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***Drosophila melanogaster* as a Model System to Study Mitochondrial Biology**

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Summary

Mitochondria play an essential role in cellular homeostasis. Although in the last few decades our knowledge of mitochondria has increased substantially, the mechanisms involved in the control of mitochondrial biogenesis remain largely unknown. The powerful genetics of *Drosophila* combined with a wealth of available cell and molecular biology techniques, make this organism an excellent system to study mitochondria. In this chapter we will review briefly the opportunities that *Drosophila* offers as a model system and describe in detail how to purify mitochondria from *Drosophila* and to perform the analysis of developmental gene expression using *in situ* hybridization.

Keywords

Drosophila; gene expression; molecular localization

1. Introduction

The fruit fly *Drosophila melanogaster*, a tiny insect about 3 mm long, was used extensively as an animal model in biology throughout the last century. In the famous Fly Room at Columbia University, T. H. Morgan and his students A. H. Sturtevant, C. B. Bridges, and H. J. Muller carried out a series of genetic analyses of *Drosophila* that led them to formulate the chromosome theory of heredity. This important achievement led to Morgan's 1933 Nobel Prize.

Between 1913 and 1930, several essential techniques required for genetic analysis were introduced. These include (1) the use of balancers, which are chromosomes with multiple inversions that cannot recombine with their homologs, thus allowing the maintenance of lethal mutations in heterozygotes without further selection; (2) the discovery of polytene chromosomes, which allow the physical mapping of genes; and (3) the introduction of x-rays as a mutagenic agent, a finding that led to Muller's 1946 Nobel Prize. Most of the techniques developed at that time are still used in genetic work and make *Drosophila* the most genetically manipulable metazoan.

In the 1970s, many powerful biochemical, molecular, and cellular techniques were developed that allowed the use of *Drosophila* as a model system to study many complex biological phenomena. Paradigmatic examples of the feasibility of *Drosophila* in biological

research were the identification and cloning of the bithorax complex by E. Lewis, D. Hogness, and their colleagues, and the genomewide mutational screen carried out by C. Nüsslein-Volhard and E. Wieschaus in 1981 that led to the discovery of dozens of genes involved directly in regulating embryonic development. Lewis, Nüsslein-Volhard, and Wieschaus shared the Nobel Prize in 1995. Another breakthrough in *Drosophila* research was the development in 1981 by A. Spradling and G. M. Rubin of efficient techniques based on P-transposons to generate transgenic flies.

During the last two decades of the 20th century, an arsenal of cellular and molecular tools have also been developed in *Drosophila* or adapted to work with this organism. The complete genome sequence was first reported in 2000, and its analysis is proceeding rapidly. The possibility to combine the power of classical genetics with a wide variety of cellular and molecular techniques has attracted more and more scientists to work with *Drosophila* in the context of many different fields, including regulation of gene expression, cell biology, neurobiology, behavior, development, aging, and more recently the physiopathology of human diseases.

However, in spite of the many advantages, *Drosophila* has not achieved priority status as an animal model in the mitochondrial field, in which scientists traditionally have been more focused on yeast and mammals. In this chapter, we present a brief introduction to the system, emphasizing some aspects that may be useful for laboratories interested in using *Drosophila* to study mitochondrial biogenesis and function. The reader is redirected to some excellent and extremely useful bench books and World Wide Web utilities that explain the genetics, biology, and manipulability of *Drosophila* in detail that is beyond the scope of this chapter. An introduction to *Drosophila* research may be found at <http://flybase.bio.indiana.edu/allied-data/introductory.html>.

1.1. The *Drosophila* Life Cycle

The *Drosophila* life cycle is short, and therefore it is easy to raise a large number of individuals for genetic, biochemical, and molecular analyses. In the laboratory, *Drosophila melanogaster* is usually cultured at 25 or 18°C (the latter mainly for maintaining stocks); we provide all the timing for 25°C, except where specifically indicated. The generation time is roughly 10 d from fertilized egg to eclosed adult, and the maximum life span ranges from 60 to 80 d depending on the culture conditions. *Drosophila* is a holometabolous insect, and its life cycle can be divided into four stages: embryo, larva, pupa, and adult (**Fig. 1**).

