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## Using the GAL4-UAS system for functional genetics in Anopheles gambiae

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**TITLE:**

Using the GAL4-UAS system for functional genetics in *Anopheles gambiae*

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**KEYWORDS:**

3xP3, Localization, Endogenous Expression, RNAi Knockdown, Microscopy, bipartite, Insecticide resistance, vectorial capacity.

**SUMMARY:**

The bipartite GAL4-UAS system is a versatile tool for modification of gene expression in a controlled spatiotemporal manner which permits functional genetic analysis in *Anopheles gambiae*. The procedures described for using this system are a semi-standardized cloning strategy, sexing and screening of pupae for fluorescent protein markers and embryo fixation.

**ABSTRACT:**

The bipartite GAL4-UAS system is a versatile and powerful tool for functional genetic analysis. The essence of the system is to cross transgenic 'driver' lines that express the yeast transcription factor GAL4 in a tissue specific manner, with transgenic 'responder' lines carrying a candidate gene/RNA interference construct whose expression is controlled by Upstream Activation Sequences (UAS) that bind GAL4. In the ensuing progeny, the gene or silencing construct is thus expressed in a prescribed spatiotemporal manner, enabling the resultant phenotypes to be assayed and gene function inferred. The binary system enables flexibility in experimental approaches to screen phenotypes generated by transgene expression in multiple tissue-specific patterns, even if severe fitness costs are induced. We have adapted this system for *Anopheles gambiae*, the principal malaria vector in Africa.

In this article, we provide some of the common procedures used during GAL4-UAS analysis. We describe the *An. gambiae* GAL4-UAS lines already generated, as well as the cloning of new responder constructs for upregulation and RNAi knockdown. We specify a step by step guide for sexing of mosquito pupae to establish genetic crosses, that also includes screening progeny to follow inheritance of fluorescent gene markers that tag the driver and responder insertions. We also present a protocol for clearing *An. gambiae* embryos to study embryonic development. Finally, we introduce potential adaptations of the method to generate driver lines through CRISPR/Cas9 insertion of GAL4 downstream of target genes.

## INTRODUCTION:

The bipartite GAL4-UAS system is the workhorse of functional characterization of genes in the insect model organism *Drosophila melanogaster*<sup>1-3</sup>. To use the GAL4-UAS system, transgenic driver lines, expressing the yeast transcription factor GAL4 under control of a regulatory sequence, are crossed with responder lines carrying a gene of interest or RNA interference (RNAi) construct controlled by an Upstream Activation Sequence (UAS) recognized by GAL4. The progeny of this cross express the transgene of interest in a spatiotemporal pattern dictated by the promoter controlling GAL4 expression (**Figure 1**). Phenotypes displayed by progeny of driver-responder crosses can be assessed to elucidate the function of candidate genes. Although *D. melanogaster* has been used to examine genes from other organisms<sup>4-7</sup>, the GAL4-UAS system has now been adapted for use in insects of medical and agricultural importance to provide direct analysis in the species of interest<sup>8-14</sup>.

In the African malaria mosquito, *An. gambiae*, the GAL4-UAS system was first tested by cell line co-transfection<sup>9</sup>. Multiple constructs were assayed for efficiency in different pairwise combinations and found that 14 tandemly repeated UAS supplemented with a small artificial intron (UAS-14i) displayed the widest range of activation potential when used with a panel of GAL4 drivers. To demonstrate in vivo functionality, these constructs were then used to create two separate transgenic *An. gambiae* lines by PiggyBac transformation<sup>8</sup>: a driver line carrying GAL4 driven by a midgut specific promoter, and a responder line containing both the luciferase and enhanced yellow fluorescent protein (eYFP) genes under regulation of UAS sequences. Gut specific luciferase activity and fluorescence in the progeny indicated that the system was efficient in *Anopheles*. Since then, driver lines have been created expressing transgenes in other tissues important for vectorial capacity and insecticide resistance, including oenocytes<sup>15</sup> and hemocytes<sup>16</sup>, and in a close to ubiquitous pattern<sup>10</sup>. Numerous UAS lines have also been generated to assay genes thought to be involved in metabolism and sequestration mediated insecticide resistance, cuticular hydrocarbon synthesis and to fluorescently tag different cell and tissue types (**Table 1**). For the responder lines, site-directed integration of the transgene is now performed by ΦC31 catalyzed recombination cassette exchange<sup>17,18</sup> to fix the genomic context of the UAS regulated genes. In this way, transgene expression is normalized regarding genomic insertion location, allowing for more accurate comparison of the phenotypic effects of different candidate genes.

The responder lines created to date are designed to express the transgene either at elevated levels or to reduce gene expression through RNA interference (RNAi). Usually cDNA clones are

fused to the UAS sequence to generate suitable expression plasmids, however full genomic sequences are also feasible assuming that they are not too large for cloning. To generate silencing constructs, we have used three different methods to obtain suitable tandem inverted sequences that form hairpin dsRNA that stimulates RNAi. These have included fusion PCR, asymmetric PCR and commercial synthesis of hairpin constructs. Common to each method is the inclusion of an intron sequence between the inverted sequences to provide cloning stability. Responder plasmids into which a gene of interest/RNAi construct can be inserted have been developed<sup>15</sup>. These plasmids also carry the required  $\Phi$ C31 *attB* sites for RMCE (described in Adolphi accompanying JoVE paper which describes the RCME technique in detail). Protocols covering the important steps required when selecting the sequence for insertion into one of these plasmids for overexpression are included in this manuscript. Additionally, two protocols for RNAi hairpin construct creation are described and illustrated.

When creating new lines, identification of rare transgenic individuals is crucial to breed from to establish and maintain transgenic colonies. Most importantly for the Gal4-UAS system there is a necessity to distinguish the responder and driver lines to establish crosses and identify individual progeny that carry both transgenes. This is achieved by using different dominant selectable marker genes linked to the driver and responder cassettes. Most commonly these are fluorescent marker genes that are clearly distinguishable using optical filters (e.g., eYFP, eCFP, dsRed). It is important that markers are expressed in a known and reliable spatiotemporal pattern as this makes identification of abnormalities and contamination easier. Fluorescent marker gene expression is routinely regulated by the synthetic *3xP3* promoter, which causes eye and ventral ganglia specific expression in all stages of *An. gambiae* development<sup>19</sup>. Fluorescent markers controlled by *3xP3* are included in all transformation plasmids described in this article. A protocol detailing the common methods used to screen fluorescent *An. gambiae* pupae GAL4-UAS lines is included here.

One of the key elements of the GAL4-UAS system is the necessity to cross the differentially marked driver and responder lines. To do this male and females from each line must be separated prior to mating. Adults are readily distinguishable by sight, however, for establishing genetic crosses it is sensible to separate the sexes prior to adult emergence to ensure that mating has not occurred. The general size difference between male and female *An. gambiae* pupae is too variable to be an efficient and dependable method of sex determination<sup>20</sup>. Instead clear morphological differences in the external genitalia provide a reliable basis for sexing in *An. gambiae*. In this article, we describe a dependable method for sexing *An. gambiae* pupae to set up appropriate crosses.

[Place Figure 1 here]

It is the use of crosses that provides the bipartite nature of the GAL4-UAS system, which has distinct advantages over more linear approaches. For example, many more combinations of driver and responder lines can be assessed than would be feasible if a new transgenic line had to be generated and maintained for promoter/gene combination. More importantly, it allows the analysis of genes that produce lethal or sterile phenotypes when their expression is perturbed

which are difficult to create/maintain in a linear system. Such lethal phenotypes can manifest at all developmental stages, depending on the gene function and spatiotemporal expression, but are most often observed during embryonic development. Visualizing mosquito embryo development requires the clearing of the opaque chorion which coats the eggs. Following methods described in Trpiš (1970)<sup>21</sup> and Kaiser et al. (2014)<sup>22</sup>, we describe the protocols we use to fix embryos, whilst maintaining structural integrity, and bleaching to clear the endochorion that allows microscopic visualization and imaging.

## PROTOCOLS:

### 1. Design and construction of UAS constructs

#### 1.1. Design and assembly of vectors for candidate gene expression

1.1.1. Determine the sequence to be used for candidate gene upregulation.

1.1.1.1. Sequence the cDNA/gDNA from the strain of interest and compare it to the published sequence to verify its identity and identify potential SNPs and restriction sites for diagnostic digest.

1.1.1.2. Ensure that the forward primer used for gene amplification covers the native Kozak sequence and start codon, where appropriate. A primer with ~10 bp binding upstream of the start codon will encompass the Kozak sequence.

1.1.1.3. Include the stop codon in the fragment amplified from the reverse primer in most circumstances. Use 3' termination sequences provided in the plasmid vectors described, or amplify from candidate gene genomic sequences.

1.1.1.4. Order commercial sequences with specific codon bias if desired.

1.1.2. Use standard subcloning procedures to insert gene cassettes in to UAS plasmid vectors, e.g., *pSL-attB-UAS14-gyp[3xP3-eYFP]*<sup>15</sup> (**Figure 1**) for both upregulation and RNAi constructs.

1.1.3. Produce transgenic mosquitoes created using ΦC31 recombination mediated cassette exchange as described in the this paper<sup>10,17,18,23</sup>.

#### 1.2. Creation of RNAi hairpin constructs: single step amplification using asymmetric PCR<sup>15,24</sup>

1.2.1. Extract genomic DNA (gDNA) from adult female *An. gambiae* carrying the desired candidate gene using the Livak method<sup>25</sup>.

1.2.1.1. Design the forward primer to bind to target exon at the 5' of the desired fragment directed towards the neighboring intron. Design the 3' end of a bridge primer to bind to the end

of the preceding exon to amplify the intron. The 5' end is complementary to a small fragment of the target exon immediately after the intron.

1.2.2. Run an asymmetric PCR reaction as is described in Xiao (2006)<sup>24</sup> (**Figure 2**).

1.2.3. Clone the purified PCR product into a suitable vector carrying the UAS promoter (e.g., *pSL-attB-UAS14-gyp[3xP3-eYFP]*<sup>15</sup>).

NOTE: Enzymes within the multiple cloning site which are appropriate for *pSL-attB-UAS14-gyp[3xP3-eYFP]* cloning<sup>15</sup> and the next steps required are indicated in **Figure 1**. Single enzyme digest is essential as only one restriction site is added. Dephosphorylation of the plasmid will improve cloning efficiency.

