Fluorescence *in situ* Hybridization to the Polytene Chromosomes of *Anopheles* Mosquitoes

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[Abstract] Fluorescence *in situ* hybridization (FISH) is a method that uses a fluorescently labeled DNA probe for mapping the position of a genetic element on chromosomes. A DNA probe is prepared by incorporating Cy-3 or Cy-5 labeled nucleotides into DNA by nick-translation or a random primed labeling method. This protocol was used to map genes (Sharakhova *et al.,* 2010) and microsatellite markers (Kamali *et al.,* 2011; Peery *et al.*, 2011) on polytene chromosomes from ovarian nurse cells and salivary glands of malaria mosquitoes. Detailed physical genome mapping performed on polytene chromosomes has the potential to link DNA sequences to specific chromosomal structures such as heterochromatin (Sharakhova *et al*., 2010). This method also allows comparative cytogenetic studies (Sharakhova *et al.,* 2011; Xia *et al.*, 2010), and reconstruction of species phylogenies (Kamali *et al.*, 2012).

Materials and Reagents

1. Early fourth instar *Anopheles* larvae
2. Female *Anopheles* mosquitoes
3. Template DNA
4. Fisherfinest* Premium Extra-Thick Frosted Microscope Slides (Double frosted coating) (Thermo Fisher Scientific, catalog number: 12-544-6)
5. Fisherfinest* Premium Cover Glasses (22 x 22 mm) (Thermo Fisher Scientific, catalog number: catalog number: 12-544-10)
6. 50% Propionic acid in water
7. Razor blade
8. Liquid nitrogen
9. Ethanol, molecular biology grade
10. Microscope slide staining jar with lid
11. Random Primed DNA Labeling Kit (Roche Applied Science, catalog number: 11004760001)

12. Random Primers DNA Labeling System (Life Technologies, Invitrogen™, catalog number: 18187-013)
13. Formamide (Super pure) (Fisher Bioreagent, catalog number: BP228-100)
14. Dextran Sulfate Sodium salt from *Leuconostoc spp.* (Sigma-Aldrich, catalog number: D8906)
15. Prolong® Gold antifade reagent (Life Technologies, Invitrogen™, catalog number: P36930)
16. Cy3-dUTP (GE Healthcare, catalog number: PA53022)
17. Cy5-dUTP (GE Healthcare, catalog number: PA55022)
18. YOYO®-1 Iodide (491/509)-1 mM Solution in DMSO (Life Technologies, Invitrogen™, catalog number: Y3601)
19. Paraformaldehyde (Sigma-Aldrich, catalog number: F8775)
20. DNA polymerase I (Fermentas, catalog number: EP0041)
21. DNase I (Fermentas, catalog number: EN0521)
22. QIAquick® Gel Extraction Kit (QIAGEN, catalog number: 28704)
23. QIAquick® PCR purification Kit (QIAGEN, catalog number: 28104)
24. Carnoy's solution (see Recipes)
25. 20x SSC (see Recipes)
26. 3 M NaAC (see Recipes)
27. 1x PBS (see Recipes)
28. Hybridization buffer (see Recipes)

Equipment

1. 1.5 ml microcentrifuge tubes
2. Forceps
3. Disposable transfer pipette
4. Dissecting needles
5. Research stereo microscope (Leica, model: VA-OM-E194-354)
6. Phase contrast compound microscope with 10x, 20x, 40x and 100x objective lenses
7. Thermal cycler
8. Vacufuge® vacuum concentrator (Eppendorf, model: 022820001)
9. Incubator
10. Water Bath
11. Vortexer
12. Confocal Microscope or Fluorescence Microscope

Procedure

A. Polytene chromosome preparation

A-1 Salivary gland chromosome preparation

1. Preserve early fourth instar larvae in Carnoy's Solution and keep at -20 °C.
2. Remove one fourth-instar larva from the vial with a pair of forceps and place it on a dust-free microscope slide with back upward, then put a drop of fresh Carnoy's solution onto it immediately (Figure 1a).

   Note: Continue adding drops of Carnoy's solution when needed to prevent drying out until dipping 50% Propionic acid onto the gland.