Females lay roughly 100 embryos per day, and embryogenesis lasts only 24 h (for a detailed description of embryonic stages, see <http://www.sdbonline.org/fly/aimain/2stages.htm> or <http://flymove.uni-muenster.de/>). The first instar larva begins to feed immediately on the surface of the medium and passes through two molts (**Fig. 2**). Second instar larvae burrow into the medium, and when the third instar larva is mature, it leaves the culture medium and wanders up the walls of the flask, searching for a place to pupariate for 24–48 h. During pupariation, a complete body metamorphosis from larva to adult takes place; most larval tissues are degraded, and adult organs develop from an undifferentiated sac of cells, the imaginal disks. In *Drosophila*, there are 10 pairs of imaginal disks, which reconstruct the entire adult body except the abdomen, and a genital disk, which forms the reproductive

organs. The abdominal epidermis forms from histoblasts, a group of specialized imaginal cells. The imaginal disks constitute cellular territories that have been extensively used to unravel the role of many genes involved directly in the morphogenesis of adult structures. Finally, the adult emerges between 9 and 10 d after egg fertilization (at 18°C, development takes twice as long).

1.2. The *Drosophila* Genome

Drosophila has four pairs of chromosomes: X/Y, II, III, and IV, with most of the gene content located on chromosomes X, II, and III. The first annotated sequence, release 1, was published in March 2000 (1). The haploid genome size is estimated to be 175 Mb by flow cytometry of propidium-stained nuclei, a value very similar to that obtained in the release 3.2 genome sequence (176 Mb). The number of protein-coding genes based on *in silico* methods of gene prediction is roughly 15,000, approx half of those predicted in the human genome. Release 4.0 was made public in April 2004 (the, last update was on March 03, 2006 [<http://flybase.net/annot/release.html>]), differing from release 3.2 with very few new annotations. Release 5.0 of genomic sequences was available on March 29, 2006 (<http://www.fruitfly.org/sequence/release5genomic.shtml>).

The mitochondrial genome of *Drosophila* shows the general features of other animal mitochondrial deoxyribonucleic acids (mtDNAs) regarding gene order, density, structure, and a genetic code that differs from the universal code, although some genes are rearranged compared to the mammalian mitochondrial genome (2). A striking difference lies in the noncoding region, which contains 90–96% deoxyadenylate and deoxythymidylate residues (the A+T-rich region) and ranges in size from 1 to 5 kb in different *Drosophila* subgroups (3). In *D. melanogaster*, the total length of the mtDNA molecule is 19,517 bp.

1.3. *Drosophila* Mitochondrial Proteins

As in other eukaryotic systems, the *Drosophila* mitochondrial genome encodes only a very small fraction of mitochondrial proteins that share a very high degree of evolutionary conservation. Many of the nuclear-encoded mitochondrial proteins are also very well conserved. An excellent analysis of the latter is presented in the MitoDrome database (<http://www.ba.itb.cnr.it/BIG/MitoDromeOLD/>), where one can find the *Drosophila* nuclear genes encoding mitochondrial proteins, their human counterparts, functions, and ontology. MitoDrome2 (<http://www2.ba.itb.cnr.it/MitoDrome/index.php>) is an enhanced version in which the authors identify, characterize, and show tools for analyzing genes encoding proteins that constitute the five large respiratory chain complexes in *D. melanogaster*, *D. pseudoobscura*, and *Anopheles gambiae* (4).

Although analysis of the mitochondrial proteome is well under way in several organisms (e.g., *Arabidopsis*, rice, yeast, mouse, and human) (5), to date no similar studies have been initiated in *Drosophila*.

1.4. Working With *Drosophila*

Working with *Drosophila* in the laboratory is relatively easy and requires neither special technical skill nor sophisticated infrastructure. Flies are generally grown in plastic vials and

bottles containing medium (fly food) (**Fig. 3**). It is also possible to culture them in mass using special containers if you need to work with large numbers of flies (e.g., to carry out protein purification. Several media have been described, all based on simple components such as agar, yeast (not yeast extract), sucrose, and propionic acid. Culture medium can be prepared in a simple kitchen or with more complex and automated facilities depending on the number of stocks and specific needs. An excellent Web page to learn in detail how to prepare a complete series of media for *Drosophila* culturing, either animals or cells, under different conditions or for different purposes is http://www.protocol-online.org/prot/Model_Organisms/Drosophila/Drosophila_Culture_Handling/.

Stocks are usually maintained in vials at 18°C with four to five generation cycles before transfer. Because fly stocks can only be maintained by live culturing, it is crucial to keep two to four different cultures for each individual stock, with alternate generations separated by 1–2 wk if it is possible. Flies in experimental use are maintained routinely at 25°C.

To carry out crosses, you must start with virgin females. Female flies do not mate within the first 8–12 h after emergence as adults from the pupae. Thus, using this window of time, flies can be collected, and females can be separated from the males and kept separately until needed. Males can be collected at any time, with the best efficiency of mating when they are between 3 and 10 d old. The number of flies needed to start a new culture varies, mainly depending on the genotype and the specific requirements of the experiment. In general terms, 4–8 virgins and a smaller number of males are required for vials, and 10–20 flies are needed for bottles of small and medium size. To collect virgins, examine phenotypic markers, and manipulate *Drosophila* stocks, CO₂ is generally used to anesthetize flies instead of the traditional ether because is safer, easier, and avoids overanesthetization of the animals.