### 1.3. Construction of RNAi hairpin constructs: Fusion PCR of cDNA and gDNA<sup>15</sup>

1.3.1. Extract genomic DNA (gDNA) from adult female *An. gambiae* carrying the desired candidate gene using the Livak method<sup>25</sup>.

1.3.1.1. Include gDNA in a PCR reaction to amplify the target area of the exon and intron sequences together (**Figure 2**).

1.3.1.2. Design the 3' end of the forward primer to bind to the reverse target exon sequence to amplify towards the target intron sequence and the 5' end to carry a restriction site to facilitate cloning.

1.3.1.3. Design reverse primer (1) to bind to the 5' end of the intron and the 5' end overhang carries the first bases of the forward sequence of the neighboring exon. This overhang is used in the fusion PCR.

1.3.1.4. Purify the desired reaction product.

1.3.2. Extract RNA, remove DNA using DNase's and prepare cDNA from adult female *An. gambiae* carrying the desired candidate gene following manufacturer's protocols.

1.3.3. Use cDNA in an PCR reaction to amplify the target area of the exon only (**Figure 2**).

1.3.3.1. Design forward primer (2) so that the 3' end binds at the 3' end of the complementary target exon sequence and the 5' end of the primer carries a restriction site for use in cloning.

1.3.3.1.1. Use the forward primer from 1.3.1.2 again in this second reaction. However, this will mean that a single enzyme digest is essential. Using a second forward primer with a different restriction site will permit double digest which may increase cloning efficiency.

1.3.3.2. Design reverse primer (2) - the 3' end binds to the 5' end of the neighboring exon amplifying the target exon. The 5' end binds to the 3' end of the introns forward strand. This overhang is used in the fusion PCR.

1.3.3.3. Purify the desired reaction product.

1.3.4. Include the products of step 1.3.1 and 1.3.2 as templates for a fusion PCR reaction using standard concentrations with Forward primers 1 and 2. Purify the desired product.

1.3.5. Digest the purified product to generate the overhangs for cloning. Clone into a suitable vector downstream of UAS promoter. Appropriate enzymes for pSL-attB-UAS14-gyp[3xP3-eYFP] cloning<sup>15</sup> and the next steps required are indicated in **Figure 1**.

[Insert Figure 2 here]

## **2. *An. gambiae* pupae screening**

### **2.1. Collection of pupae for microscopic characterization**

NOTE: Throughout these protocols water refers to distilled water supplemented with 0.01% pond salt.

2.1.1. Rear *An. gambiae* mosquitoes using standard protocols (e.g., MR4<sup>26</sup>) to pupal stage.

CAUTION: Take care not to injure pupae throughout this process.

2.1.2. Collect pupae onto a clear flat dish suitable for use with a stereomicroscope (e.g., a 100 x 15 mm plastic Petri dish, avoiding the edges).

NOTE: To collect pupae we use a 3 mL plastic Pasteur pipette with about 10 mm cut from the end to widen the end and prevent injury to the mosquitoes. Screening and sexing can be completed on individuals, however, this is very slow. It is recommended to carry out screening and sexing on groups of 50-200 pupae (the size of group possible is limited by the size of dish used and is subject to personal preference). If a large number are being screened, efficiency can be increased by first aligning pupae about 4 to 5 deep in lines, and moving the target pupae out of this line.

2.1.3. Using a Pasteur pipette, carefully remove almost all the water from around the pupae. Leave just enough water around pupae so they are effectively immotile but can be moved easily with a fine brush. If they become difficult to move, then add more water.

NOTE: When enough water is removed, the pupae will lie on their side, permitting visualization of eyes for fluorescence detection and identification of dimorphic genitalia (**Figure 4DE**).

CAUTION: Ensure pupae do not desiccate. If only a very small volume of water is left it can reduce further with the heat from the lamp of the microscope and when split between pools of pupae. Additional water sometimes must be added during the process using a 3 mL Pasteur pipette to the desired group(s).

## 2.2. Identification of fluorescent markers in pupae

NOTE: The use of a low magnification stereoscope allows wide field screening, sorting can be done on an inverted compound microscope, but has to be done individually.

2.2.1. When screening for a fluorescent marker it is first crucial to know the expected patterns of expression and inheritance. Consider the following:

2.2.1.1. Color(s): determine which filter(s) to visualize the expression.

2.2.1.2. Spatiotemporal expression pattern: Understand where and in what life stage you expect to see expression.

2.2.1.3. Ratio of different phenotypes: establish what percentage of the population should carry the markers of interest.

2.2.2. Conduct fluorescent screening in darkness, as even low light can interfere with resolution of fluorescence. However, use a lamp beside the stereoscope when light is required for other manipulations.

CAUTION: Ensure that the workspace around the fluorescent stereoscope is clear prior to turning off the lights.

2.2.3. Turn on fluorescent bulb and leave to warm for manufacturer's recommended period (Normally 10-15 min). Select the required filter on the fluorescent stereoscope and check that there is a colored beam of light visible that is directed at the center of the stage plate. If this is not visible or is very faint the fluorescent bulb may not have fully warmed, the shutter is closed, or the microscope optics are not well aligned.

2.2.4. Using white light, center the pupae in the field of view and bring them into focus. This magnification may need to be changed when switching between different filters depending on the fluorescence intensity.

2.2.5. Using a fine detailing paint brush ensure that the pupae examined do not overlap.

2.2.6. Turn off the white light of the stereoscope and use the fine focus to bring the area of the pupae carrying the phenotype of interest into focus. The fluorescent pattern should be visible. Examples of *3xP3* promoter-controlled fluorescence are provided in **Figure 3**.



2.2.6.1. Use the lowest magnification at which the expected fluorescent phenotype from individuals with no fluorescence can be reliably distinguished.

2.2.6.2. For strains with bright fluorescence use a low intensity brightfield light as well while screening, if the fluorescent signal is still clearly identifiable.

2.2.6.3. When finished primary screening, rapidly scan populations under other filters to detect potential contamination.

[Insert Figure 3 here]

CAUTION: Ensure that there is a clear distance between the groups of sorted pupae to prevent contamination by pupae movement. Be aware that the size of the groups will change as pupae are sexed and that distances can appear bigger when looking under magnification. Take particular care when the pools are not within the field of view.

## 2.3. Sexing Pupae

2.3.1. Collect pupae. Remove excess water, but provide sufficient so that the anal paddles part slightly from the genitalia to aid visualization and morphological characterization (**Figure 4D,E**).

2.3.2. If any pupa/e are not on their side, use a fine detailing paint brush to gently turn the pupa and move the anal paddles so that external genitalia can be identified.

2.3.3. Separate pupae based on distinctive external genitalia; males have a long tube that extrudes from the final dorsal segment approximately half the length of the anal paddles (**Figure 4B**). The external genitalia of female pupae are considerably shorter and bifurcate (**Figure 4A**).

NOTE: On occasion, if the 4<sup>th</sup> instar larval exoskeleton remains attached or the external genitalia are damaged (**Figure 4C**), confident identification of the sex is more difficult. When the sex of a pupa is not clear, it is best practice to discard it. If the individual is to be kept, the pupa should be allowed to emerge in isolation and its sex determined using adult morphological features. It is likely that if its genitalia are damaged the individual may not mate successfully.

2.3.4. Make a pool for each sex at the opposite end of the dish to the unsexed pool, moving identified pupae across the dish using a fine detailing paint brush. Label the underside of the dish where the two pools will be gathered to identify them later.

2.3.5. When both sexing and fluorescent screening is required, perform fluorescent screening first, since it is the quicker process of the two.

[Insert Figure 4 here]

## 2.4. Sex confirmation as adults

2.4.1. Until a very low error rate has been demonstrated, confirm pupae sexing by adult morphology after emergence. Separate sexed pupae into groups of 10 or less in a clear 20 mL tube with a few mL of water, sealing with a ball of cotton wool, labelled with the expected sex and allow to emerge overnight.

NOTE: As adults are transferred the following morning it is not necessary to supply emerging adults with food.

2.4.2. Confirm the sex of emerged adults using morphological features the following day.

2.4.3. If any males are present in the female collections, discard the females, in case mating has already occurred.

2.4.4. If any females are present in male collection, remove the female/s and keep the males for crossing.

## 2.5. Setting Up GAL4-UAS system crosses

2.5.1. Aspirate the desired number of male and female adults from the tubes in step 2.4 into a cage or small bucket set up in the standard manner for *An. gambiae* rearing.

NOTE: Take care not to damage the adults during this transfer.

2.5.2. Use approximately 50 females with an equal number of males, when ~2000 adults are required from the progeny.

NOTE: Where a cross is to be fed multiple times to generate multiple batches up to 200 of each sex may be set up in 30cm x 30cm x 30cm cages. When only a small number of females (<20) are available for the cross, we add ~4x the number of males to increase the likelihood of successful mating.

2.5.3. Blood feed the crossed females and rear progeny to appropriate stage, following standard protocols<sup>26</sup>, to conduct phenotypic assessment (e.g., insecticide resistance, vectorial capacity and fitness cost assays).

2.5.4. Where maternal effect of transgene expression is likely, set up reciprocal crosses of the driver and responder lines and assay expected phenotype.

NOTE: Crosses using 'heterozygous' or mixed populations of driver and responder lines, produce progeny with each of 4 possible genotypes. This provides wild type, UAS only and GAL4 only controls, as well as the transheterozygotes GAL4-UAS with which to analyze phenotype. If homozygous populations are crossed, set up additional crosses to provide appropriate controls

to compare phenotypes. The progeny should be screened as above separating progeny carrying both or only either marker, as well as negatives, for phenotypic assessment.

## **2.6. Establishing homozygous populations from lines generated through RCME carrying alternative fluorescent markers**

NOTE: It is essential that the fluorescent marker of both lines is present at the same genomic location and they are completely distinguishable.

2.6.1. Set up a parental cross of approximately 200 adults with equal numbers of differentially marked males of one line and females of the other line following screening to select individuals displaying correct fluorescence and sex, as described above. Around one-week later blood feed the cross using established protocols<sup>26</sup>.

2.6.1.1. Rear the F1 progeny to pupae using standard protocols and collect pupae as described previously.

2.6.2. Screen for fluorescence selecting those carrying both parental markers (transheterozygous). Set up a F1 intercross with these pupae.

