Video Article

Fluorescent *in situ* Hybridization on Mitotic Chromosomes of Mosquitoes

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Abstract

Fluorescent *in situ* hybridization (FISH) is a technique routinely used by many laboratories to determine the chromosomal position of DNA and RNA probes. One important application of this method is the development of high-quality physical maps useful for improving the genome assemblies for various organisms. The natural banding pattern of polytene and mitotic chromosomes provides guidance for the precise ordering and orientation of the genomic supercontigs. Among the three mosquito genera, namely *Anopheles*, *Aedes,* and *Culex*, a well-established chromosome-based mapping technique has been developed only for *Anopheles*, whose members possess readable polytene chromosomes [1]. As a result of genome mapping efforts, 88% of the *An. gambiae* genome has been placed to precise chromosome positions [2,3] . Two other mosquito genera, *Aedes* and *Culex,* have poorly polytenized chromosomes because of significant overrepresentation of transposable elements in their genomes [4, 5, 6]. Only 31 and 9% of the genomic supercontings have been assigned without order or orientation to chromosomes of *Ae. aegypti* [7] and *Cx. quinquefasciatus* [8], respectively. Mitotic chromosome preparation for these two species had previously been limited to brain ganglia and cell lines. However, chromosome slides prepared from the brain ganglia of mosquitoes usually contain low numbers of metaphase plates [9]. Also, although a FISH technique has been developed for mitotic chromosomes from a cell line of *Ae. aegypti* [10], the accumulation of multiple chromosomal rearrangements in cell line chromosomes [11] makes them useless for genome mapping. Here we describe a simple, robust technique for obtaining high-quality mitotic chromosome preparations from imaginal discs (IDs) of 4th instar larvae which can be used for all three genera of mosquitoes. A standard FISH protocol [12] is optimized for using BAC clones of genomic DNA as a probe on mitotic chromosomes of *Ae. aegypti and Cx. quinquefasciatus,* and for utilizing an intergenic spacer (IGS) region of ribosomal DNA (rDNA) as a probe on *An. gambiae* chromosomes. In addition to physical mapping, the developed technique can be applied to population cytogenetics and chromosome taxonomy/ systematics of mosquitoes and other insect groups.

Video Link

The video component of this article can be found at https://www.jove.com/video/4215/

Protocol

1. Chromosome Preparation

Mosquito larvae were reared using a standard protocol described in Methods in Anopheles Research available at the website of the Malaria Research and Reference Reagent Resource Center (MR4) [13]. The temperatures of mosquito rearing were modified to provide the highest number of chromosomes in imaginal discs and lowest mortality of the larvae. The stages of mosquito larvae development were determined based on the sizes of their head capsules [13].

1. Hatch mosquito eggs at 28 °C, and after 2-3 days, transfer 2nd or 3rd instar larvae to 16 °C for *Ae. aegypti* and *Cx. quinquefasciatus* and to 22 °C for *An. gambiae.*
2. Place 4th instar larvae on ice for several minutes for immobilization.
3. Transfer larva to a slide with a drop of cold hypotonic solution (0.5% sodium citrate or 0.075 M potassium chloride), and place it under the stereo microscope.
4. Select larva with oval IDs (**Figure 1B**) for further dissection.
5. Decapitate larva, and cut the cuticle from the ventral side of the larval thorax using dissecting scissors (**Figure 2A**). Make additional cut in second or third abdominal segment to dissect the gut from the larva. The directions of the cuts are shown by arrows.
6. Open the cuticle, and remove the gut and fat body from the larva. Remove the hypotonic solution from the slide using filter paper, and add a fresh drop of hypotonic solution directly to the IDs (**Figure 2B**). Keep larva in hypotonic solution for 10 min at RT.
7. Remove hypotonic solution using filter paper, and apply Carnoy's solution (ethanol/acetic acid in 3:1 ratio). After adding fixative solution, IDs immediately turn white and become easily visible under the microscope (**Figure 2C**).
8. Using dissecting needles, remove IDs from the larva (**Figure 2D**), and transfer them to a drop of 50% propionic acid. Remove any other tissues, such as the gut and fat body, from the slide. Cover IDs with an unsiliconized 22x22 cover slip, and keep for 10 min at RT.
9. Cover the slide with filter paper, and squash the tissue by tapping the eraser of a pencil on the perimeter of the cover slip.