It is important to note the striking conservation of biological processes from flies to mammals. When a *Drosophila* homolog of an essential but poorly understood mammalian gene is identified, as happens with a large number of mitochondrial genes, powerful genetic and molecular techniques available in *Drosophila* can be applied to its characterization. These techniques include those discussed next.

1.4.1. Loss of Function Phenotype

1.4.1.1. Use of Mutagenic Agents: Mutagenic agents are used, followed by analysis of the different phenotypes produced and characterization of those caused by the mutation in the gene of interest.

1.4.1.2. Gene Disruption Mediated by P-Elements: This method is based on the insertion of a DNA flanked by transposase target sequences (the so-called P-element). The DNA inserts randomly into the genome. There are thousands of *Drosophila* lines available with P-elements inserted in different locations. In addition, a project to disrupt each gene in the *D. melanogaster* genome is under way (P-Element Screen/Gene Disruption Project; 6). Excellent information can be found at <http://flypush.imgen.bcm.tmc.edu/pscreen/>.

The power of these techniques can be increased using deletion mutants. Detailed information about the *Drosophila* Deletion Project such as construction, maps, and available stocks can be found at <http://www.drosdel.org.uk/>.

1.4.1.3. Ribonucleic Acid Interference (see Chapter 15): Knockdown of *Drosophila* genes by ribonucleic acid interference (RNAi) either in cells or in animals is described in detail in Chapter 15. An excellent Web page to visit in relation to ribonucleic acid interference is <http://flyrnai.org/>.

1.4.1.4. Homologous Recombination: Although historically it was thought that *Drosophila* lacks the homologous recombination process, the method developed by Golic and collaborators demonstrated that it does occur (7). This technique allows precise substitution of a specific DNA region of the *Drosophila* genome by another homologous, although not identical, sequence.

1.4.2. Overexpression Phenotype (see Chapter 15)—By a relatively easy transgenesis, one can introduce extra copies of a complementary DNA or a gene under the control of a selected promoter. In addition, the UAS/GAL4 system is a powerful and extensive transgenesis-based method described in detail in Chapter 15. There are collections of transgenic flies available for this technique, such as those found at <http://flystocks.bio.indiana.edu/Browse/misc-browse/gal4.htm>.

1.4.3. Developmental Pattern of Expression—Conservation of the developmental pattern of expression of a gene in different organisms may be an initial indicator of a similar function of the gene or of the process in which it is involved. An excellent Web site to access this method as a first approach is <http://www.ceolas.org/VL/fly/protocols.html>. In addition, we describe here a protocol for visualizing gene expression during *Drosophila* embryogenesis that we use in our laboratory and suggest a visit to <http://www.fruitfly.org/cgi-bin/ex/insitu.pl>. During *Drosophila* embryonic development, many nuclear-encoded mitochondrial genes involved in mtDNA replication, mtDNA maintenance, transcription, and translation share the midgut as a common territory of transcription (**Fig. 4**). Transcription of the genes encoding the mitochondrial ribosomal proteins mRpS17 and mRpL22 and the mtDNA maintenance factor TFAM (Mitochondrial Transcription Factor A) is also active in the midgut, as one can see at <http://www.fruitfly.org/cgi-bin/ex/bquery.pl?qpape=entry&qtype=summary>.

1.4.4. Phylogenetic Footprinting—Myriad computer programs have been developed to assist in the analysis of sequence data. Availability of genome sequences from other *Drosophila* species such as *Drosophila yakuba*, *Drosophila simulans*, or *Drosophila pseudoobscura* and other insects such as *Anopheles* or *Apis* open the possibility of using phylogenetic footprinting for identification of common regulatory elements that might suggest functional relationships among genes or groups of genes.

One notable example of this approach is the work of Caggese and collaborators (8), which revealed the presence of a putative regulatory element termed NRG (nuclear respiratory gene) in 100% of respiratory chain genes and in many other nuclear-encoded mitochondrial

genes in *D. melanogaster*. These are 90% conserved in *D. pseudoobscura* and in the respiratory chain complex V genes of *A. gambiae*. These authors also identified and annotated the *D. melanogaster*, *D. pseudoobscura*, and *A. gambiae* orthologs of 78 nuclear genes encoding mitochondrial proteins involved in oxidative phosphorylation by comparative analysis of their genomic sequences and organization.