2.6.3. One week later, blood feed the F1 females and rear progeny to pupal stage following standard protocols.

2.6.4. Screen the F2 pupae selecting those which display **ONLY** one of the markers. These will be homozygous for the insertion. Only 25% of the progeny will be homozygous for each insertion, so ensure that enough progeny are reared to provide a stock cage (400-500).

NOTE: The selection of transheterozygous progeny must be entirely rigorous otherwise the process becomes contaminated, and complete homozygosity may not be achieved. Double check all the progeny selected for the G1 intercross.

## **3. *An. gambiae* embryo clearing protocol**

### **3.1. Blood feeding and maintenance**

3.1.1. Rear *An. gambiae* mosquitoes to adults following standard protocols (e.g., MR4).

3.1.2. Blood feed 5-7 day old female adults, ensuring most are fully engorged.

CAUTION: Throughout this protocol working quickly is essential to ensure that eggs are not allowed to desiccate.

### **3.2. Induced egg laying**

3.2.1. 3 days after blood feeding collect eggs through induced laying.

3.2.2. Assemble the oviposition chamber.

3.2.2.1. Fill oviposition pot with water to a depth of approximately 5 mM. Attach the pot to one end of a 50 mL polypropylene tube, previously cut with a hacksaw so that both ends are open. (We use a plastic disc for a pot (**Figure 5**); however, the original lid of the tube can be used instead).

3.2.2.2. Cover the other end of the cut polypropylene tube with material (hose/tights) or sections of latex glove secured with an elastic band, so adults can be introduced but cannot escape (**Figure 5**). Other alternative oviposition chamber designs exist and can be used<sup>26</sup>.

3.2.3. Carefully introduce 10-15 females (blood fed in step 3.1.2) to the oviposition chamber. Cover the oviposition chamber to produce darkness and leave for 20 minutes.

[Insert Figure 5 here]

CAUTION: Avoid moving the oviposition pot once eggs have been laid to prevent stranding and desiccation of eggs.

3.2.4. Carefully detach the 50 mL polypropylene tube from the oviposition pot, whilst ensuring not to release the mosquitoes. White eggs should be visible. Check that sufficient have been laid for proscribed purpose. Repeat if necessary.

3.2.5. Cover the pot (for dust protection) and allow eggs to mature to the developmental stage of interest.

3.2.6. Use a fine detailing paint brush to pick up eggs from the pot and place them on water in a 40 mm<sup>2</sup> excavated glass block.

### 3.3. Embryo Fixing

CAUTION: Perform all fixing steps (step 3.3) in a fume hood due to use of formaldehyde.

3.3.1. Prepare FAA solution as described in Kaiser et al. (2014)<sup>22</sup>. FAA comprises 3.6 M formaldehyde, 0.87 M acetic acid and 8.5 M absolute ethanol made up to volume with distilled water (dH<sub>2</sub>O).

3.3.1.1. For 10 mL of FAA combine 2.68 mL of 13.42 M formaldehyde, 4.96 mL of 17.14 M ethanol and 0.5 mL of 17.4 M acetic acid with 1.86 mL of distilled H<sub>2</sub>O. Fixative can be kept for at least 3 months in a tightly sealed glass container, kept in a designated chemical cupboard.

3.3.2. Carefully remove the water from the glass block with a micropipette and cover eggs in 500  $\mu$ L of FAA and oscillate gently (~25 RPM) on an orbital shaker at room temperature for 30 minutes. No color change is visible at this point.

3.3.3. Rinse eggs thoroughly with distilled water. Perform rinsing 15 times to remove all traces of formaldehyde. Using a 1000  $\mu$ L micropipette, add and then remove 1 mL of dH<sub>2</sub>O at a time ensuring not to damage the eggs while doing so.

3.3.4. Store wastewater from rinses in a designated formaldehyde discard container for disposal according to safety guidelines.

3.3.5. At this point fixed, store eggs at 4 °C overnight in water to keep them hydrated.

### 3.4. Embryo Bleaching

CAUTION: Perform all bleaching steps (step 4) in a fume hood due to the potential release of chlorine gas when sodium hypochlorite and acetic acid are combined.

3.4.1. Prepare bleaching solution (Trpiš solution - described in Trpiš (1970)<sup>21</sup> and modified according to Kaiser et al. (2014)<sup>22</sup>). Trpiš solution is 0.59 M sodium hypochlorite and 0.35 M acetic acid dissolved in distilled H<sub>2</sub>O.

3.4.1.1. For a 10 mL ovolume of Trpiš solution, combine 2.68 mL of 2.2 M sodium hypochlorite and 0.2 mL of 17.4 M acetic acid with 7.12 mL of distilled H<sub>2</sub>O.

NOTE: Trpiš solution can be stored for at least 3 months in a tightly sealed glass container and kept in a secure chemical cupboard. Solution may need to be vortexed after storage and should always be opened in a fume hood in case of release of chlorine gas.

3.4.2. Cover fixed eggs with 1 mL of Trpiš solution and incubate at room temperature for 30 minutes. Eggs will start to develop pale patches after around 5 minutes of incubation, eventually reaching a milky white color once cleared.

3.4.3. Rinse eggs as in step 3.3.3 to remove Trpiš solution.

3.4.4. Store wastewater in a designated waste container and dispose with excess water down the drain.

### 3.5. Storage

3.5.1. Store in 500  $\mu$ L of dH<sub>2</sub>O and keep between 2-8 °C for a few days. Remove most of the water carefully ahead of viewing and imaging on mass but avoid desiccation of the eggs by leaving a small volume of water in the watch glass. This will not disrupt photographing the eggs. Individual eggs can be placed on microscope slide for higher magnification imaging.

## REPRESENTATIVE RESULTS:

3xP3 expression of eYFP, dsRed and eCFP provides reliable, readily distinguishable identification of individuals possessing the marker genes producing expression in eyes and ventral ganglia of *An. gambiae* pupae (**Figure 3**). The differential morphology observed in male and female external genitalia used for sexing and an example of an unidentifiable pupae are highlighted in **Figure 4**. Removal of all water from pupae increases sexing difficulty as anal paddles obscure visualization of genitalia (**Figure 4D,E**). The bipartite GAL4-UAS system permits modification of gene expression in a controlled spatio-temporal manner that can be visualized by crossing the oenocyte (**Figure 6A,C**) and ubiquitous driver (**Figure 6B,D**) lines with the responder line UAS-mCD8:mCherry<sup>10</sup>. The morphological characteristics observed in different stages of embryonic development at 12, 24 and 36 h post laying are clear following completion of protocol number 3 (**Figure 7**).

[Insert Figures 6 and 7 here]

## FIGURE AND TABLE LEGENDS:

Table 1 – Table listing the available lines in the Lycett Laboratory, short descriptions and the reference in which the creation methodology is described. The catalog numbers for lines currently stored in the MR4 repository are noted in the description.

**Figure 1 – Diagrammatic representation of process for using the bipartite GAL4-UAS System in *Anopheles gambiae*.** (A) The major components of an example vector (pSL-attB-UAS14-gyp[3xp3-eYFP]) are depicted, detailing the available restriction sites (EcoRI, NheI, XhoI and NcoI) within the multiple cloning sites that are suitable for use to insert the hairpin construct or coding sequence for the gene of interest. The structure of the docking line is also depicted. (B) The crossing step is illustrated indicating the use of males from the driver line (carrying GAL4 driver by a promoter of interest and eCFP driven by the 3xP3 promoter) and females from the responder line (carrying the gene of interest or hairpin construct controlled by a UAS promoter and an eYFP marker controlled by the 3xP3 promoter). (C) A diagrammatic representation of GAL4 driving expression of the gene of interest in the progeny of the cross in B and a list of some of the typical phenotypes that are assessed. Abbreviations: Multiple Cloning Site (MCS), Recombinase mediated cassette exchange (RMCE), Upstream Activator Sequence (UAS), enhanced yellow fluorescent protein (eYFP), enhanced cyan fluorescent protein (eCFP).

**Figure 2 – Diagrammatic representation of the creation of RNAi constructs for insertion into pSL-attB-UAS14-gyp[3xP3-eYFP] by two methods:** (A) Single step asymmetric PCR (adapted from Xiao. Y H et al (2006) and (B) multiple step fusion PCR.

**Figure 3 – *Anopheles gambiae* pupae expressing fluorescent markers driven by the 3xP3 promoter (A) eYFP, (B) dsRed and (C) eCFP.** Magnification: A=16X, B,C=20X.

**Figure 4 – Sexing *Anopheles gambiae* pupae.** Individual pupae indicating the external genitalia of (A) a female (B) a male and (C) an individual which cannot be readily identified due to

incomplete detachment of the larval exoskeleton. Enlarged images below highlighting the external genitalia. Pupae with ♀ (female) and ♂ (male) indicating the external genitalia of pupae with (D) ~50% of the pupa submerged in water and with (E) all water removed highlighting the difference in ease of visualization of the external genitalia. Magnification: A,B,C=40x, D,E=30x.

**Figure 5 – Example of an Oviposition Chamber (A) dismantled to highlight the components and (B) assembled.**

**Figure 6 – Tissue Specific Expression Visualization. (A, B) Larvae and (C, D) adults expressing mCherry driven by (A, C) ubiquitous and (B, D) oenocyte specific driver lines. Magnification: A,B=32X, C=25X, D=40X.**

**Figure 7 – Embryo Clearing Example Results.** Images of embryos following the described protocol (A) 12, (B) 24 and (C) 36 hours post oviposition and (D) an unsuccessful attempt using household bleach where over bleaching caused discoloration and bursting of embryos. Magnification: 50x.

## DISCUSSION:

Understanding mosquito gene function is vital to develop new approaches to control *Anopheles* and impact malaria transmission. The GAL4-UAS system described is a versatile and powerful system for functional analysis of candidate genes and to date we have used the system to examine the genetic basis of insecticide resistance<sup>17</sup> and cuticular hydrocarbon production<sup>15,23</sup>, as well as to fluorescently tag different mosquito cell populations<sup>8</sup>. So far responder constructs have been generated through restriction enzyme cloning into a plasmid, carrying a multiple cloning site downstream of the UAS (**Figure 1, Table 1**). However, due to the modular nature of the constructs carrying multiple elements (attB sites, 3xP3-YFP and gypsy insulator sequences), more contemporary techniques are feasible for constructing new responder plasmids. Typically sequences for overexpression can be amplified from cDNA or gDNA using PCR, but commercial synthesis can sometimes reduce the labor and time involved in generating new constructs. It is important to sequence the target genes once amplified from respective templates to validate identity and identify potential SNPs. This is particularly relevant when commercially synthesizing DNA as it is not recommended to rely solely on database annotations.