10. Briefly analyze the quality of the slide using the phase-contrast microscope at 100x or 200x magnification (**Figure 3**). Preparations with >50 chromosome spreads can be considered suitable for FISH.
11. Dip and hold the slide in liquid nitrogen until it stops bubbling. Remove the cover slip from the slide using a razor blade, and transfer the slide immediately to a container of 70% ethanol chilled at -20 °C. Store at 4 °C for at least 1 hr for the best dehydration result (if necessary, slides can be stored at this step from several minutes to several days).
12. Dehydrate slides in a series of ethanol (70%, 80%, 100%) at 4 °C for 5 min each, and air dry at RT.
13. Store dry slides at -20 °C before utilizing them for FISH.

2. Extraction of Repetitive DNA Fractions

Performing FISH of the BAC clone DNA probe on chromosomes from Ae. aegypti *and* Cx. quinquefasciatus *requires using unlabeled repetitive DNA fractions to block unspecific hybridization of the DNA repeats to the chromosomes. The reassociation of single-strand DNA fragmented into pieces of several hundred bp follows a C_0t curve where C_0 is the initial concentration of single-stranded DNA and t is the reannealing time. DNA fractions with C0t values equal to 10^{-4}-10^{-1} or 10°-10^{2} are considered as highly and moderately repetitive, respectively.*

1. Extract 400-500 µg of the genomic DNA from entire adult mosquito using Qiagen Blood and Cell Culture Maxikit, and prepare 100-1,000 ng/µl DNA solution in 1.2x SSC.
2. Denature DNA by placing a safe-lock tube with genomic DNA into a heating block prewarmed to 120 °C for 2 min. High temperature helps to range DNA into 200-500 bp fragments.
3. Depending on the DNA concentration, reassociate DNA by placing the tube at 60 °C for 15-150 min to obtain C_0t DNA fractions up to C_0t3 (Table 1).
4. Place the tube with DNA on ice for 2 min.
5. Transfer the DNA to 42 °C, add preheated 10x S1 nuclease buffer and S1 nuclease to a final concentration of 100 U per 1 mg of DNA, and incubate for 1 hr.
6. Precipitate DNA by adding 0.1 volume of 3 M sodium acetate and 1 volume of isopropanol at RT.
7. Centrifuge at 14,000 rpm for 20 min at 4 °C.
8. Wash DNA in 70% ethanol, and centrifuge again at 14,000 rpm for 10 min at 4 °C.
9. Air-dry and dissolve DNA pellet in TE buffer.
10. Measure the DNA concentration, and visualize by gel electrophoresis. Usually the final quantity of repetitive DNA fractions represents 35-50% of the original DNA amount.

3. DNA Probe Labeling

Two different protocols were used for the labeling BAC clone DNA probe and IGS rDNA probe.

3.1 BAC clone labeling using nick-translation

1. Extract BAC clone DNA from the BAC library using Qiagen Large Construct Kit.
2. Prepare reaction mixture for nick-translation labeling on ice with final volume of 50 µl: 1 µg isolated BAC clone DNA, 0.05 mM each of unlabeled dATP, dCTP, and dGTP and 0.015 mM of dTTP; 1 µl of Cy3-dUTP (or another fluorochrome); 0.05 mg/ml of BSA, 5 µl of 10x nick-translation buffer, 20 U of DNA-polymerase I, and 0.0012 U of DNase.
3. Incubate at 15 °C for 2.5 hr.
4. Stop reaction by adding 1 µl of 0.5 M EDTA.
5. Store probe at -20 °C in a dark place.

3.2 IGS rDNA labeling using PCR

1. Prepare reaction mixture on ice with final volume of 50 µl: 200 ng of genomic DNA; 0.05 mM each of unlabeled dATP, dCTP, and dGTP; 0.015 mM of dTTP; 1 µl of Cy3-dUTP (or another fluorochrome); 5 µl of 10x PCR-buffer; 50 pmol of forward; UN (GTGTGCCCCTTCCTCGATGT) and reverse; GA (CTGGTTTGGTCGGCACGTTT) primers for IGS amplification; and 10 U of Taq DNA polymerase [14].
2. Perform PCR reaction using standard PCR parameters for IGS amplification: 95 °C /5 min x 1 cycle; (95 °C /30 sec, 50 °C /30 sec, 72 °C /30 sec) x 30 cycles; 72 °C /5 min x 1 cycle; and 4 °C hold [14].
3. Store probe at -20 °C in a dark place.

