Chapter 8

A Multiplex Fluorescent In Situ Hybridization Protocol for Clonal Analysis of *Drosophila* Oogenesis

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Abstract

Fluorescent in situ hybridization (FISH) is a common inmunohistochemical method used to examine the distribution of RNAs in tissue samples. In mosaic tissues composed of a mixed population of wild-type and loss-of- or gain-of-function mutant cells, FISH allows comparison of the effect of the perturbation on gene expression patterns in a mutant cell and its wild-type neighbors. Here, we provide a protocol for the detection of RNA in *Drosophila* mosaic follicular epithelia, where the mosaic analysis with a repressible cell marker (MARCM) technique is used for expression of transgenes.

Key words *Drosophila melanogaster*, Oogenesis, Follicle cells, Mosaic analysis with a repressible cell marker (MARCM), Fluorescent in situ hybridization (FISH)

1 Introduction

Drosophila melanogaster oogenesis is a classic model for the analysis of patterning and morphogenesis regulation by extracellular signaling pathways. In addition to the simple anatomy of the egg chamber, the large availability of transgenic tools for its manipulation allows the study of a number of developmental processes, many of which are conserved between flies and vertebrates. Moreover, this tractable system shares many of the morphogenetic events of larger organs under the control of some of the same regulatory networks, and has already provided great insight into the molecular mechanisms coordinating morphogenesis [1, 2].

One useful tool to prove the function of genes is the GAL4/UAS binary system for transgene expression. GAL4 encodes a protein identified in the yeast *Saccharomyces cerevisiae* as a regulator of genes induced by galactose. In *Drosophila*, many lines (known as "drivers"), where the GAL4-coding sequence has been stably incorporated into the genome under the control of *Drosophila* regulatory sequences (with tissue- and stage-specific expression), are reported and publicly available. GAL4 regulates transcription by

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binding to four related 17 bp sites known as upstream activating sequences (UAS), and thus promoting transcription of nearby genes [3, 4]. Many lines with the coding sequences of *Drosophila* genes under the control of UAS are also publicly available.

An additional level of control of transgene expression can be achieved with the MARCM method [5, 6], which combines the FLP/FRT [7] system and the dominant GAL80 protein to inhibit activation of the GAL4/UAS system. The MARCM method can be used to prevent lethality due to off-target effects, and to allow comparison of gene expression in a mutant cell and its wild-type neighbors within the same tissue.

The FLP-recombinase and its target, the FRT sequence, can induce high-frequency mitotic recombination between FRT sites located on homologous chromosome arms to produce mosaics of homozygote mutant, heterozygote, and homozygote wild-type cells [7]. If a GAL80 sequence is incorporated distally to an FRT site, FLP-induced mitotic recombination removes GAL80 in a random group of cells which allows the transcription of UAS-regulated transgenes by GAL4 (Fig. 1a). Furthermore, the choice