Generating sequences for RNAi constructs is often more complex due to the requirement to clone an inherently unstable inverted repeat of the target sequence for dsRNA formation of the hairpin product. This cloning is often achieved by including an intron sequence between the repeats to enable stable replication in *Escherichia coli*, and efficient splicing in the mosquito<sup>27</sup>. Amplification of hairpin constructs using asymmetric PCR is efficient due to the single step nature. In early cycles of asymmetric PCR, the strand amplified by the forward primer is preferentially amplified as this primer is supplied to the reaction in excess (2x concentration of bridge primer). This product accumulates as single strands. The 3' end of these strands is complementary to the beginning of the target exon. In mid phase cycles, a loop forms as the complementary 3' end anneals to the start of the target exon. When this occurs, the complementary sequence of the target exon is amplified from the 3' end generating a product [inverted-exon: intron: exon]. In late cycles this final product is amplified by the forward primer and can be readily isolated and

cloned. A similar inverted repeat construct can be produced by fusion PCR<sup>28</sup> as indicated in **Figure 2**. However for both methods, a suitable target site that fulfils the required properties for RNAi<sup>24</sup> must be directly adjacent to an intron of appropriate size for cloning. When a suitable target is not available, a modified fusion PCR or commercial synthesis can be used where the inverted sequences are fused with an intron sequence as three separate fragments in a PCR reaction or *in silico* respectively. We have successfully used the 4<sup>th</sup> intron of the *Drosophila white* gene as a cleaved spacer sequence<sup>23</sup>. In this case, the company required a spacer (intron) of at least equal length to the target repeat sequences to allow efficient synthesis. As the cost of commercial synthesis increases with the size and complexity of the fragment, typically only the hairpin or gene fragment is synthesized then cloned into the desired vector as opposed to synthesis of the entire plasmid. When using commercial synthesis to generate constructs for transgenesis, it is important to keep in mind that the expected sequence annotation could have differences, such as SNPs, compared to sequences present in lab strains. Additionally, it is important to consider the impact of Kozak sequences and ensure that they are included in the final construct where required<sup>29</sup>.

As described above, separation of the GAL4 and UAS components expands the potential of each line permitting evaluation in combinations and analysis of lethal or detrimental phenotypes. For example, vastly different resistance phenotypes were observed when expressing metabolic enzymes ubiquitously, rather than in the gut or oenocytes, which were previously thought to be some of the main tissues conferring insecticide resistance<sup>17</sup>. Moreover, tissue specific expression/knockdown of genes involved in cuticular hydrocarbon production has highlighted the role of oenocytes in larval development and adult eclosion<sup>15,23</sup>. Moving forward, the generation of driver lines with alternative spatial-temporal tissue specific GAL4 expression may well be simplified by CRISPR-Cas technology. Previous driver lines were generated with limited knowledge of promoter sequences to regulate expression and were often 'hit and miss' in their activity, which is often compounded by position effects. This may be overcome by targeted insertion of GAL4 with a self-cleaving peptide (such as T2A)<sup>30</sup> downstream of a target gene which should produce expression of GAL4 in the spatiotemporal manner of the targeted gene<sup>31</sup>. This strategy has been effectively used in *D. melanogaster*<sup>32</sup> including the generation of a library of stocks expressing GAL4 under control of different endogenous promoters<sup>33</sup>. Although not tested extensively in mosquitoes yet, and there are anecdotal questions surrounding the efficiency of co-expression, the versatility and adaptability of this targeted approach is exciting.

Even when using such methods for driver production, sexing and screening of pupae in an efficient and reliable manner is fundamental to the use of the GAL4-UAS system in mosquitoes. The protocols described here are robust methods for small scale analysis. When higher mosquito numbers are required it becomes highly time consuming to manually screen for genotype. This can be overcome by crossing homozygous driver and responder lines so that all progeny will be transheterozygous, and so do not require rigorous screening and separation. It is often possible to distinguish homozygous mosquitoes based on the intensity of marker fluorescence and thus to intercross these to produce stable homozygous lines. However, the efficacy of this method is somewhat dependent on user familiarity with expression patterns. Moreover, some lines do not have consistent, unambiguous differences in expression in respect to marker copy number. An



easier way to generate homozygous lines is described in protocol 2.6, and is applicable when two lines have been generated with different fluorescent markers in the same genomic locus (e.g., through  $\Phi$ C31 site directed transformation into the same docking line). Either or both lines can be reliably made homozygous through simple crossing and screening. The method relies on being able to distinguish between the different fluorescent markers, and of the most commonly used markers, eCFP, eYFP and dsRED can be unambiguously discriminated using appropriate filter sets. Unfortunately, the emission spectra of eGFP overlaps considerably with eCFP and eYFP and cannot generally be used in conjunction with these markers, although dsRED and eGFP make an appropriate pairing.

Higher throughput of analysis can also be achieved by automatic sorting of fluorescently tagged larvae. This has been described with the Complex Object Parametric Analyzer and Sorter (COPAS) machine<sup>34</sup>, which operates in similar fashion to a fluorescence-activated cell sorter to gate individual mosquitoes based on their marker expression. The method has also been used to isolate differentially tagged males and females<sup>34</sup>. Due to size, larger stages such as pupae, have not yet been successfully screened using the COPAS system. Automated adult sex-sorting using video and machine learning algorithms has also been developed for large scale field releases<sup>35</sup>, however this is not yet cost efficient for the scale on which GAL4-UAS system transgenics are typically used. Differentially tagged sexes are not yet available for GAL4-UAS mosquitoes and so manual sexing as pupae is still necessary to perform specific crosses. The method described here for sexing pupae based on morphological differences in external genitalia is robust. However, it is subject to operator error and for trainees it is good practice to confirm the accuracy of sexing by observing if the correct adult sex has been isolated. Once confident of 100% accuracy, the pupae can be placed directly in the mating cage to save time.

We also describe an adapted method for analysis of embryo development through fixation and clarification of eggs following precisely timed collection. Our previous attempts to clear embryos using household bleach as described in Goltsev, Rezende, et al.<sup>36</sup>, Rezende, Martins, et al.<sup>37</sup>, Vargas, Farnesi, et al.<sup>38</sup> and Chang, Liu, et al.<sup>39</sup>, caused burning of the embryos as indicated in **Figure 7D**. The chlorine concentration in household bleach may vary substantially, so we opted to use high quality chemicals. We have used this method to observe the extent of embryo development following genetic modification of hormonal activation. Through observation of the developing embryo it became clear that the absence of larval hatching was due to developmental changes rather than fertility problems in the adults. Similar techniques have been used for immuno-histochemistry and RNA hybridization as described by Clemons, Flannery, et al.<sup>40</sup> and Juhn and James<sup>41</sup> respectively. As we have shown, the GAL4-UAS system in *An. gambiae* has proven invaluable for examining particular aspects of mosquito biology and development, and the basic protocols described for transgene design, fluorescent screening, manual pupae sexing and embryo clearing are required for efficient utilization of this important system.

#### **ACKNOWLEDGMENTS:**

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Wellcome Postdoctoral fellowship to LG: 215894/Z/19/Z) that have incorporated Gal4UAS analysis in the proposals.

#### DISCLOSURES:

The authors have nothing to disclose.