4. Fluorescent *in situ* Hybridization

This FISH protocol includes two variations: the first for using BAC clone DNA as a probe on mitotic chromosomes of Ae. aegypti *and Cx. quinquefasciatus and the second for using IGS rDNA on mitotic chromosomes of An. gambiae. If using BAC clone DNA probes, skip RNase treatment steps 4.3, 4.4, and simultaneous slide/probe denaturation step 4.19. If using IGS rDNA probe, prepare hybridization mixture without C_0t DNA fractions, and skip separate slide/probe denaturing steps 4.10, 4.11, 4.16, and 4.17.*

1. Incubate slides in 2x SSC for 30 min at 37 °C.
2. Dehydrate slides in series of 70%, 80%, and 100% ethanol for 5 min each at RT, and air dry. *If performing FISH with BAC clone DNA, proceed directly to step 4.5.*
3. Incubate chromosome preparation in 0.1 mg/ml RNase solution under parafilm for 30 min at 37 °C**.**
4. Wash twice in 2x SSC for 5 min each at 37 °C.
5. Put slides in a jar with 0.01% pepsin and 0.037% HCl solution, and incubate for 5 min at 37 °C.
6. Wash slides in 1x PBS for 5 min at RT.

7. Fix chromosome preparation in a jar with 1% formalin in 1x PBS prepared from 10% neutral-buffered formalin for 10 min at RT.
8. Wash slides in 1x PBS for 5 min at RT.
9. Dehydrate slides in series of 70%, 80%, and 100% ethanol for 5 min each at RT, and air dry preparations at 37 °C. *If performing FISH with IGS, proceed directly to step 4.12*
10. Denature slides in a jar with prewarmed 70% formamide for 2 min at 72 °C.
11. Dehydrate slides in series of cold (-20 °C) 70%, 80%, and 100% ethanol for 5 min each, and air dry at 37 °C.
12. Prepare hybridization mixture: 5 µl of labeled probe DNA from step 3, 10 µl of C_0t DNA from step 2 with final concentration of 0.5 ng/µl, and 5 µl of 1 µg/µl sonicated salmon sperm DNA. *For FISH with IGS rDNA, prepare hybridization mixture without C_0t DNA fractions.*
13. Precipitate DNA by adding 0.1 volume of 3 M sodium acetate and 2 volumes of ethanol. Keep at -20 °C for 1-3 hr.
14. Centrifuge at 14,000 rpm at 4 °C for 20 min, remove the ethanol, and air dry the pellet at RT.
15. Thoroughly dissolve the pellet in 10 µl of hybridization buffer: 50% formamide, 20% dextran sulfate, 2x SSC. *If performing FISH with IGS, proceed directly to step 4.18*
16. Denature hybridization mixture for 7 min at 97 °C, and immediately put on ice for 1 min.
17. Prehybridize mixture at 37 °C for 30 min to prevent unspecific hybridization of repetitive DNA to the chromosomes.
18. Place 10 µl of the hybridization mixture on the slide, and cover with a 22x22 cover slip. Prevent bubble formation - air bubbles should be removed with gentle pressure to the coverslip. *If performing FISH with BAC clone DNA, proceed directly to step 4.20*
19. Denature the probe and chromosome DNA simultaneously using a heating block at 75 °C for 5 min.
20. Glue cover slip around the perimeter using rubber cement.
21. Perform overnight hybridization in a humid chamber at 37 °C.
22. Remove rubber cement and coverslip from the slide.
23. Wash slide 2 min in prewarmed Solution 1 (0.4x SSC, 0.3% Nonidet-P40) at 73 °C.
24. Wash slides in Solution 2 (2x SSC, 0.1% Nonidet-P40) for 5 min at RT.
25. Counterstain slide using 0.001 mM YOYO-1 in 1x PBS for 10 min in humid chamber at RT.
26. Mount in a small amount of Prolong Gold antifade reagent with a cover slip.
27. Analyze preparations under a fluorescent microscope using appropriate filter sets at 1,000x magnification (**Figure 4**).