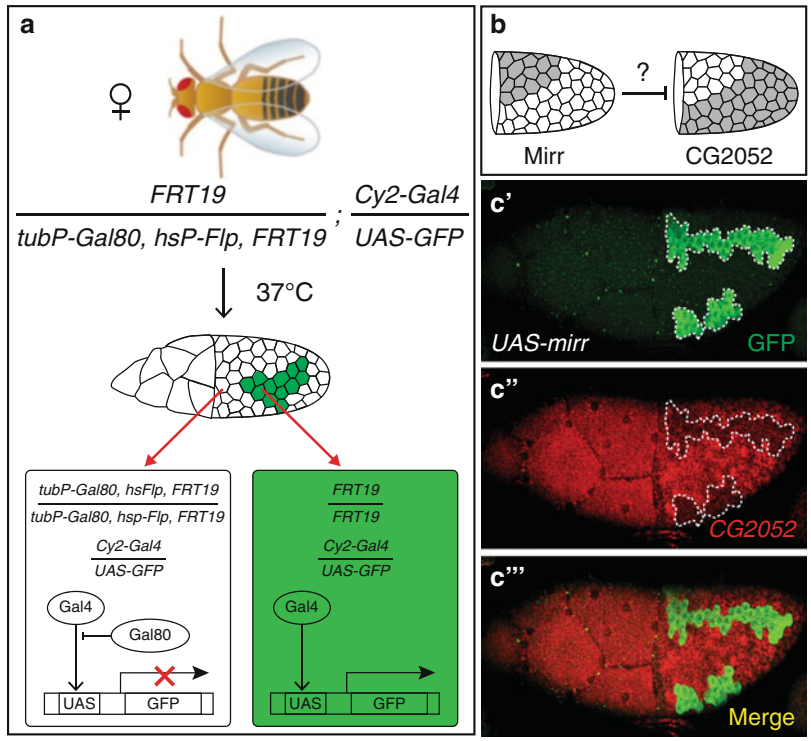

Fig. 1 The effect of a gene on a potential target can be tested by ectopic expression of the gene under UAS control. (**a**) Females with a *tubP-GAL80*, *FRT* in *trans* to an *FRT* site, an *hsP-FLP* recombinase, a *UAS-GFP*, and the oogenesis-specific *Cy2-GAL4* driver produce GFP-marked clones upon heat shock. (**b**) The effect of the transcription factor mirror (*mirr*) on another gene with complementary expression pattern can be assayed by introducing a *UAS-mirr* transgene [12, 13]. (**c**–**c**′′′) Ectopic expression of *mirr* in ventral follicle cells produces downregulation of *Lin-29* (*CG2052*) RNA in GFP-marked clones (*dashed line*)

of oogenesis-specific Gal4 drivers [8] allows a more temporally restricted transgene expression.

In this chapter, we describe a procedure to induce MARCM clones in follicle cells during oogenesis, and to analyze the resulting changes in RNA expression using fluorescent in situ hybridization (FISH). The FISH protocol described below was adapted from that developed for *Drosophila* embryos, with further modification suggested in the author's laboratory website [9, 10]. This protocol can successfully detect medium-to-high levels of RNA. Other techniques allowing the detection of low levels of RNA through the use of amplification steps [11] are not covered here.

2 Materials

Prepare all solutions using nuclease-free water and molecular biology-grade reagents.

2.1 Labeled RNA Probe Preparation

1. Hydrolysis buffer: Dissolve sodium carbonate (Na_2CO_3) and sodium bicarbonate ($NaHCO_3$) in water to concentrations of 1.2 M and 0.8 M, respectively. Check for a final pH of 10. The solution can be prepared in advance and stored in aliquots at −20 °C.
2. Stop buffer: Dilute sodium acetate buffer solution (3 M, pH 5, Sigma) to a final concentration of 0.2 M in water. Use sodium acetate buffer solution (3 M, pH 7, Sigma) to adjust the final pH to 6. The solution can be prepared in advance and stored in aliquots at −20 °C.
3. Hybridization buffer: Mix 25 mL of formamide, 12.5 mL of SSC buffer (20×, Sigma), 12 mL of water, 500 μL of Salmon sperm DNA (10 mg/mL, Sigma), 50 μL of Tween-20 (Sigma), and 50 μL of a 50 mg/mL stock of heparin. To prepare the heparin stock, dissolve heparin sodium salt (Sigma) in water. The hybridization buffer can be prepared in advance and stored at −20 °C.
4. in vitro transcription (IVT) reaction mix: Combine 2 μL of transcription buffer (10×, Roche Applied Science), 2 μL of DIG or Biotin RNA labeling mix (Roche Applied Science), 2 μL of RNase inhibitor (Roche Applied Science), 2 μL of nuclease-free water, and 2 μL of T3, T7, or SP6 RNA Polymerase (Roche Applied Science). Select the RNA polymerase that will produce antisense RNA from the template DNA. Use immediately after preparation.
5. DNAse I (Roche Applied Science).
6. RNeasy Mini Kit (Qiagen).
7. Microcentrifuge.

2.2 Ovary Sample Preparation

1. Isotherm incubator at 37 °C.
2. Baker's yeast (MP Biomedicals).
3. Stereo dissecting microscope, carbon dioxide pad, Pyrex glass spot plate, and Dumont forceps for dissection.
4. Wide-bore pipet tips.
5. Dissection solution: Dissolve Tween-20 in Grace's Insect Medium (1×, Invitrogen) to a final concentration of 0.02 %. The solution can be prepared in advance and stored at 4 °C.
6. PBST wash solution: Dissolve Tween-20 in 1× Dulbecco's phosphate-buffered saline (PBS) to a final concentration of 0.02 %. The solution can be prepared in advance and stored at room temperature.
7. Nutating mixer.
8. 8 % formaldehyde fix solution: Mix 43 μL of formaldehyde (37 %), 157 μL of 1× PBS, and 600 μL of heptane. Use immediately after preparation.
9. Methanol.