2. Materials

2.1. Partially Purified Mitochondria

The ionic strength of buffers is determined using a radiometer conductivity meter.

1. Phenylmethylsulfonyl fluoride (PMSF) is prepared as a 0.2 *M* stock solution in isopropyl alcohol. Store aliquots at -20°C .
2. Sodium metabisulfite: 1.0 *M* stock solution at pH 7.5. Store aliquots at -20°C .
3. Leupeptin (Peptide Institute, Inc., code 4041): 1 mg/mL stock solution in 50 mM Tris-HCl, pH 7.5, 2 mM EDTA (ethylenediaminetetraacetic acid). Store aliquots at -20°C .
4. 0.5 *M* EDTA, pH 8.0.
5. 1 *M* Sucrose, ultrapure.
6. 1 *M* HEPES-KOH, pH 8.0; store at 4°C .
7. 1 *M* Dithiothreitol (DTT). Store aliquots at -20°C .
8. 3 *M* Potassium chloride.
9. 1 *M* Calcium chloride.
10. 10% (v/v) Triton X-100.
11. Homogenization buffer: 15 mM HEPES-KOH, pH 8.0, 5 mM KCl, 2 mM CaCl_2 , 0.5 mM EDTA, 0.5 mM DTT, 0.28 *M* ultrapure sucrose, 1 mM PMSF, 10 mM sodium metabisulfite, 2 $\mu\text{g/mL}$ leupeptin.
12. Dounce tissue grinder (homogenizer) (Wheaton), 7 mL, with tight and loose pestles.
13. Oak Ridge centrifuge tubes (screw-capped polypropylene copolymer tubes), 50 mL and 10 mL (Nalge Nunc International).
14. 25-mL Glass graduated cylinder.
15. Small plastic funnel.
16. Camel hair or similar brush with bristles clipped to approx 5 mm long.
17. 75- μm Nitex screen (Sefar America, Inc.), four 15-cm squares.

2.2. Mitochondrial Extraction

1. 20% (w/v) Sodium cholate. Cholic acid is dissolved in hot ethanol, filtered through Norit A (J. T. Baker Chemical Co.), and recrystallized twice before titration to pH 7.4 with sodium hydroxide.
2. Extraction buffer: 25 mM HEPES-KOH, pH 8.0, 10% (v/v) glycerol, 0.3 M NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 10 mM sodium metabisulfite, 2 µg/mL leupeptin.
3. Stabilization buffer: 25 mM HEPES-KOH, pH 8.0, 2 mM EDTA, 80% (v/v) glycerol.
4. 5 M Sodium chloride.
5. 1.5-mL Microcentrifuge tubes.
6. Other materials are as in **Subheading 2.1**.

2.3. Visualizing Mitochondrial Messenger RNAs in *Drosophila* Embryos

2.3.1. Preparation of the Probe

1. For transcription, we use the in vitro labeling kit from Roche (DIG RNA Labelling Kit SP6/T7; cat. no. 1175025). Although not included, T3 RNA polymerase can be used with this kit.
2. Phenol/chloroform (1:1).
3. Carbonate buffer: 120 mM Na₂CO₃, 80 mM NaHCO₃, pH 10.2. Store at −20°C.
4. Degradation stop solution: 0.2 M NaAc, pH 6.0.
5. 4 M LiCl.
6. Transfer RNA (tRNA) from baker's yeast (Sigma, cat. no. R5636).
7. 3 M Sodium acetate.
8. 100% Ethanol.
9. 70% (v/v) Ethanol in water.
10. Hybridization solution: 50% (v/v) deionized formamide, 5X SSC (Saline–Sodium Citrate), 50 µg/mL heparin, 100 µg/mL tRNA, and 0.1% (v/v) Tween-20.
11. Heparin sodium salt (Sigma- Aldrich, **ref.** H-3393).
12. 20X SSC: 3 M NaCl, 0.3 M sodium citrate, pH 7.

2.3.2. Preparation of the Embryos

1. 2.25% (w/v) Sodium hypochlorite.
2. A small spatula, a soft brush, and a filter to retain embryos.
3. Fixing solution: 1.3 mL 37% (v/v) formaldehyde, 5 mL heptane, 0.5 mL 10X phosphate-buffered saline (PBS), and 3.2 mL water.