5. Representative Results

Insect IDs are located in each segment of the larva. Depending on the position, they transform into different tissues at the adult stage of the insect. The IDs, which are used for the chromosome preparation in this protocol, develop into legs at the adult stage of the mosquito. These IDs are located at the ventral side of the larval thorax and are clearly visible through the cuticle under the microscope (**Figure 1**). At the early 4th instar larval stage, IDs have a round shape (**Figure 1A**). The largest numbers of mitosis, ~175 in one ID [9], are accumulated at a later "oval shaped" stage (**Figure 1B**), which must be considered the optimal stage for slide preparation. At this time, the intermediate ID splits into two: one transforms into a leg and another one transforms into a wing. We prefer using the large leg IDs at the "oval-shaped" stage for the chromosome slide preparation. **Figure 1C** represents IDs at the latest stage of 4th instar larva development. At this stage, the IDs are already developed into legs and wings, and contain a significant amount of differentiated tissues and a low number of mitosis. This stage of ID development should be avoided for chromosome slide preparation. We also recommend rearing mosquito larvae at low temperatures: 16 °C for *Aedes* and *Culex* and 22 °C for *Anopheles.* This helps to increase the amount of mitosis in IDs [9].

Figure 2 illustrates ID dissection from the thorax of 4th instar larva. Because the cuticle of a live insect is hard to dissect, we recommend using dissecting scissors instead of the needles commonly used for larva preparation. The most crucial procedure for obtaining high-quality chromosome preparation is the hypotonic solution treatment. For best results, we remove the gut and fat body from the larval thorax before this treatment. Swelling of the ID cells during this procedure helps to spread chromosomes on a slide (**Figure 3A**). The appropriate quality of the hypotonic solution treatment can be easily recognized by the round shape of cells in the preparations (**Figure 3A, B**). Cells with an oval shape indicate insufficient hypotonic solution treatment (**Figure 3C**). To be selected for FISH, chromosome preparation should contain at least 50 high-quality chromosome spreads. Normally, ~90% of the slides prepared using this protocol have sufficient quality for FISH [9].

We present two slightly different FISH protocols: an advanced protocol for FISH using genomic BAC clone DNA probe on mitotic chromosomes of *Aedes* and *Culex* and a simple FISH protocol for IGS rDNA probe on mitotic chromosomes of *Anopheles*. The genomes of *Aedes* and *Culex* are highly repetitive because of the overrepresentation of transposable elements [7,8]. Thus, performing FISH, which utilizes genomic BAC clone DNA as a probe, requires adding unlabeled repetitive DNA fractions to the probe to block unspecific hybridization of the DNA repeats to chromosomes. For the extraction of the repetitive DNA fractions, genomic DNA is denatured at 120 °C for 2 min. Boiling DNA at a high temperature also helps to obtain DNA in fragments of 200-500 bp. DNA is allowed to reassociate after this treatment. The highly repetitive DNA fragments tend to find their mate for reassociation faster than DNA with unique sequences does. As a result, the reassociation of DNA follows a $C_0 xt$ curve where C_0 is the initial concentration of single-stranded DNA, and t is the reannealing time. DNA fractions with C_0t values equal to 10-4--10-1 or 100-102 are considered highly and moderately repetitive, respectively. The time of the reassociation for different C_0t DNA fractions can be calculated using the formula $t = C_0tX \times 4.98/C_0$, where t - time of incubation, C_0tX - C_0t fraction ($C0t1$=1, C_0t2=2, etc.) and C_0 - initial DNA concentration in µg/µl[15] (Table 1). After reassociation, the single-stranded DNA is digested using S1 nuclease. We prefer using all C_0t DNA fractions up to C_0t3 together instead of the commonly used C_0t1 DNA fraction. These C_0t fractions include some of the moderately repetitive DNA sequences and together usually represent 35-50% of the original amount of the genomic DNA in *Ae. aegypti*. The correct proportion between labeled DNA probe and unlabeled C_0t DNA fraction depends on the repetitive DNA component in each particular BAC clone. On average, we use 1:20 probe to C_0t DNA fraction proportion for obtaining an acceptable signals/background ratio of the FISH result. Prehybridization of the DNA probe with C_0t DNA fractions in a tube for 30 min before the actual hybridization on the slide also helps to reduce background. Labeling, hybridization itself, and washing in this protocol are performed using standard conditions [12].