   While firmly holding the larva with one dissecting needle, gently pull the head away from thorax with another needle (Figure 1b). Insert a needle from the middle rear of the thorax just underneath the cuticle, and gently move forward to break the thorax cuticle along the mid dorsal line (Figure 1c). Carefully open up the thorax and separate the salivary gland from connecting tissue (Figure 1d and 1e). Remove the carcass and other tissue from slide and put one drop of fresh 50% Propionic acid onto the gland (Figure 1f).

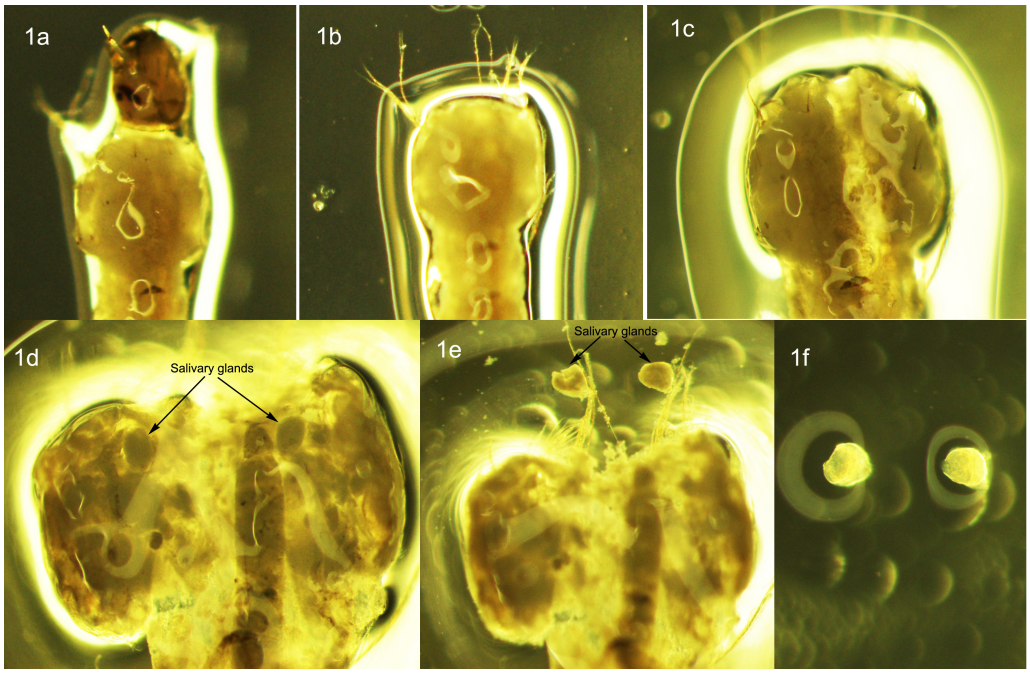

Figure 1. Dissection of salivary glands in 4th instar larva of *An. Sinensis*

3. Cover gland with a dust-free coverslip and leave them for about 5 min. After 5 min, place a piece of filter paper over the coverslip, hold the four edges still with fingers. Gently tap it with a pencil eraser to release the polytene chromosomes from the salivary gland.

4. Examine the banding pattern and spread of polytene chromosomes using a phase-contrast microscope.
5. Place slides with good chromosomal preparations in a humid chamber with 4x SSC in the bottom of the chamber, at 60 °C for 15-20 min. After heating, put slides at 4 °C overnight or until immersing the slides in liquid nitrogen. Heating can be done on the Thermobrite machine with the absorbent strips soaked in distilled water.
   Note: Slides can dry out if left at 4 °C for extended periods of time. Leaving them at 4 °C for longer than overnight is not recommended.
6. While holding one corner of the slide with forceps or a gloved hand, dip chromosome preparation into the liquid nitrogen so that the coverslip is completely immersed. Hold slide in liquid nitrogen until the bubbling stops (usually 10-15 sec). Take it out of the liquid nitrogen and immediately remove the coverslip with a razor blade from one corner. It sometimes helps to put the slide on a flat surface when trying to remove the cover slip. Put slide in a slide jar with prechilled 50% Ethanol (-20 °C) and keep at 4 °C for at least 2 h.
7. Dehydrate the preparations in slide jar with an ethanol series of 70%, and 90% for 5 min each at 4 °C and then 100% ethanol for 5 min at room temperature. Air dry, and keep slides in slide box until ready for use in *in situ* hybridization (Figure 2).
   Note: Slides that are kept protected from dust and debris can be used for FISH at least within a year after the preparations are made.

