additional ones were added (line 60).

62 The insertion size limitation may be a tricky one. One would imagine that the 10-15 Kb limit indicated for TE transformation may be surpassed by using a different donor vector which could accommodate larger fragments (e.g. cosmids) and on the other hand you would expect the same limitation applicable also for attB carrying plasmids. I would recommend the authors to expand/clarify this aspect.

Transformation efficiency using piggyBac is greatly affected by the size of the construct with transformation efficiency declining with the size of the insert, thus the use of larger vectors would/may not solve the problem of integrating larger fragments.

The sentence 'their limited size of DNA cargo (10-15 Kb in length) with transformation efficiency declining with increasing plasmid size¹⁰⁻¹²' was added in the introduction (line 63).

65 "sites in the genome" I would suggest replacing with "sites naturally found in the"
Modified as 'occurring naturally' (line 69).

68 "no longer amenable to the action of the integrase." Is there any evidence about this, add relative reference if yes or address in the text if not tested.

The text was modified to 'The recombination of such hybrid sites (leading to DNA excision) would require not only the presence of an active viral integrase but also another phage-encoded recombination factor^{16,17}.' (line 71).

69 "potential undesired remobilization" remobilisation of pBac transgene insertions seems to vary across insects and very low or not active in *A. gambiae*. I would probably be careful and clearer about this aspect, providing additional references, and address the current/limited experimental knowledge about pBac and attP/B sites reactivation in *Anopheles*.

Evidence in *Anopheles stephensi* suggests that remobilisation can occur, at variable rates, when a source of transposase is provided. This was incorporated in the text as 'as observed in the germline of *Anopheles stephensi* when providing a source of *piggyBac* transposase¹⁰⁻¹²' (line 62).
The issue of attR/L excision was addressed in the previous comment.

73 "regulated within the same genomic context." Should probably be "neighbouring genomic context"
Modified as suggested (line 80).

103 "loss of the plasmid backbone" there is no real loss as the backbone is never inserted, suggest rewriting.
Modified to 'without integration of the plasmid backbone' (line 110).

148-151 and Fig 3 - It would be useful to show the essential attP/B sequence and resulting attL/R after recombination
Sequences were added in Fig 3 panel C.

154 reinforces the utility of attP/B sequences
Sequences were added in Fig 3 panel C.

Fig.1B bottom right shows two attL sites, is this intentional or typo?
This is intentional. The recombination of the second inverted attP site leads to the creation of an inverted attL site rather than an attR site. If the second attP site were not inverted, then an attR site would have been formed.

163 "selectable marker" specificity as it may be confused with fluorescent markers
Modified to 'a marker for plasmid selection *in vitro*' (line 178).

180 I have noticed different plasmid concentrations being used for microinjections; it would be good to give a recommended concentration of range. This may be particularly useful for constructs that may cause dosage-dependent toxicity/embryo lethality.

A range of published concentrations of integrase and donor plasmid is now reported as 'Note'.
'NOTE: Integrase helper plasmid concentrations of 60-500 ng/μL and donor plasmid concentrations of 85-200 ng/μL have also been reported^{21,22,26,41}' (line 202).

208 "Nanodrop" - probably any spectrophotometric quantification system would do the job
Modified to 'on a spectrophotometer (e.g., Nanodrop)' (line 225).

217 Probably useful to add a step for this as described previously - e.g. "filter through a 0.2 micron Millipore Ultrafree-MC filter"
This was included as alternative method in the relevant note (line 234).

231 "Arm feeding or feeding on mice increases the quality and quantity of eggs" probably true but I would not encourage scientists to do this (in consideration of Animal Welfare and 3Rs) unless there is evidence that is strictly necessary for specific applications, which I'm not aware of.
We clarified that these methods are not encouraged, and we had stated that ethical approval was required (lines 249-252).

253 suggest replacing "trigger" with "helps" or similar
Modified as suggested (line 272).

265 Different promoters may alter the typical pattern of transient expression in some cases (e.g. 3xP3 vs Actin5C) therefore I would specify the promoter used, and provide a fluorescent image if available.
Clarified as suggested as part of the 'NOTE: The pattern of transient expression is dictated by the promoter used. When using the 3xP3 promoter, transient expression of the fluorescence marker is visible of the fluorescence marker in the anal papillae/nerve cord in a stereoscope (see Figure 6 in Pondeville et al., 2014)' (lines 287-289).

286 "Blood feed G0 females multiple times" specify the deposition step between feedings
Modified to 'Blood feed and collect eggs from G₀ females multiple times' (line 309).

293 Probably not essential to wait for L3-L4 larvae for the screening, but it may depend on the promoter and insertion?
A note was added to clarify 'NOTE: Fluorescence driven by the 3xP3 promoter is visible in all postembryonic stages and the screening may be performed on younger larvae, however these are more fragile and have to be handled relatively carefully. Pupae can also be screened (lines 322-324).

295 Would microscope slides be also ok?
Option of using microscope slides was added (line 318).

299 This may not be true for all the single-attB designs (see Bernardini et al. 2014)
A note was added indicating modified screening strategy when using single-attB marker-less docking lines or docking lines where the integration of the new cassette disrupts the function of the pre-existing marker.
'NOTE: Screening exception for single attB designs - When using marker-less docking lines²³, screen for the presence of the new marker only. When using docking lines where integration results in the inactivation of the pre-existing marker²², screen for the presence of the new marker and the loss of the pre-existing one.' (lines 330-333).

304 Suggest replacing "old" with "pre-existing"
Modified as suggested (line 336).

385 check writing

Rephrased for clarity as 'The protocol illustrated here enables to generate...' (line 418).