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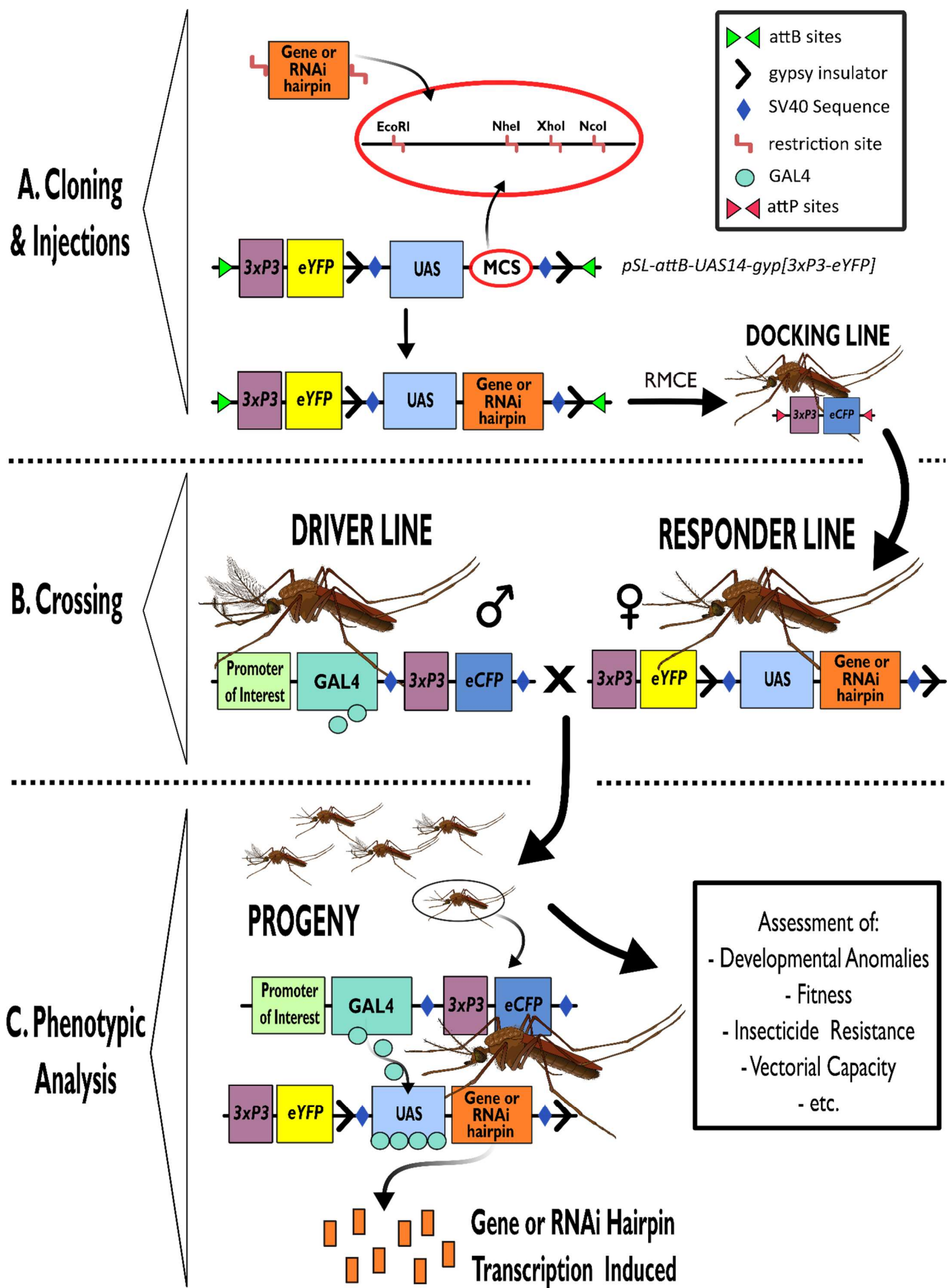
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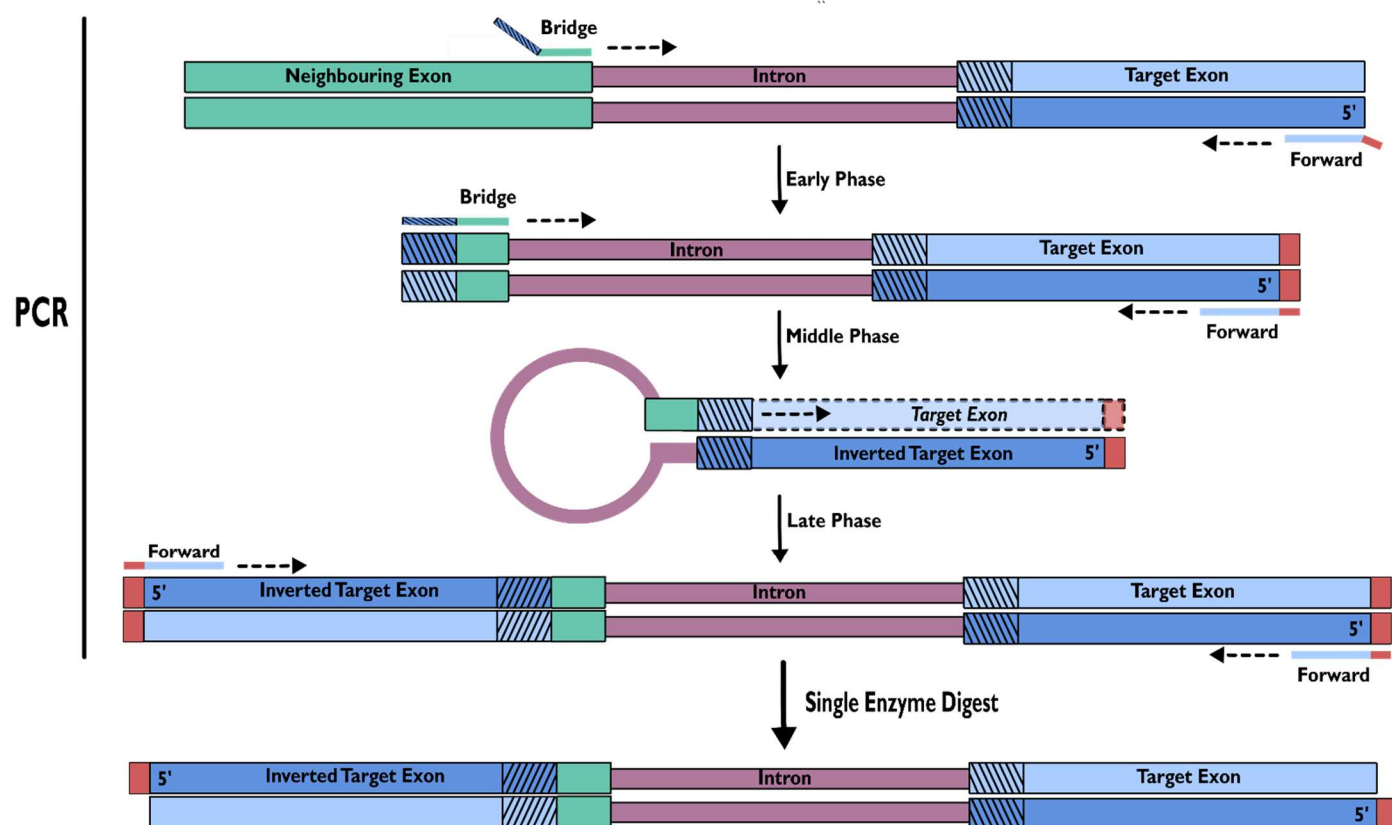
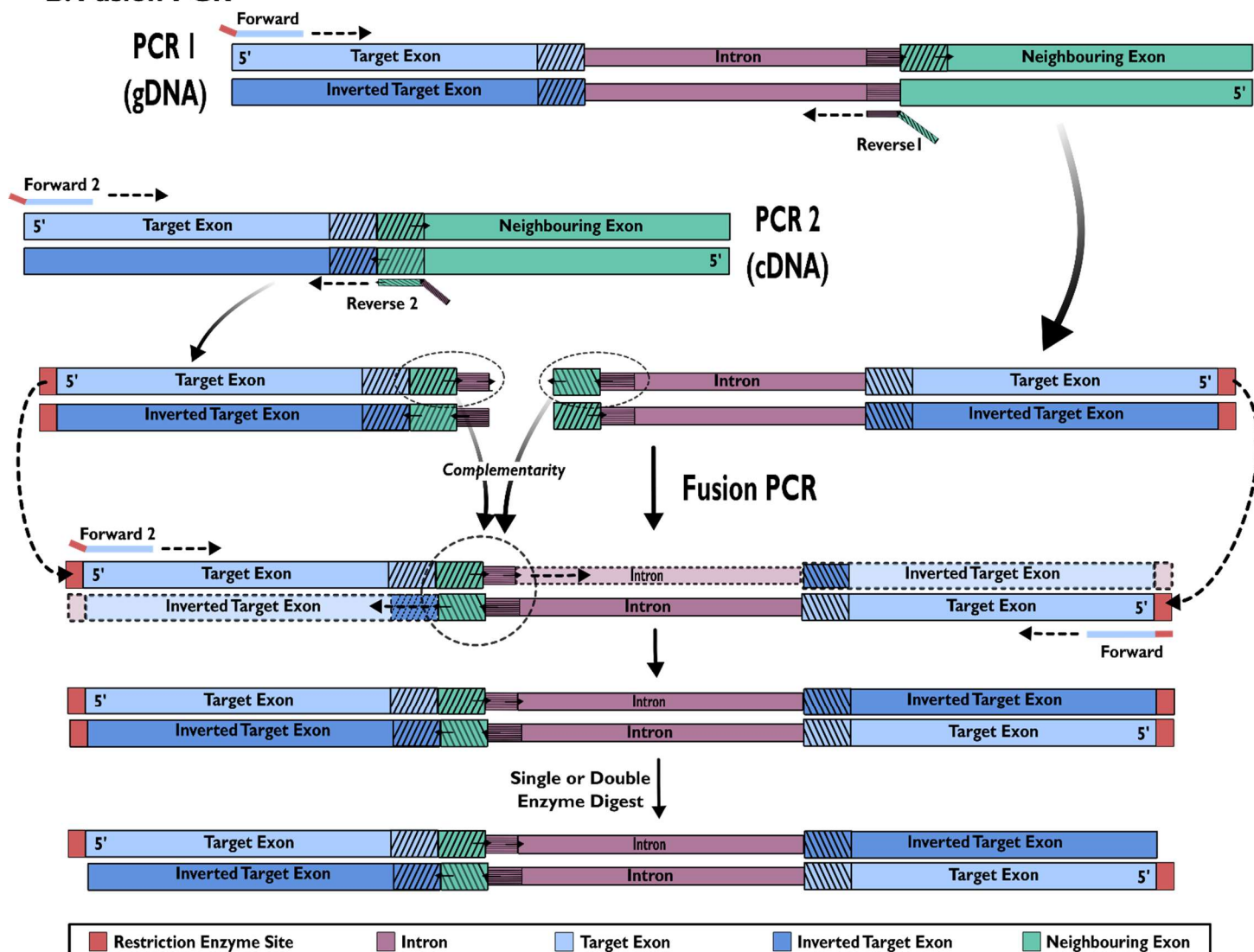
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**A. Asymmetric PCR (Adapted from Xiao.Y H et al (2006))****B. Fusion PCR**