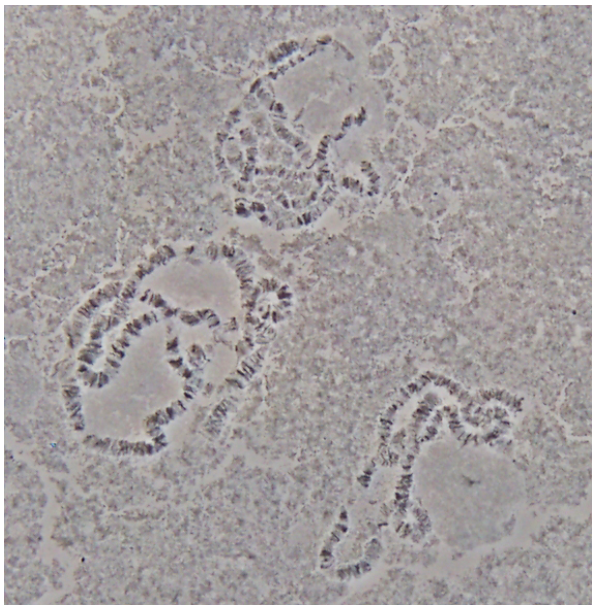

Figure 2. Polytene chromosomes from salivary glands of *An. sinensis*

A-2 Chromosome preparation from *Anopheles* ovaries

1. Dissect the ovaries of *Anopheles* mosquitoes from half gravid females 18-33 h after 2nd or 3rd blood feeding (Christophers' III stage) and keep 4-5 ovaries in a vial with 1 ml of Carnoy's solution. After fixing the ovaries for 24 h at room temperate, transfer the vials to -20 °C for storage.
   Note: Females should be bloodfed and lay eggs at least once before bloodfeeding again and dissecting ovaries for chromosomal preparations.
2. To make the chromosome preparations, take one ovary out of the vials with a pair of forceps (or a transfer pipet) and place it into a drop of Carnoy's solution on microscope slide. After carefully removing tissues, trachea and blood, quickly separate the follicles from one ovary into 2-4 pieces. Up to four preparations can be made from one ovary.
   Note: While dissecting, the ovaries should never be allowed to dry. Continue adding drops of Carnoy's solution when needed to prevent drying of the ovaries.
3. On 4-8* microscope slides, add each of the pieces of divided ovary and one drop of 50% propionic acid on a separate slide. Let the pieces of ovary rest in propionic acid for about 5 min until follicles become clear, and swell to about twice their original size.
   **number of slides you need depends of how many pieces each ovary is divided into.*
4. For each slide, use a dissecting microscope to separate the cleared follicles from each other and any other tissue or debris on the slide. Remove tissue and debris by wiping it away with a piece of paper towel, and apply a fresh drop of 50% propionic to the separated follicles.
5. Do the same as steps 3-7 in "*Salivary gland chromosome preparation".*

B. Probe preparation and labeling

If using PCR products as probe, purify the PCR product from an agarose gel or from the PCR reaction using a QIAquick® Gel Extraction Kit or QIAquick® PCR purification Kit. Similar kits that remove excess nucleotides can also be used. However, when using a kit, dissolve the DNA in double distilled water instead of the elution buffer suggested in the final step.

B-1 Random Primer labeling protocol for fragments shorter than 1 kb (Random Primed DNA Labeling kit from Roche)

1. Add 25 ng template DNA into double distilled water to a final volume of 13.5 µl in a microcentrifuge tube.
2. Denature the DNA by heating in a boiling water bath for 10 min at 95 °C and chilling quickly in an ice bath.
3. Add the following to the freshly denatured probes on ice:

dGTP, 1.0 mM	1 µl
dCTP, 1.0 mM	1 µl
dATP, 1.0 mM	1 µl
Reaction Mixture (Vial 6)	2 µl
Klenow enzyme (Vial 7)	1 µl
Cy3 or Cy5-dUTP, 1.0 mM	0.5 µl

Mix and centrifuge briefly.