4. PBS: 136 mM NaCl, 2 mM KCl, 8 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4.
5. 100% methanol.

2.3.3. Hybridization, Developing, and Visualization

1. PBT: PBS, 0.01% (v/v) Tween-20.
2. Rotator mixer.
3. 70, 50, and 30% (v/v) Methanol in PBT.
4. 4% (v/v) formaldehyde in PBT.
5. Hybridization solution/PBT (8:2 and 1:1) (*see item 10 in Subheading 2.3.1.*).
6. Antidigoxigenin antibody from Roche (cat. no. 1093274).
7. Developing solution: 4 M NaCl, 50 mM MgCl₂, 100 mM Tris-HCl, pH 9.0, 0.1% (v/v) Tween-20.
8. *p*-Nitroblue tetrazolium chloride from Roche (cat. no. 1383213).
9. 5-Bromo-4-chloro-3-indolyl phosphate from Roche (cat. no. 1383221).
10. 70, 50, and 30% (v/v) Ethanol in PBT.
11. Xylene.
12. Permount SP15-500 (Fisher Chemicals).
13. Glass slides and coverslips.
14. Clear nail polish.
15. Other materials as in **Subheading 2.3.2.**

2.4. Visualizing Mitochondrial Proteins With Fluorescent Antibodies in *Drosophila* Embryos

1. 1.25% (w/v) Sodium hypochlorite.
2. AbFixing solution (fixing solution for using antibody): 0.6 mL 37% (v/v) formaldehyde, 8 mL heptane, 2.8 mL water, and 0.6 mL 5X buffer B (50 mM potassium phosphate buffer, pH 6.8, 225 mM KCl, 75 mM NaCl, 65 mM MgCl₂).
3. 10% (w/v) bovine serum albumin (BSA) in PBT.
4. Vectashield H-1000 (Vector Laboratories, Inc., Burlingame, CA).
5. Other materials are as in **Subheading 2.3.2.**

3. Methods

3.1. Partially Purified Mitochondria

1. Collect *D. melanogaster* (Oregon R) embryos (average age 9 h) immediately before use by rinsing from agar collection plates using 0.1% (v/v) Triton X-100 and 0.7%

(w/v) NaCl, brushing with a camel hair brush, and collecting onto a 75- μ m Nitex screen (9).

2. Dechorionate embryos by incubation in 2.25% (w/v) sodium hypochlorite for 2 min with stirring, then rinse embryos thoroughly using Triton-NaCl solution (9).
3. Settle embryos for 15 min in 20 mL Triton-NaCl solution in a 25 mL graduated cylinder to remove remaining chorions, yeast, and fly fragments; aspirate supernatant; and repeat settling twice (*see Note 1*) (9).
4. Collect dechorionated settled embryos onto a tared 75- μ m Nitex screen and blot until damp between layers of paper towels; then, weigh embryos.
5. Suspend processed embryos at a ratio of 4 mL/g (*see Note 2*), wet weight, in homogenization buffer containing 15 mM HEPES-KOH, pH 8.0, 5 mM KCl, 2 mM CaCl₂, 0.5 mM EDTA, 0.5 mM DTT, 0.28 M ultrapure sucrose, 1 mM PMSF, 10 mM sodium metabisulfite, and 2 μ g/mL leupeptin; homogenize in approx 7-mL portions in a standard (7-mL) Dounce homogenizer using six strokes of the loose pestle followed by six strokes of the tight pestle (*see Note 3*).
6. Filter the homogenate through a 75- μ m Nitex screen into a 50 mL Oak Ridge centrifuge tube.
7. Rehomogenize the sample retained on the Nitex screen as in **step 5** using the same buffer (1 mL/g), filter as in **step 6**, and combine with the original filtrate (*see Note 4*).
8. Centrifuge the combined filtrate at 1000g for 7 min at 3°C to pellet nuclei and cellular debris (*see Note 5*).
9. Remove the supernatant using a 10-mL pipet, transfer to a fresh centrifuge tube, and repeat centrifugation as in **step 8** (*see Note 5*).
10. Repeat **step 9** once.
11. Pellet mitochondria by centrifugation at 7400g for 10 min at 3°C (*see Note 6*). Aspirate supernatant and discard.
12. Resuspend the mitochondrial pellet at a ratio of 2 mL of homogenization buffer per gram of starting embryos, transfer suspension into a 10-mL Oak Ridge centrifuge tube, centrifuge at 8000g for 15 min at 3°C, and aspirate supernatant and discard.
13. Repeat **step 12** once.

¹All operations are performed at 0–4°C.

²The procedures from **step 5** through **step 15** are designed for 5 g starting material and may be adjusted proportionally.

³Push the pestle slowly through the sample. To prevent sample loss, try wrapping parafilm around the top of the homogenizer and the pestle.

⁴The sample retained on the Nitex screen is rehomogenized to break any remaining intact embryos.

⁵The nuclear and cellular debris pellet is pale yellow and somewhat loose; try to remove supernatant without disturbing the pellet.