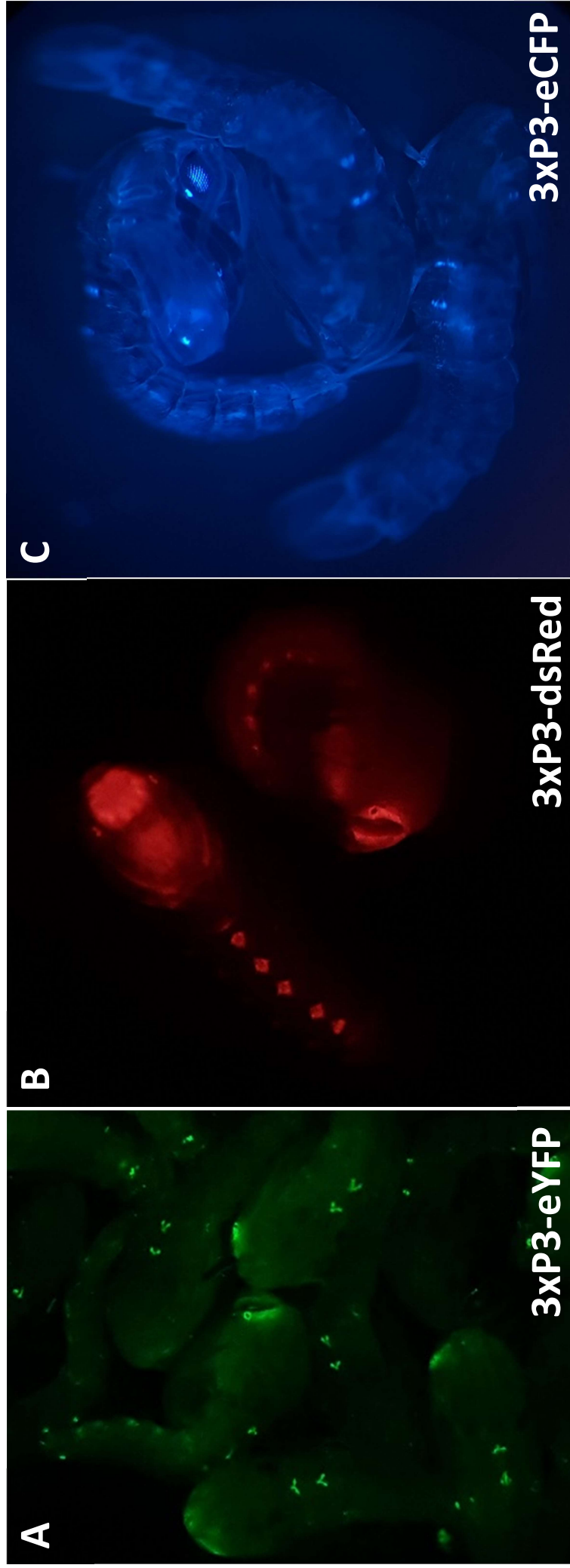
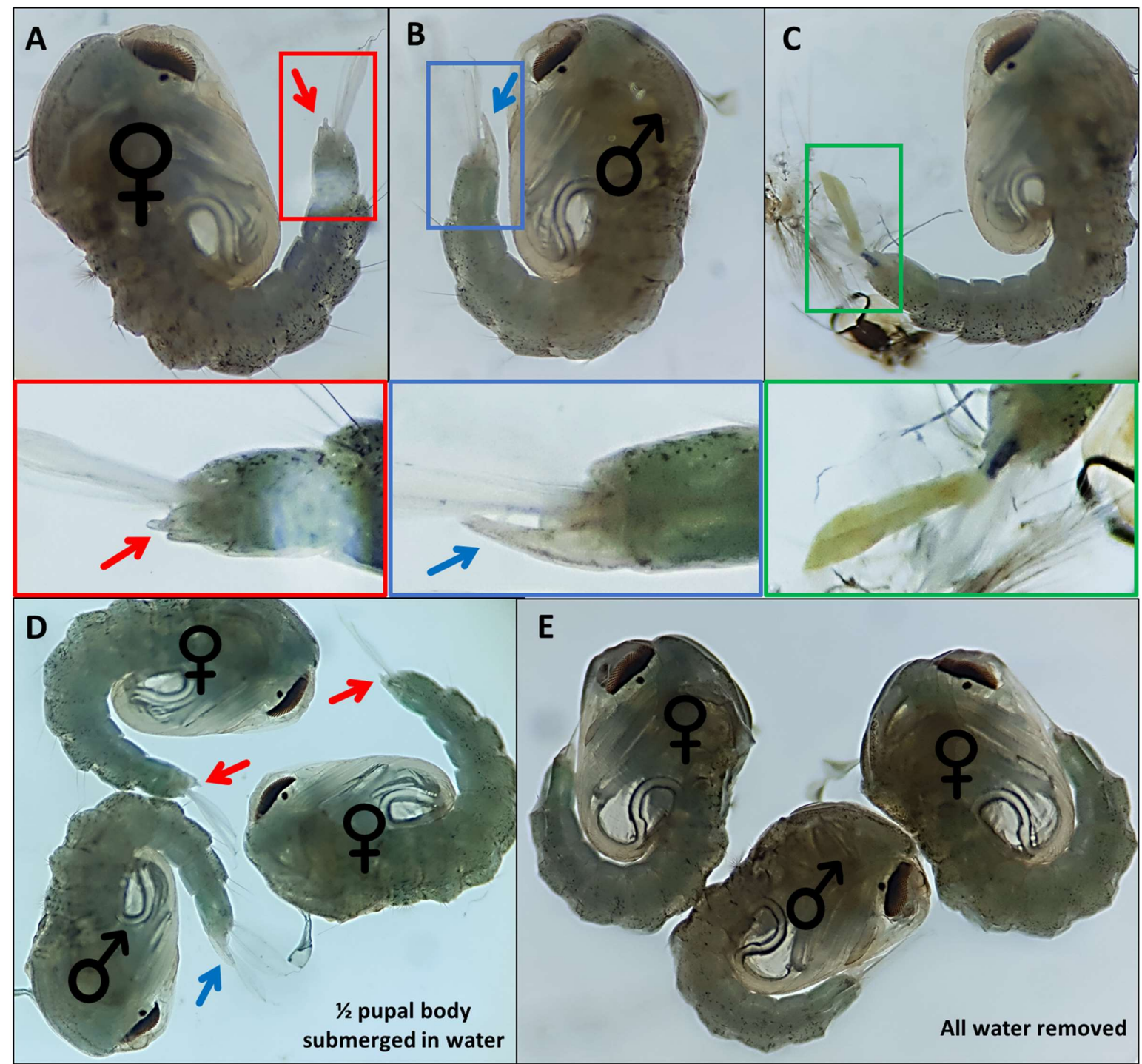
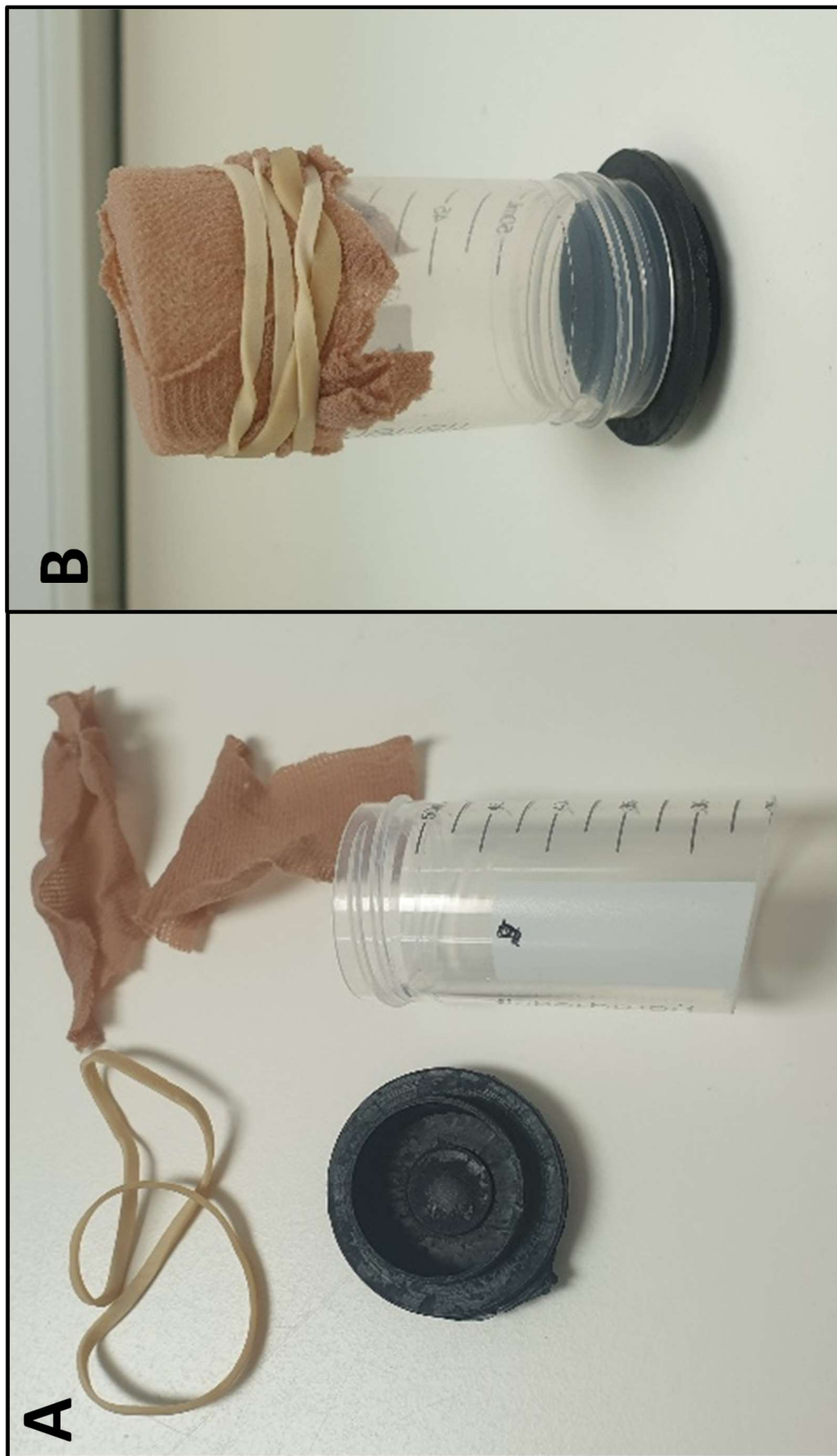