The FISH results of two differently labeled BAC clone DNA probes on mitotic chromosomes of *Ae. aegypti* and *Cx. quinquefasciatus* are shown in **Figures 4A** and B, respectively. The BAC clone DNA probes produce strong signals in a single position on the chromosomes. Chromosomes shown in **Figure 1** are counterstained with YOYO-1 iodide. This dye produces the best banding patterns on *Ae. aegypti* chromosomes [9]. Alternatively, other fluorescent dyes, such as DAPI or propidium iodide, can be utilized for the chromosome counterstaining. For suppressing

photobleaching of the slides, we use Prolong Gold antifade mounting medium. This reagent has good signal preservation abilities and also can be easily removed from the slide by rinsing in 1x PBS if it is necessary to use the same slide for several hybridizations.

A simple version of the FISH protocol is designed for hybridization of IGS rDNA probe on mitotic chromosomes of *Anopheles*. Ribosomal genes in *Anopheles* are represented as a polymorphic cluster of genes located on sex chromosomes [16]. A DNA probe in this protocol is labeled using standard PCR reaction by adding fluorescently labeled Cy3 or Cy5 dNTPs. Because blocking unspecific hybridization of repetitive DNA in euchromatin is not needed, all steps related to using C_0t DNA fractions are omitted. Instead, chromosome preparations are pretreated with RNase for preventing hybridization of the IGS rDNA probe to the nucleolus. Chromosomes and the DNA probe are denatured simultaneously by heating the slide together with a probe in a hybridization system at 75 °C for 5 min. Hybridization and washing in this protocol are also performed using standard conditions for FISH [12]. The result of FISH is demonstrated in Figure 4C: the polymorphism of the IGS rDNA hybridization between two X chromosomes is clearly visible.

	DNA concentration µg/µl	**Reannealing time, min**
C_0t 2	0.1	100
	0.3	33
	0.5	20
	0.7	14
	0.9	11
	1	10
C_0t 3	0.1	150
	0.3	50
	0.5	30
	0.7	21
	0.9	17
	1	15

Table 1. DNA concentration and reannealing times for preparation of C_0t2 and C_0t3 fractions.

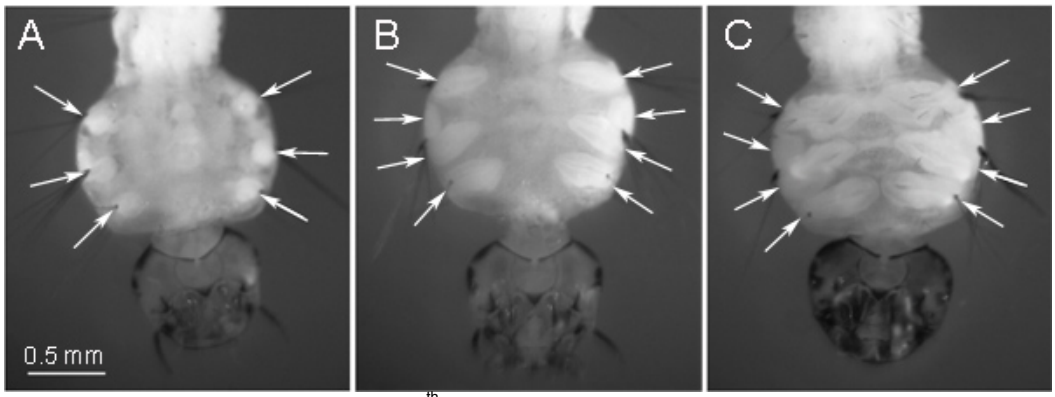

Figure 1. Stages of the ID development in 4th instar larva: A) an early "round shape" stage; B) an intermediate "oval shape" stage - optimal for the chromosome preparation; C) a late stage - inappropriate for chromosome preparations. The positions of IDs are indicated by arrows on the ventral side of the larval thorax.