⁶The mitochondrial pellet is beige and forms a tighter pellet than that observed in **step 8**.

14. Resuspend the third pellet at a ratio of 0.5 mL homogenization buffer per gram, combine sample into one 10-mL tube or distribute into two 1.5-mL microcentrifuge tubes, and centrifuge as in **step 12**.
15. Freeze the final mitochondrial pellet in liquid nitrogen and store at -80°C .

3.2. Mitochondrial Extraction

1. Thaw frozen, partially purified mitochondria from freshly harvested and dechorionated *Drosophila* embryos (5 g) on ice for at least 30 min.
2. Resuspend mitochondria at a ratio of 0.5 mL/g of starting embryos (*see Note 7*) in extraction buffer containing 25 mM HEPES-KOH, pH 8.0, 10% (v/v) glycerol, 0.3 M NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 10 mM sodium metabisulfite, and 2 $\mu\text{g/mL}$ leupeptin.
3. Add sodium cholate to a final concentration of 2% (v/v) (*see Note 8*) and incubate the suspension on ice for 30 min with gentle mixing by inversion at 5-min intervals.
4. Centrifuge the resulting extract at $96,000g$ for 30 min at 3°C .
5. Recover the supernatant fluid (*see Note 9*) and add an equal volume of stabilization buffer containing 25 mM HEPES-KOH, pH 8.0, 2 mM EDTA, and 80% (v/v) glycerol.
6. Store the mitochondrial extract (fraction I) at -20°C .

3.3. Visualizing Mitochondrial Messenger RNAs in *Drosophila* Embryos

3.3.1. Preparation of the Probe—Smaller probes penetrate the embryo more readily. Thus, after transcription, the probe is usually degraded by alkali treatment and purified (*see Note 10*).

1. The fragment to be labeled must be previously cloned by standard methods in an *Escherichia coli* vector (i.e., pBluescript) flanked by a T7, T3, or SP6 RNA polymerase promoter (*see Note 11*).
2. Digest 2–3 μg of the plasmid with a suitable restriction enzyme. This must produce a linear fragment containing the promoter and the gene or fragment of the gene (*see Note 12*).
3. Check the digestion by agarose gel electrophoresis. If it is complete, then treat it three times with phenol/chloroform (*see Note 13*).

⁷Resuspend the mitochondrial pellet in one-third of the total extraction buffer volume, using the remaining two-thirds in two aliquots to wash out residual mitochondria and combine.

⁸Before adding sodium cholate, make certain that the mitochondria are completely suspended. Immediately following the addition of sodium cholate, the sample should become slightly viscous.

⁹The top of the supernatant will have a white lipid layer, and the top of the pellet is somewhat loose. Remove the supernatant carefully, avoiding the lipid layer and leaving behind approx 5% of the supernatant near the pellet.

¹⁰This is for probes bigger than 300 bp.

¹¹It is not necessary to clone a complete complementary DNA; a fragment of approx 300–400 bp is sufficient.

¹²Only 1 μg of the digestion is used for the transcription reaction, and the remainder may be stored. The promoter and the insert must be arranged in order to transcribe antisense molecules to hybridize with the mRNA of interest.

4. Precipitate the DNA with sodium acetate and ethanol by standard procedures and resuspend it to yield a 1-mg/mL concentration in water.
5. Make the riboprobe using the appropriate RNA polymerase (e.g., use an *in vitro* labeling kit following manufacturer's recommendations). A standard reaction includes 1 µg DNA; digU-NTP mix (labeling mix containing the four rNTPs plus digoxigenin-UTP); ribonuclease inhibitor; buffer; and RNA polymerase in a final volume of 10 µL.
6. After 2 h at 37°C, check 1 µL of the transcription reaction on a 1% agarose gel. You should see a single band of the expected size, although more diffuse than a DNA band (*see Note 14*).
7. To degrade the probe, add first 15 µL water (*see Note 10*).
8. Add 25 µL carbonate buffer and keep at 65°C for 40 min.
9. Add 50 µL degradation stop solution.
10. To purify the already degraded riboprobe, precipitate the RNA by adding 10 µL 4 M LiCl, 5 µL tRNA (20 µg/µL), and 300 µL ethanol.
11. Incubate 30 min at −20°C.
12. Centrifuge at 12,000g for 20 min at 4°C.
13. Wash twice with 70% (v/v) ethanol in water and resuspend in 100 µL of hybridization solution. Check 5 µL by agarose gel electrophoresis. Degradation must be observed.
14. Store at −20°C for days or up to several months.