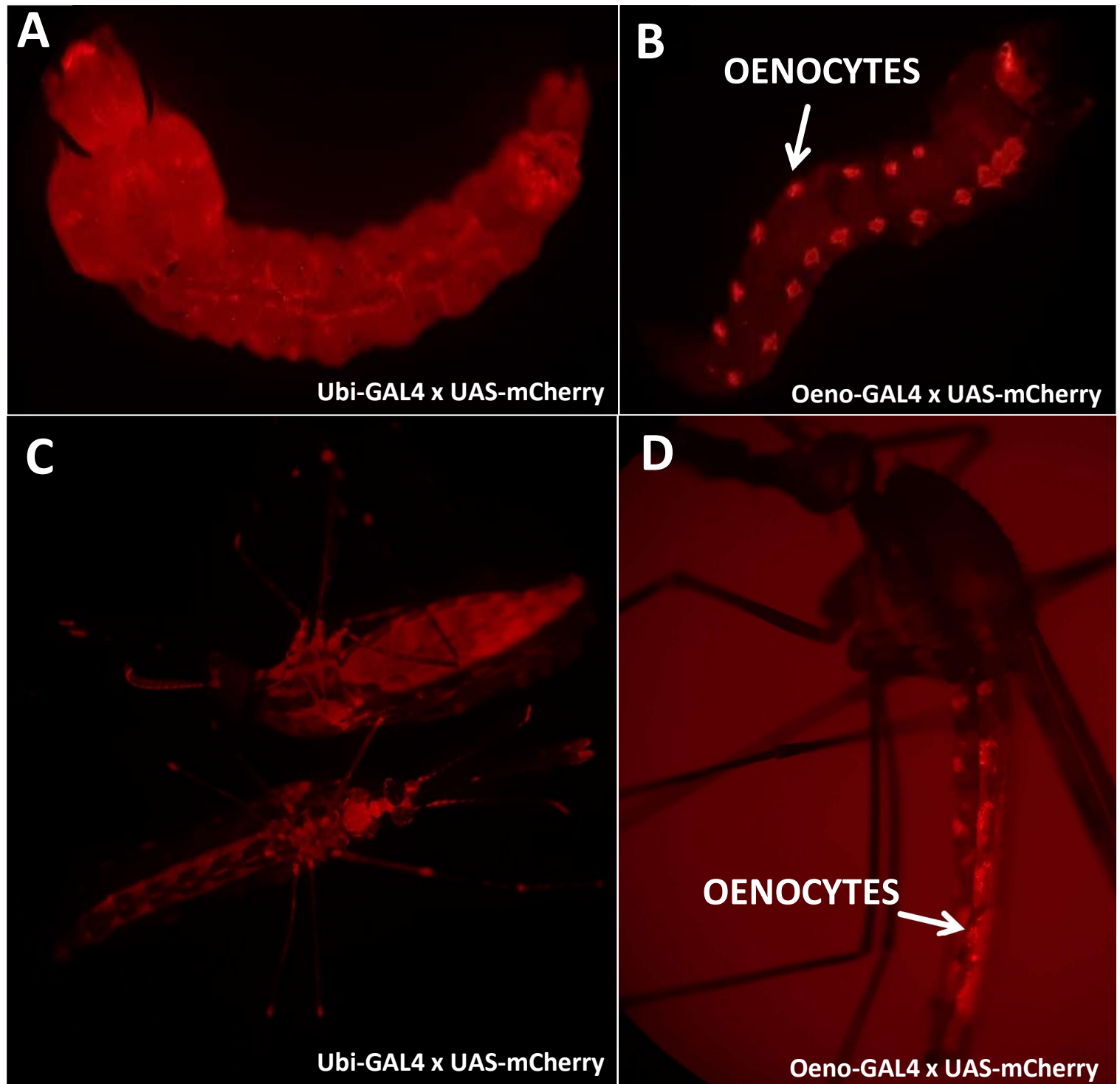


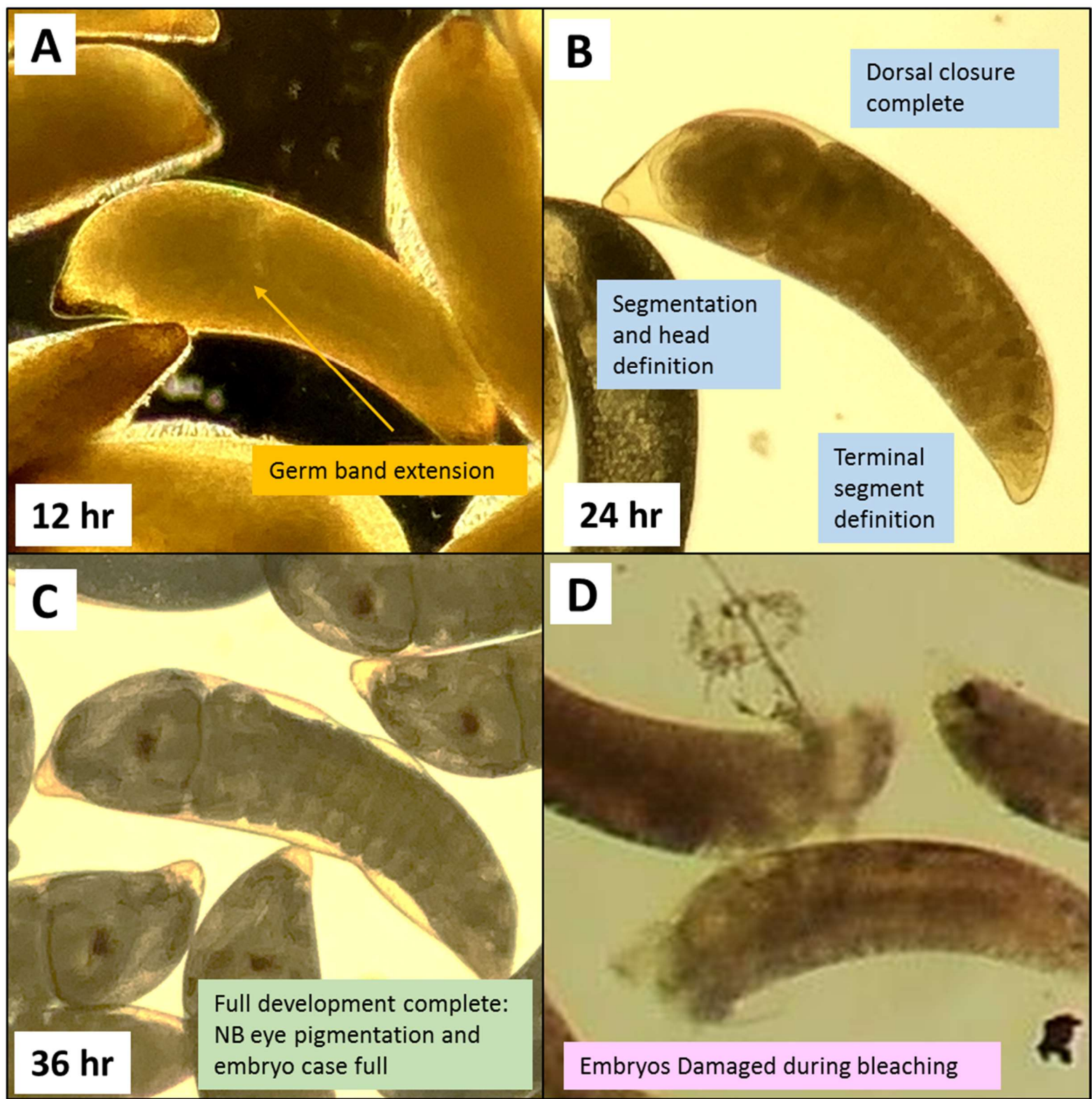
Figure 4











Type	Line	Content	3xP3 Marker	Description	Source
Responder	mCherry	UAS-mCD8:mCherry	eCFP, eYFP	Expresses the mCherry coding sequence when crossed with a driver line	Adolfi. A et al (2018)
	16i	UAS-RNAi <i>cyp4g16</i>	eYFP	Contains an RNAi hairpin construct which targets <i>cyp4g16</i> which is expressed and causes knockdown of <i>cyp4g16</i> when crossed with a driver line	Lynd. A et al (2020)
	17i	UAS-RNAi <i>cyp4g17</i>	eYFP	Contains an RNAi hairpin construct which targets <i>cyp4g17</i> which is expressed and causes knockdown of <i>cyp4g17</i> when crossed with a driver line	Lynd. A et al (2020)
	M2	UAS- <i>cyp6m2</i>	eYFP	Expresses <i>cyp6m2</i> when crossed with a driver line	Adolfi. A et al (2019)
	P3	UAS- <i>cyp6p3</i>	eYFP	Expresses <i>cyp6p3</i> when crossed with a driver line	Adolfi. A et al (2019)
	e2	UAS- <i>GSTe2</i>	eYFP	Expresses <i>GSTe2</i> when crossed with a driver line	Adolfi. A et al (2019)
	SAP2	UAS- <i>Sap2</i>	eYFP	Expresses <i>Sap2</i> when crossed with a driver line	Ingham. V et al (2019)
	FAS1899i	UAS-RNAi <i>FAS1899</i>	eYFP	Contains an RNAi hairpin construct which targets <i>FAS1899</i> which is expressed and causes knockdown of <i>FAS1899</i> when crossed with a driver line	Grigoraki. L et al (2020)
	3050i	UAS-RNAi <i>Desat3050</i>	eYFP	Contains an RNAi hairpin construct which targets <i>Desat3050</i> which is expressed and causes knockdown of <i>Desat3050</i> when crossed with a driver line	Grigoraki. L et al (2020)
	Wnd	UAS-eYFPnls-luc	eCFP	When crossed with a GAL4 line eYFP is expressed. Generated using <i>piggybac</i> (MR4 code: MRA-1165)	Lynd. A et al (2011)
	Mbl	UAS-eYFPnls-luc	eCFP	When crossed with a GAL4 line eYFP is expressed. Generated using <i>piggybac</i> (MR4 code: MRA-1164)	Lynd. A et al (2011)

Docking	A11	VG-LRIM1	eCFP	Carrying the required attP docking sites required for ΦC31 mediated cassette exchange	Lynd.A et al (2019)
Driver + Docking	A10	Ubi-A10 GAL4	eCFP	Contains the transcription factor GAL4 coding sequence controlled by the <i>An. gambiae</i> <i>Polyubiquitin-c (PUBc)</i> gene. Also carries the attP docking sites required for ΦC31 mediated cassette exchange	Adolfi. A et al (2018)
Driver	Gareth	Oenocyte enhancer-GAL4	dsRed	Expresses GAL4 controlled by an oenocyte specific enhancer	Lynd. A et al (2012)
	<i>hml</i> - GAL4	<i>hml</i> -GAL4- <i>hml</i> -GAL80	eYFP-nls	Expresses GAL4 and GAL80 driven by a hemocyte specific promoter	Pondeville. E et al (2020)
	F	carboxypeptidase promoter - GFY-GAL4	dsRed	Expresses GAL4 in the midgut and when crossed with a UAS line will express YFP. Generated using <i>piggybac</i> (MR4 code: MRA-1167)	Lynd. A et al (2011)
	Dgl	carboxypeptidase promoter - GFY-GAL4	dsRed	Expresses GAL4 in the midgut and when crossed with a UAS line will express YFP. Generated using <i>piggybac</i> (MR4 code: MRA-1166)	Lynd. A et al (2011)