4. Incubate for 1 h to 20 h (overnight) at 37 °C.
5. Add 1/10 volume of 3 M NaAC and 2.5-3 volume of 100% ethanol. And mix by inverting the tubes. Keep at -80 °C or -20 °C for at least 3 h or until probes are needed for hybridization. If necessary, probes can be left in the freezer for long-term storage.

B-2 Random Primer labeling protocol for fragments shorter than 1 kb (Random Primers DNA Labeling System from Invitrogen)

1. Mix 1 µl DNA and 10 µl 2.5x Random Primer Solution and 2.5 µl sterile water well.
2. Denature 5 min in boiling water or heating block, immediately cool on ice.
3. Add 1.25 µl 1.0 mM dNTP mix (without a labeled dNTP), 8.75 µl water and 1 µl Klenow Fragment, mix gently but thoroughly.
4. Add 0.5 µl Cy3 or Cy5-dUTP fluorescent nucleotide to each tube, when finished, tube must be covered immediately to protect from light. Mix well and incubate at 37 °C for 1.5 h.
5. Do the same as step 5 in Section B-1 "Random Primer labeling protocol for fragments shorter than 1 kb (Random Primed DNA Labeling kit from Roche)".

B-3 Nick Translation labeling for fragment longer than 1 kb (1-150 kb)

1. Prepare the following reaction mixture on ice:

10x buffer for DNA Polymerase I	5 µl
1.0 mM dATP, dCTP, dGTP and 0.3 mM dTTP mixture	5 µl
DNase I freshly diluted to 0.02 units/µl	4 µl**
DNA Polymerase I	1 µl**
Template DNA	1 µg
Cy3- or Cy5-dUTP	1 µl
BSA diluted to 0.5 mg/ml	5 µl
Add water to final volume 50 µl	

Note: This protocol can be scaled down by ½ to accommodate 500 ng of template DNA.

***Final concentrations of DNase I and DNA Polymerase I have to be optimized based on*

factors including initial size of template DNA, template DNA concentration and reaction time. Larger template size and greater template concentration generally require more DNase.

2. Incubate the mix at 15 °C for 2-3 h.
3. Run 3 µl of reaction mixture on an agarose gel to determine the size of digested fragments. Fragments should be 100-600 bp for best hybridization results. If fragments are still larger than this, incubate at 15 °C for additional time.
4. To terminate the reaction and precipitate labeled probes, do the same as step 5 in Section B-2 “Random Primer labeling protocol for fragments shorter than 1 kb (Random Primed DNA Labeling kit from Roche)”
   Note: Fluorescently labeled probes should be protected from light! In the steps following, even where it is not explicitly stated, make efforts to protect probes from light.

C. Chromosomal fixation
1. Do step C-2 and C-3 if slides are more than two months old. Otherwise, go to step C-4.
2. Fix slides in 1:3 glacial acetic acid: methanol at RT for 10 min and air-dry.
3. Dehydrate slides in 100% ethanol for 10 min and air dry again.
4. Immerse slides in 1x PBS for 20 min at RT.
5. Fix slides at room temperature in 4% paraformaldehyde for 1 min.
   Note: Paraformaldehyde is hazardous and should be handled carefully. Avoid breathing gas or dust during preparation: wear gloves and other PPE when handling. Paraformaldehyde solution should not be dumped down drains.
6. Dehydrate the slides through an ethanol series of 50%, 70%, 90%, 2x 100% for 5 min each at RT.
7. Air-dry the slides.

D. *In situ* hybridization
1. Centrifuge the tubes of labeled probes at 20,817 *x g* for 10 min. Carefully remove the supernatant and vacumfuge the tubes for 20 min to dry pellets.
2. Dissolve dry probes in hybridization buffer pre-warmed to 37 °C. The amount of hybridization buffer used to dissolve depends on the total amount of DNA you are dissolving. Dissolve 1 µg of DNA in 20-40 µl of warmed hybridization buffer.
3. In a clean microcentrifuge tube, combine at least 250 ng each of one blue (Cy5 labeled) and one red (Cy3 labeled) probes. *In situ* hybridization is efficient if at least 500 ng of DNA is hybridized on the slide. Vortex and centrifuge the tube of combined probe briefly.
4. Transfer the above prepared solution of combined probes to a chromosome preparation

slide and cover with a 22 x 22 mm coverslip. Remove any large air bubbles with gentle pressure.