3.3.2. Preparation of Embryos

1. Harvest embryos from 8 h collection using a soft brush, water, and a small filter that permits liquid to pass through but retains the embryos (*see Note 15*).
2. Submerge the filter in 2.25% (w/v) sodium hypochlorite for 2 min to remove the chorion (*see Note 16*).
3. Rinse exhaustively with water (*see Note 17*).
4. Fix the embryos by taking them with a spatula and submerging in 10 mL fixing solution (*see Note 18*).

¹³From this point, take care to protect the RNA (use gloves, aqueous solutions treated with diethyl pyrocarbonate, and sterilized materials).

¹⁴The reaction may be stopped by deoxyribonuclease I treatment. For *in situ* experiments, this is not strictly required because of the high number of transcribed RNA vs DNA template molecules.

¹⁵Because *Drosophila* can store embryos in the abdomen several hours, the time of laying is delayed. Thus, two or three consecutive layings are discarded, and the fourth laying period is harvested with few old embryos. For visualizing RNA, embryos can be stored at −20°C so that a large number can be harvested for several experiments.

¹⁶Some researchers prefer 1.25% (w/v) sodium hypochlorite for 4 min or other combinations.

¹⁷Some consider inserting a final wash with 0.7% (w/v) NaCl/0.02% (v/v) Triton X-100.

¹⁸We use small glass vials that are sold for scintillation counting. Embryos go to the interface. It is important that they form a monolayer. If there are too many embryos, then they aggregate and do not fix properly.

5. Mix vigorously for 20 min (i.e., 300 rpm in a shaker).
6. Remove the formaldehyde phase (the lower phase).
7. Add 10 mL methanol and mix vigorously by hand for 60 s. This removes the embryonic vitelin membrane.
8. Embryos sediment in a few seconds. Those with vitelin membranes remain suspended.
9. Remove everything except embryos at the bottom.
10. Add 5 mL methanol, mix gently, and remove it. Repeat twice.
11. Add 1 mL methanol, transfer embryos carefully with a cut pipetor tip to a 1.5-mL tube, and store at 4°C or –20°C (*see Note 19*).

3.3.3. Hybridization, Developing, and Visualization—Although embryos are already fixed, we recommend fixing the embryos again after storage. This requires a previous hydration, which is made as follows (*see Note 20*):

1. Use approx 50 µL of embryos in a 1.5-mL tube.
2. Remove the methanol and add 1 mL 70% methanol in PBT. Mix gently for 10 s.
3. Remove the methanol and add 1 mL 50% methanol in PBT. Mix gently for 10 s.
4. Remove the methanol and add 1 mL 30% methanol in PBT. Mix gently for 10 s.
5. Remove the methanol and add 1 mL PBT. Mix gently for 2 min. Repeat.
6. To refix the already hydrated embryos, remove the PBT and add 1 mL PBT/4% (v/v) formaldehyde.
7. Mix on a rotator mixer for 20 min at room temperature.
8. Remove the PBT/formaldehyde solution and wash with 1 mL PBT on a rotator mixer for 5 min. Repeat five times.
9. Wash with 1 mL PBT/hybridization solution (1:1).
10. For hybridization, remove the PBT/hybridization solution and prehybridize by adding 1 mL hybridization solution.
11. Incubate at 55°C for 60 min (no rotator mixer required) (*see Note 21*).
12. Prepare the probe: 1 µL of probe is added to 50 µL of hybridization solution and heated to 80°C for 10 min. Place on ice for 5 min (*see Note 22*).
13. Remove the prehybridization solution from the embryo tube and add the probe.
14. Incubate at 56°C overnight.

¹⁹Some prefer to store embryos in ethanol. This requires extra washes with ethanol before storage.

²⁰Incubation of non-refixed embryos with hybridization solution may break many structures in the embryo. For refixing, previous rehydration is required.

²¹Prehybridization saturates the nonspecific nucleic acid-binding elements in embryos.

²²This treatment denatures the RNA so that hybridization is facilitated.