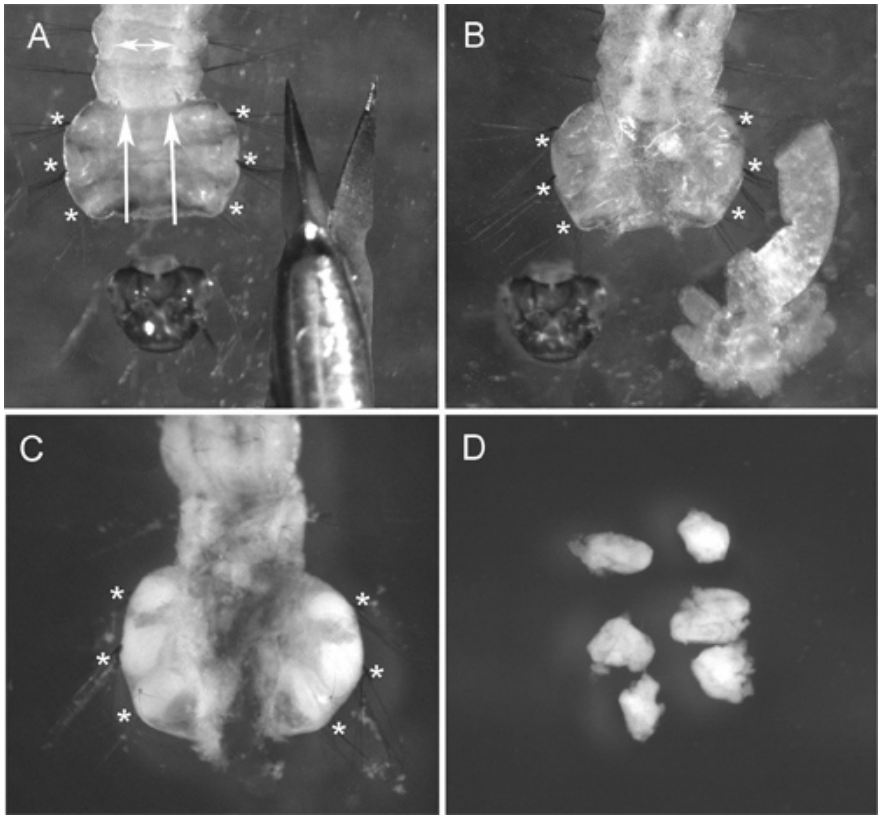

Figure 2. Steps of ID dissection: A) decapitated larva (the direction of cuts are indicated by arrows); B) larvae with dissected gut under hypotonic solution treatment (IDs swell and become almost invisible); C) larva after Carnoy's solution application (IDs become white and clearly visible); D) dissected IDs in Carnoy's solution. Positions of IDs in larva are indicated by asterisks.

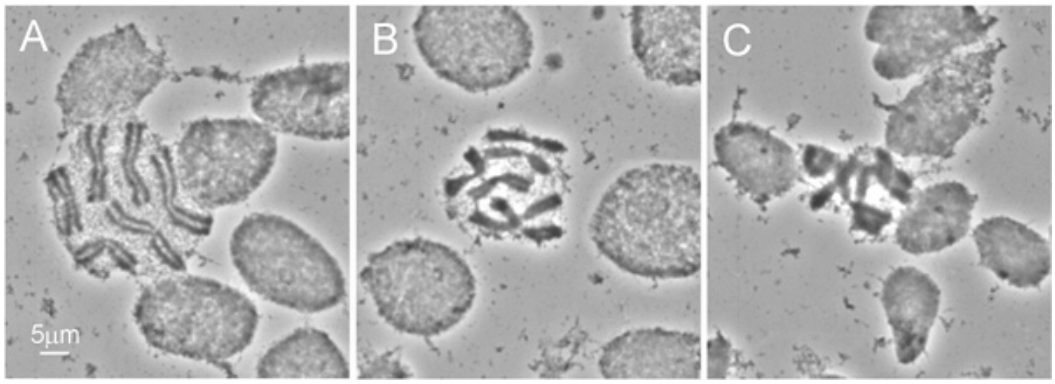

Figure 3. Different qualities of the chromosome spreads: A) a perfect chromosome spread - round shape of the cells demonstrates sufficient treatment of the IDs in hypotonic solution; B) a perfect hypotonic treatment - chromosomes are slightly undersquashed; C) a poor chromosome spread - the result of insufficient hypotonic treatment is indicated by oval shape of the cells.