5. Denature the target and probe DNA by placing the slides on the Thermobrite machine at 90 °C for 10 min. Thermobrite machine does not need to be humid.
6. Seal edges of cover slip with rubber cement.
7. Transfer the slides to pre-warmed humid chambers with 4x SSC at the bottom of the chambers and incubate at 39 °C for interspecies (*e.g An. gambiae* probe to *An. stephensi* chromosomes) or 42 °C for intraspecies hybridization for 3-18 h (usually overnight).
   Note: Because there are fluorescently labeled probes on the slide, humid chambers should be impermeable to light.

E. Washing

1. Carefully remove rubber cement with forceps and coverslip.
2. In a slide jar covered with aluminum foil, wash the slides with 1x SSC at 39 °C after interspecies or 0.2x SSC at 42 °C after intraspecies hybridization for 20 min in 50 ml without shaking.
3. Wash the slides with 1x SSC after interspecies or 0.2x SSC after intraspecies hybridization at RT for 20 min in 50 ml without shaking.
4. Dilute fluorescent dye YOYO-1 100 times in 1x PBS to make a stock solution. Mix 10 µl of 100x diluted YOYO-1 with 90 µl 1x PBS for each slide that you want to stain. The working solution of YOYO-1 is 1,000x diluted relative to original concentration.
5. After washing in SSC for 20 min at room temperature, rinse slide in 1x PBS, and add 100 µl of YOYO-1 in PBS on each slide. Cover with parafilm. Leave at RT for 10 min inside of a slide box or somewhere dark.
6. Rinse in 1x PBS and add 10 µl Prolong Gold antifade reagent, place coverslip on slide and blot out bubble. Keep in the slide box at 4 °C.

F. Signal detection

Detect the signals using a confocal or fluorescence microscope and map them to the polytene chromosomes of *Anopheles* mosquitoes (Figure 3).

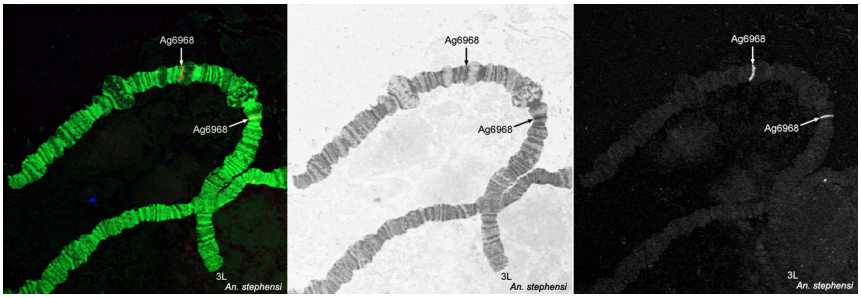

Figure 3. Fluorescence *in situ* hybridization and mapping of DNA probes on polytene chromosomes from ovarian nurse cells of *An. Stephensi*

Recipes

1. Carnoy's solution: Methanol: Glacial Acetic Acid = 3:1

   Note: Carnoy's solution should be used with good ventilation or in a fume hood. Gloves should also be worn while using Carnoy's solution and it should not be disposed of down the drain.

2. 1x PBS (1 L)

NaCl	8.01 g
KCl	0.20 g
NaH_2PO_4 (anhydrous)	1.15 g
KHP_2O_4 (anhydrous)	0.20 g

3. 20x SSC (500 ml)

Sodium chloride	87.5 g
Sodium citrate	44 g
Add 1 N HCl to pH 7.0	

4. Hybridization buffer (2 ml)

20x SSC	120 µl
Dextran sulfate	0.2 g
Formamide	1.2 ml
Water	580 µl

5. 3 M NaAC

   Dissolve 24.61 g of Sodium Acetate (anhydrous) in 100 ml water.

Acknowledgments

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