390 "hatching rates of $\geq 20\%$ are generally sufficient to yield transformants" the success will probably be relative to the overall number of injected embryos as well. Maybe a minimum number of transient would be a better indication of what may be the minimum requirement to obtain transformed insects?

We inserted an example with numbers in the discussion 'We suggest injecting at least 500 embryos, which should lead to the hatching of at least 100 G_0 larvae and to 2-7 G_0 adult founders from which stably transformed progeny can be obtained. if screening for transient expression in G_0 larvae, up to 40 positive larvae can be expected.' (lines 435-438).

404-407 The promoter (3xP3 I believe) should be indicated. I don't think "(A.A. unpublished)" is needed being published here. Same for (G.J.L. unpublished) in 415

Promoter was specified and reference to unpublished work removed

413 Also the RMCE involves integration events, I would specify e.g. "single integration events" rather than just "integration". Please replace also where applicable in the manuscript.

Modified as suggested throughout the text.

499 "careful handling" and use of RNase free reagents

Added to the text as suggested (line 550).

552 DNA extraction after deposition and confirmed hatching may also be advisable

Added in the protocol (lines 368-370) and in the discussion (line 604).

- Please check that "unidirectional" is a correct definition here. I don't think you can envision a different (e.g. bidirectional) recombination considering plasmid-chromosome DNA exchange
'Unidirectional' was eliminated from the text (line 471).

-Considering that 3xP3-CFP is also used as an example, it may be useful to indicate the possible background (particularly high when using CFP filters for screening of larvae)

A figure was included to show CFP background. This is now Figure 6.

Text was added in the Representative Results section too (lines 454-459): 'When screening for the presence of a fluorescent marker it is crucial to distinguish its signal from possible background autofluorescence. This is particularly important when using CFP as Anopheles larvae display natural blue autofluorescence (Figure 6A). Increasing the magnification and focusing on the tissues and organs where fluorescence is expected to be driven by the promoter is necessary to identify true CFP-positive individuals as illustrated in Figure 6B using the 3xP3-CFP marker'.

-It would be good to give the specs of fluorescence filters to use, at least for the fluorescent markers indicated in the examples

Specifications for DsRed, YFP and CFP filter used were included in the list of Materials.

-Fig. 6 A simplified version of Fig 3 schematic, showing primer position, may be useful here
Figure 6 (now figure 7) was modified to accommodate schematic as suggested.

Reviewer #2:

Manuscript Summary:

The manuscript submitted by Adolphi et al, entitled "Site-directed ϕ C31-mediated integration and cassette exchange in Anopheles vectors of malaria" describes a protocol to achieve site directed insertions into the mosquito genome by using existing AttP mosquito lines.

Major Concerns:

The protocol provided by the authors describes the insertion genes into specific AttP loci in transgenic mosquitoes. While some of these transgenic lines are referenced, the protocol lacks useful data about existing AttP mosquito lines.

1. The authors should provide a table detailing available AttP lines. The information should include: Description, lab of origin, availability, insertion locus, fluorescent marker, and sequence of insertion locus. This would be critical for anyone interested in creating transgenic mosquitoes using this method.

Table 1 was added with a list of available Anopheles docking lines. We specified that the lines created by the authors of this manuscript will be available upon request.

2. The authors should provide full annotated sequences of all the plasmids used as examples in this protocol. The authors should also be prepared to provide these plasmids, (or deposit them in a plasmid bank).

Full annotated plasmid sequences will be available upon request. A note was added to the legend of Figure 3 where a schematic representation of such plasmids is illustrated.

Minor Concerns:

1. Please do not reference unpublished data (lines 53,62, etc). Line 453, remove the reference.

All references to unpublished work were removed.

2. Figure 6: Please number your primers 1-4 and not 5-8. Please provide the sequence of these primers.

Primers are numbered sequentially as reported in the panels of Fig. 3. Renaming them as suggested would create duplicate primers numbers. Primer sequences were published as part of previous work, the appropriate reference was added in the figure legend.

3. Line 268: As the authors mention later, the lack of transient expression in injected mosquitoes is not an indication as to the integration of injected plasmid into the genome. Authors should make clear that all G0 injected larvae should be reared.

A line has been added in the discussion with the recommendation of rearing all surviving G0s (line571).

4. Line 402: Please translate to numbers - a good number of eggs to inject (500-600) will yield 50-100 larvae, and 2-7 transgenic individuals.

Numbers were incorporated in the discussion text (lines 435-438). 'We suggest injecting at least 500 embryos, which should lead to the hatching of at least 100 G0 larvae and to 2-7 G0 adult founders from which stably transformed progeny can be obtained. If screening for transient expression in G0 larvae, up to 40 positive larvae can be expected.'

Reviewer #3:

Manuscript Summary:

PhiC31-mediated integration and cassette exchange is a powerful method for facilitating generation of transgenic animals. It allows integration into specific sites that have been characterized as being suitable for insertion of foreign DNA (for instance, insertion sites that are not in genes causing deleterious mutations). By using one site for integration of different transgenes, it controls for

chromosomal position effects for comparison of the different transgenes. This manuscript describes using this method to generate transgenic mosquitoes of the medically important *Anopheles* genus. This is a very well written protocol with comprehensive description of background as well as methods. I have only two comments.

Major Concerns:

None.

Minor Concerns:

Line 187: In my experience, unless working with less than 1 ng/ul DNA, the DNA immediately precipitates after adding EtOH. No need for the recommended incubations. Also, centrifugation and the 70% EtOH wash can be done at room temp. This simplifies this part of the protocol.

[Suggested modifications were incorporated in the protocol at steps 2.3 and 2.5.](#)

Line 452, Fig 4: Showing a comparison larvae negative (autofluorescence) and positive for the fluorescent protein would be informative. This is also true for Fig 5.

[A figure was included to show CFP background. This is now Figure 6.](#)