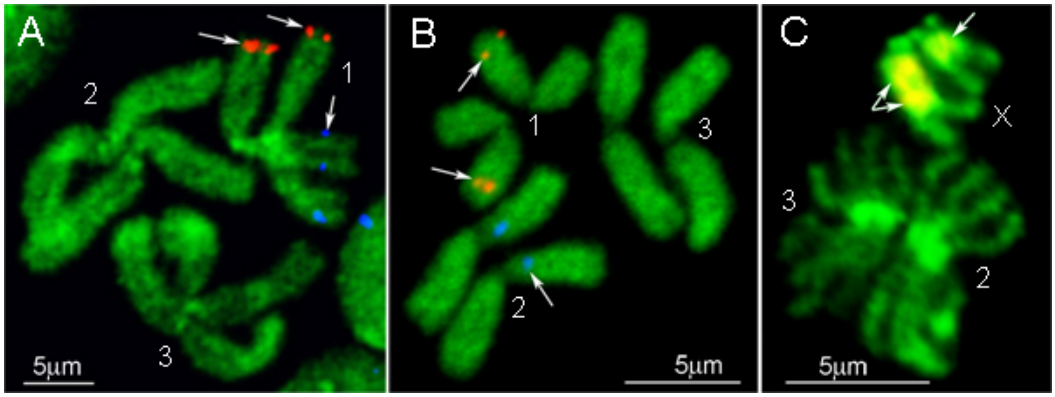

Figure 4. Examples of FISH with BAC clones (A, B) and IGS rDNA (C) in the chromosomes of *Ae. aegypti* (A), *Cx. quinquefasciatus* (B), and *An. gambiae* (C). 1, 2 and 3 - are numbers of chromosomes; X - female sex chromosome in *An. gambiae.*

Discussion

Nonfluorescent *in situ* hybridization on mitotic chromosomes of mosquitoes was performed for the first time in 1990 by A. Kumar and K. Rai [17]. In that study, 18S and 28S ribosomal DNA genes, cloned together in one plasmid, were placed to the chromosomes of 20 species of mosquitoes. The DNA probe was radioactively labeled and hybridized to the chromosomes from brain ganglia. Among three mosquito genera, a FISH technique has been developed only for mitotic chromosomes from the cell line of *Ae. aegypti* [10,18,19] and has never been performed on mitotic chromosomes from live mosquitoes. Recently, we developed a simple, robust technique for obtaining high-quality chromosome preparations from IDs of 4th instar larvae [9]. This method allows a high number of chromosomes to be obtained in one slide and can be universally used for all species of mosquitoes. The necessity of using only larval, not pupal or adult stages of mosquitoes, for slide preparation is probably the only limitation of the method. The standard FISH method [12] was optimized for using genomic BAC clone and IGS rDNA as probes for the mitotic chromosomes of *Aedes, Culex,* and *Anopheles*.

In addition to these specific applications, the FISH protocols described here can also be used for other purposes. The advanced FISH protocol, which utilizes C_0t DNA fractions for blocking unspecific hybridization, can also be applied for the hybridization of BAC clones or any other large DNA fragments in heterochromatic regions of *Anopheles*. Heterochromatic regions are enriched with transposable elements and other repeats, and probes from these regions normally produce strong background on the chromosomes[3]. Using unlabeled C_0t DNA fractions will help to reduce unspecific hybridization of the probe to the chromosomes. The simple version of the FISH protocol can be used for any rDNA or repetitive DNA probes on mitotic chromosomes of mosquitoes and other insects. In addition, it also can be applied for the hybridization of BAC clone DNA in species with low repetitive DNA content in euchromatic regions such as *Anopheles* or *Drosophila*. The protocol proposed here will help to obtain highly-finished chromosome-based genome assemblies for mosquitoes and can be broadly used for various cytogenetic applications in other groups of insects.

Disclosures

No conflicts of interest declared.

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