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
100 x 15 mm plastic Petri dish	SLS	2175546	Pack of 10
1000 $\mu$ L Gilson Pipette	Gilson	F144059P	
20/25 mL Universal Tubes	Starlab	E1412-3020	Pack of 400
3 mL Pasteur Pipettes	SLS	G612398	Greiner Pasteur pipette 3 mL sterile individually wrapped
50 mL Falcon Tubes	Fisher Scientific	11512303	
Absolute Ethanol	Fisher Scientific	BP2818-500	500 mL
Acetic Acid	SLS	45726-1L-F	1 L
Cages	SLS	E6099	30x30x30 with screen port
Fine Paint Brushes	Amazon	UKDPB66	KOLAMOON 9 Pieces Detail Painting Brush Set Miniture Brushes for Watercolor, Acrylic Painting, Oil Painting (Wine Red)
Fish food	Amazon		Tetra Min Fish Food, Complete Food for All Tropical Fish for Health, Colour and Vitality, 10 L
Formaldehyde Solution	Sigma Aldrich	F8775	
Mouth Aspirator	John Hock	612	
Pond Salt	Amazon		Blagdon Guardian Pond Tonic Salt, for Fish Health, Water Quality, General Tonic, pH Buffer, 9.08 kg, treats 9,092 L
Pupae Pots	Cater4you	SP8OZ	250 pots with lids
Small Plastic Buckets	Amazon		2.5 L White Plastic Pail Complete with White Lid (Pack of 10)
Sodium Hypochlorite	Fisher Scientific	S25552	

9<sup>th</sup> November 2020



Re: Revision of manuscript “Using the GAL4-UAS system for functional genetics in *Anopheles gambiae*” by Beth Poulton and colleagues.

**To the Editor in Chief, Journal of Visualized Experiments, USA**

We are pleased to send the revision of the above titled accompanying manuscript for consideration to publish in JoVE.

We thank the editor and the two anonymous reviewers for their very positive evaluation of our manuscript, and for providing excellent suggestions for modifications that have improved and clarified the text and figures. We have addressed all of their points below and indicated the lines at which these modifications can be found. The editorial and reviewers’ comments and questions are in regular type and our replies are in italics.

Yours faithfully,

Beth Poulton

## **Modifications made to address editorial comments:**

### **Editorial comments:**

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

*Corrected*

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

*No figures have been reused from previous publications.*

3. Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legend.

*We have added the magnification used to the figure legends as we do not have a scale bar for the stereoscope.*

4. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the Materials Table alphabetically by the name of the material.

*Corrected*

5. 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.

*Corrected*

6. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

*Corrected*

7. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

*Corrected*



## Modifications made to address reviewers' comments:

### Reviewer 1 Text

1. The integration of the constructs will be by RCME as alluded to in the protocol (I couldn't see the reference additional protocol). How is this integration site marked? It would be helpful if that is at least marked in Figure 1, as this might influence 3xP markers to be used.

*The markers used in the docking lines has been added to Figure 1*

2. How many docking sites are available? The Table shows 2? Is this enough? How were these integration sites validated?

*There are two RCME docking lines suitable for GAL4/UAS analysis as far as we are aware, which we have indicated in the table. As the reviewer alludes to, more may be desirable, particularly if differentially marked. We have indicated this in the text.*

*How they were validated is not really in the scope of this manuscript and is included in the accompanying paper (Adolfi et al, Site-directed  $\phi$ C31-mediated integration and cassette exchange in Anopheles vectors of malaria) which we refer in line 106. For information, the integration sites were both validated by inverse PCR and sequencing, and for the first docking line made and RCME transformation we also verified by Southern blot analysis.*

3. Typo line 210: his-> this

*Corrected*

4. Line 227. Please clarify what is meant by "0.5% of a mosquito".

*This was removed for clarity, it was supposed to refer to 1/200<sup>th</sup> of a mosquito equivalent.*

5. Are there strategies recommended for viewing expression patterns given the fluorescent markers for the UAS and GAL4 lines? For example, will a UAS-GFP work in this setup, or will the GFP be too similar to eYFP and be hard to distinguish GAL4 driven expression from 3xP-YFP expression? In other words, when does one need to be concerned about the eye markers interfering with the experimental setup?

*We have added a section in the discussion to address this.*

*Line 687 - The method relies on being able to distinguish between the different fluorescent markers, and of the most commonly used markers, eCFP, eYFP and dsRED can be unambiguously discriminated using appropriate filter sets. Unfortunately, the emission spectra of eGFP overlaps considerably with eCFP and eYFP and cannot generally be used in conjunction with these markers, although dsRED and eGFP make an appropriate pairing.*

6. Lines 360-366. This section says males are shown in Figure 4A? But the figure legend later says females are shown in this figure? Please check and clarify.

*Corrected*

7. Line 420, section 5.3. What is meant by "look for maternal effects"? How might this be visualized? Are the authors saying that it matters if the father/mother brings in the GAL4 vs UAS? If maternal effects are predicted to be a problem, do the authors recommend that the GAL4 be from the mother or the father?

*Sorry for the confusion, this isn't what we were referring to. The gal4 system can be used for many different types of phenotypic analysis – and in cases where phenotype may be a property of maternal deposition, it would be necessary to analyse this by reciprocal crosses.*

*To clarify we have added the line*

*Where maternal effect of transgene expression is likely, set up reciprocal crosses of the driver and responder lines and assay expected phenotype.*

8. Line 440, section 6.2. This section was a bit confusing. Why would this situation arise? Is this to generate homozygous lines of the GAL4 and the UAS lines after they were first tested? Otherwise, wouldn't one make homozygous lines after they were first generated? Or is it easier to make homozygous lines in this manner (which I suspect is the case). Please clarify.

*Section added to discussion to clarify:*

*It is often possible to distinguish homozygous mosquitoes based on the intensity of marker fluorescence and thus to intercross these to produce stable homozygous lines. However, the efficacy of this method is somewhat dependent on user familiarity with expression patterns. Moreover, some lines do not have consistent, unambiguous differences in expression in respect to marker copy number. An easier way to generate homozygous lines is described in protocol 2 section 6, and is applicable when two lines have been generated with different fluorescent markers in the same genomic locus (e.g. through  $\Phi$ C31 site directed transformation into the same docking line). Either or both lines can be reliably made homozygous through simple crossing and screening.*

9. If a user wanted to make a homozygous line that combined a UAS and a GAL4 insertion, how would this be done? If both are inserted at the same docking site, what are the strategies recommended by the authors?

*If both are in the same locus generated by cassette exchange then it would not be possible to have each of them in homozygosity, since there are only two possible alleles – that is the basis of selection procedure in protocol 2: section 6. ^ homozygotes through crossing described.*

*However, it is possible to generate lines carrying both Gal4 and UAS during crossover events during  $\phi$ C31 integration at a single AttP site. These will carry both Gal4 and UAS on one allele, and so can*

*be made homozygous for both elements by selecting for fluorescent intensity or, if you have a line with a third marker at that allele, by the crossing strategy in protocol 2 part 6.*

Or is maintaining a stock that has both together not recommended (and why would that be?)

*If the GAL4/UAS transgene expression is detrimental to the mosquito, over multiple generations there may be selection for reduced expression if the two elements are kept together.*

*We have not included making single integration lines and feel that to include a discussion around it would unnecessarily complicate this manuscript.*

10. Line 450. Step 6.3/6.4. Traditionally, G0/G1/G2 tends to designate transgenic made directly from injections, whereas P/F1/F2 designate lines generated from existing transgenes. So in this case, I think P/F1 would be the proper nomenclature.

*Corrected*

11. Line 555. Should just be 4.1.2

*Corrected*

12. Line 622. This differs from what is said in the main text.

*Corrected*

## **Reviewer 1 Figures**

Figure 1. Are gypsy insulators used before the SV40 sequences in these constructs? I would have thought the insulators would flank the genetic elements (i.e., UAS-promoter-geneX-sv40 or enhancer/promoter-GAL4-SV40). Please check that the diagrams are accurate.

*Corrected*

Figure 1. Please indicate the marker used by the docking line as this contributes to the genotype/marker of the integrated line.

*This has been added to figure 1.*

Figure 2. This was alluded to in the text, but what is the recommended size of the intron? Is there a

range? The authors mentioned in the text generating an artificial intron as necessary, but that is not reflected in the figure for Fusion PCR. Is there a template the authors can use for this?

*Clarification on these points has been added to the discussion section as below.*

*A similar inverted repeat construct can be produced by fusion PCR<sup>28</sup> as indicated in Figure 2. However, for both methods, a suitable target site that fulfils the required properties for RNAi<sup>24</sup> must be directly adjacent to an intron of appropriate size for cloning. When a suitable target is not available, a modified fusion PCR or commercial synthesis can be used where the inverted sequences are fused with an intron sequence as three separate fragments in a PCR reaction or in silico respectively. We have successfully used the 4th intron of the Drosophila white gene as a cleaved spacer sequence<sup>23</sup>. In this case, the company required a spacer (intron) of at least equal length to the target repeat sequences to allow efficient synthesis.*

Figure 3. Please add the genotypes (or at least the marker genotype) onto these figures. Are they all 3xP-XFPs?

*Added*

Figure 4. Please add the male/female symbol onto panels A and B. Please add an arrow pointing to the distinguishing features in A,B and also in D.

*Added*

Figure 6. Please add genotype to this figure. This will make it easier to appreciate what is being shown. It might make more sense to label panel C as panel B so that its clearer that these two mosquitoes are matched (same genotype). Right now, it looks like A and B are representing the same information.

*Genotypes added to the figure. This makes mosquito pairing clear so we have not changed the letter labels.*

Figure 7. It would be helpful if the embryos in D were taken at the same magnification as the other embryos in A-C. To the untrained eye, the embryos in D look similar to the embryos in B. It might be helpful to point out what makes the authors say that the embryos in D are 'damaged'.

*Image corrected as recommended and details of damage added to the figure legend.*

*Line 606 - (D) an unsuccessful attempt using household bleach where over bleaching caused discoloration and bursting of embryos.*

**Reviewer 2**

*All typographic errors and suggestions indicated in the reviewer attached PDF have been corrected as advised.*