2.3 Hybridization of RNA Probes

1. Ethanol.
2. Xylene.
3. Methanol.
4. 6 % formaldehyde fix solution: Dilute the formaldehyde (37 %) in 1× PBS to a final concentration of 6 % formaldehyde. Use immediately after preparation.
5. Acetone.
6. Benchtop incubating orbital shaker with Eppendorf tube holder.

2.4 Antibody Staining

1. Blocking solution: Mix 1 mL of the Western blocking reagent (WBR, 10×, Roche Applied Science) and 4 mL of 1× PBS. Store at 4 °C for up to 1 day.
2. Sheep anti-DIG antibody (Roche Applied Science) or mouse anti-Biotin antibody (Jackson ImmunoResearch Lab) depending on the particular probe used.
3. Alexa Fluor 488-conjugated rabbit anti-GFP antibody (Molecular Probes).
4. Alexa Fluor-conjugated secondary antibodies (Molecular Probes).

2.5 Sample Mounting

1. Aqua Poly-Mount (PolySciences).
2. Glass slide and glass cover slip.
3. Wide-bore pipet tips.
4. Clear nail polish.

3 Methods

3.1 Labeled RNA Probe Preparation

1. Amplify DNA template for the gene of interest by polymerase chain reaction (PCR) according to the instructions for the particular polymerase used. Add 10 μL of the PCR product to 10 μL of the IVT reaction mix. Incubate at 37 °C for 3 h.
2. Add 2 μL of DNase I and incubate at 37 °C for 30 min.
3. Purify the probe using the Qiagen RNeasy Mini Kit following the instructions for RNA cleanup.
4. Elute RNA from the Qiagen column with 40 μL of nuclease-free water.
5. Add 2 μL of hydrolysis buffer, and incubate at 60 °C for a time (in minutes) equal to $(L - 0.2)/(0.022 \times L)$ where L is the length of the DNA template in kb.
6. Add 40 μL of stop buffer to quench the hydrolysis.
7. Dilute the RNA probe solution in 200 μL of the hybridization buffer and store at −20 °C.

3.2 Ovary Sample Preparation

1. Prepare heterozygote flies carrying a *tubP-GAL80*, *FRT* in *trans* to an *FRT* site, as well as an oogenesis-specific *GAL4* driver, an *hsP-FLP* recombinase, and a *UAS-GFP* marker. Additionally, *UAS*-transgenic constructs can be incorporated to study their function (for example as in Fig. 1, *see* **Note 1**).
2. Heat-shock the flies for two consecutive days. Place vials with adult females and some male flies in an isotherm incubator set at 37 °C for 5 h each time (*see* **Note 2**). Transfer the flies to a fresh vial with standard cornmeal food.
3. Let the flies recover at room temperature, and on the fourth day add Baker's yeast to their food to promote oogenesis. Wait until the next day for dissection.
4. Anesthetize 10–20 female flies on a carbon dioxide pad.
5. Dissect ovaries in a glass spot plate with cold dissection solution. Separate each ovary in half, and transfer them to an Eppendorf tube using a wide-bore pipet tip (*see* **Note 3**).
6. Remove as much liquid from the sample as possible, and add the 8 % formaldehyde fix solution. Incubate for 20 min on a nutating mixer (*see* **Note 4**).
7. Rinse once and wash the fixed ovaries in PBST three times for 5 min each (*see* **Note 5**).
8. Dehydrate ovaries by washing successively in solution of 25, 50, and 75 % methanol in 1× PBS for 5 min each on a nutating mixer. Rinse once and store the ovaries in 100 % methanol at −20 °C for up to 3 months.

3.3 Hybridization of RNA Probes

1. Rinse ovaries once and wash with ethanol for 5 min.
2. Incubate in a solution of 90 % of xylene in ethanol for 1 h.
3. Rinse once and wash with ethanol for 5 min.
4. Rinse once and wash with methanol for 5 min.
5. Fix the dissected ovaries in an equal parts mixture of 6 % formaldehyde solution and methanol for 5 min.
6. Fix again in 6 % formaldehyde solution for 25 min.
7. Rinse once, and wash in PBST three times for 5 min each.
8. Separate ovaries into individual egg chambers (*see* **Note 6**).
9. Incubate in a solution of 80 % acetone in nuclease-free water at −20 °C for 10 min. Invert the tube once during incubation to improve permeabilization.
10. Rinse once, and wash in PBST three times for 5 min each.
11. Fix in 6 % formaldehyde solution for 25 min.
12. Rinse once, and wash in PBST three times for 5 min each.
13. Incubate in an equal parts mixture of hybridization buffer and PBST for 10 min.
14. Incubate sample in hybridization buffer at 60 °C for 2 h.
15. Dilute the RNA probe in hybridization buffer. Try several concentrations starting with a 1:10 dilution. Denature the probe by heating this solution to 80 °C for 4 min, and then quenching in ice for 1 min. Immediately after, add the probe solution to the sample and incubate at 60 °C overnight in an orbital shaker.

3.4 Antibody Staining

1. Rinse once, and wash in hybridization buffer three times at 60 °C for 1 h each.
2. Incubate in an equal parts mixture of hybridization buffer and PBST for 10 min.
3. Rinse once, and wash in PBST three times for 5 min each.
4. Incubate the sample in blocking solution for 30 min.
5. Dilute the primary antibodies in blocking solution. Try several concentrations starting with a 3:500 dilution for sheep anti-DIG, 1:100 for mouse anti-biotin, and 1:100 for rabbit anti-GFP. Add this antibody solution to the sample, and incubate at 4 °C overnight in a nutation mixer (*see* **Notes 7–9**).
6. Rinse once, and wash in PBST three times for 5 min each.
7. Incubate in blocking solution for 30 min.
8. Dilute the secondary antibodies in blocking solution. Try several concentrations starting with a 1:500 dilution. Add the probe solution to the sample and incubate for 2 h. Protect the sample from light (*see* **Note 10**).

9. Rinse twice, and wash in PBST two times for 20 min each.
10. Wash the sample in PBS for 20 min, and then remove as much of the wash liquid as possible.

3.5 Sample Mounting

1. Add an equal parts mixture of PBS and Aqua-Poly-Mount (i.e., in one drop of each for a 22 × 60 mm cover slip), and pipet several times to suspend the ovaries using a wide-bore pipet tip. Transfer the sample to a glass slide, and remove any bubbles. Place cover slip genteelly on the sample, and seal the edges with nail polish (*see* **Notes 11** and **12**). Store the slide at 4 °C, and protect from light. Wait for 1 day before imaging.

4 Notes

1. It is convenient to keep one stock with the *tubP-GAL80 FRT*, the *hsP-FLP* recombinase, the *GAL4* driver, and the *UAS-GFP* marker, and another stock with the FRT and the UAS-transgenic construct to be analyzed, that can be crossed multiple times to easily repeat experiments.
2. The duration and frequency of the heat shock affect the number and size of clones. The particular conditions described here produce several medium-size (2–10 cells) clones when combined with the *FRT19* site.
3. Keep the 0.02 % Tween-20 in Grace's medium solution in ice.
4. All fixation, wash, and incubation steps are done at room temperature unless otherwise specified.
5. In each of the rinse steps in this protocol, rinse refers to removing as much of the previous solution as possible, replacing it with PBST, and then removing the PBST, while in the wash steps, the samples are placed in nutation mixer for the indicated time before removal of the PBST. The duration of the wash steps is recommended, but can be extended.
6. The separation into individual egg chamber can also be performed after **step 7** of Subheading 3.2, before the dehydration step. If samples from different dissections will be combined into a single FISH, separating the egg chamber after **step 7** decreases the workload for the hybridization procedure.
7. Fluorescein-labeled probes can also be incorporated to monitor the expression of a third gene, combined with a commercial rabbit anti-fluorescein antibody. Because of fluorescein's green fluorescence, the secondary antibody used for its detection should be Alexa Fluor 488 conjugated. In our hands, dinitrophenyl-labeled probes produce nonspecific fluorescence.

8. Samples treated in this protocol retain negligible fluorescence from GFP; thus GFP can be detected with appropriate primary and secondary antibodies not conjugated to Alexa Fluor 488.
9. Because of the extensive sample treatment, the concentration of antibodies used for protein detection in this protocol is approximately double that required in conventional inmunostaining.
10. The DNA-binding dye DAPI can be added to this step to stain nuclei.
11. If the sample size is too small and will be analyzed with confocal microscopy, it is possible to mount the sample in between two cover slips to allow imaging of the sample from both sides. Place the sample on a cover slip and genteelly lay another cover slip of equal size on top. Let the liquid spread between the cover slips and wipe the edges to remove excess liquid. Seal the edges with nail polish, and set on a support that avoids contact with the nail polish. When imaging, tape the cover slip setup to a glass slide. As limitations, this arrangement is not adequate for epifluorescence or light microscopy, breaks easily, and should be handled with care.
12. If the ovaries show signs of shrinking, it is possible that the ratio of Aqua-Poly-Mount to PBS is too high. Increase the amount of PBS in subsequent experiments.

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