15. For washing, remove (and store) the probe containing hybridization solution and add 1 mL 55°C preheated hybridization solution. Incubate 20 min at 55°C. Repeat twice (*see Note 23*).
16. Remove solution and wash with hybridization solution/PBT (8:2). Rotator mix for 1 min.
17. Remove solution and wash with hybridization solution/PBT (1:1). Rotator mix for 1 min.
18. Remove solution and wash with PBT. Rotator mix for 20 min. Repeat four times.
19. For developing, remove PBT and add 400 µL PBT containing 10 µL pretreated antidigoxigenin antibody (*see Note 24*).
20. Incubate 60 min in a rotator mixer at room temperature.
21. Remove antibody solution and wash 5 min with 1 mL PBT in a rotator mixer. Repeat four times.
22. Remove PBT and wash twice with 1 mL freshly prepared developing solution (*see Note 25*).
23. Remove and add 1 mL developing solution containing 9 µL p-nitroblue tetrazolium chloride and 7 µL 5-bromo-4-chloro-3-indolyl phosphate.
24. When embryos are colored, stop reaction by washing with PBT (*see Note 26*).
25. Finally, to prepare embryos for the microscope, we dehydrate them by washing with 30% (v/v) ethanol in PBT and leave 2 min on the bench.
26. Wash with 50% (v/v) ethanol in PBT. Leave 2 min on the bench.
27. Wash with 70% (v/v) ethanol in PBT. Leave 2 min on the bench.
28. Wash with 100% ethanol. Leave 2 min on the bench. Repeat twice (*see Note 27*).
29. Remove ethanol and add 1 mL xylene, which removes all possible traces of water (*see Note 28*).
30. Remove xylene and add 200 µL Permount.
31. Remove the embryos carefully with a cut pipetor tip and place on a glass slide; try to separate individual embryos. Add a glass coverslip and seal with clear nail polish.
32. Take good pictures under the microscope.

²³After hybridization, probes can be used several times, although this is often unnecessary because of the excess of unused probe stored at -20°C. From here, steps are at room temperature.

²⁴To avoid unspecific interactions with embryos, a 1:50 dilution in PBT of the antidigoxigenin antibody is incubated overnight at 4°C with rehydrated embryos. Thus, final dilution of the antibody is 1/2000.

²⁵Sometimes, this solution crystallizes at room temperature. Discard in this case.

²⁶After the developing reaction, the staining will be partially removed, so it is better to overdevelop than underdevelop. This choice is usually difficult to make because one tends worry about overdevelopment. Embryos not stained in 2 h will remain as such.

²⁷Sometimes, keeping overnight at 4°C increases the contrast by eliminating nonspecific staining.

²⁸If embryos collapse or aggregate, then repeat the 100% ethanol washes.

3.4. Visualizing Mitochondrial Proteins With Fluorescent Antibodies in *Drosophila* Embryos

The preparation of the embryos is the same as for riboprobes (see **Subheading 2.3.2.**) except for the following:

The chorion is removed with 1.25% (v/v) sodium hypochlorite treatment. Substitute fixing solution for AbFixing solution.

Do not store embryos. Depending on the antibody, fresh embryos are crucial.

Thus, after embryo hydration, we incubate with primary antibody as follows:

1. Incubate embryos with 10% (w/v) BSA in PBT. Incubate 60 min in a rotator mixer at room temperature (see **Note 29**).
2. Remove the solution and wash with 1 mL PBT in rotator mixer for 10 min. Repeat three times.
3. Add primary antibody in PBT (see **Note 30**).
4. Incubate overnight at 4°C in a rotator mixer.
5. To incubate with the secondary antibody, we remove primary antibody solution and wash four times for 10 min with PBT in a rotator mixer at room temperature.
6. Add 200 µL of secondary fluorescent antibody 1:200 in PBT. Incubate at least 60 min in a rotator mixer at room temperature.
7. Wash 5 min with PBT. Repeat three times.
8. Remove PBT and add 3 drops Vectashield.
9. Remove the embryos carefully with a cut Pipetman tip and put on a glass slide. Place a glass coverslip and seal with clear nail polish.
10. Take good pictures. (*Avoid immersion oil contacts with Vectashield.*)
11. Store in dark at 4°C. Embryos will remain fluorescent for approx 1 mo.

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²⁹This is for BSA to block the nonspecific protein–protein interaction points in the embryos.

³⁰Make a dilution of serum at 1:100 to 1:500. If the antiserum is particularly poor, then use a 1:50 dilution; if excellent, then use 1:1000. For monoclonal antibodies, use a 1:10 dilution.

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Fig. 1.

The *Drosophila* life cycle is divided into four stages: embryo, larva, pupa, and adult. The time length of the stages is approximate and is shown in hours for embryos and days for larvae and pupae.

Fig. 2.

The different stages of *Drosophila* life cycle growing in a bottle. First instar larvae feed on the surface of the medium. Second instar larvae burrow into the medium to feed (small black dots are the jaws of second instar larvae). Third instar larvae wander up the walls of the bottle, where they will pupariate. Adults are at the top of the bottle.

Fig. 3.
Plastic vial containing medium for growing flies. The vial is covered with hydrophobic cotton to avoid condensation of humidity that could interfere with air supply.

Fig. 4.

Pattern of transcription of some nuclear-encoded mitochondrial genes during *Drosophila melanogaster* embryogenesis. All encode proteins involved in mtDNA metabolism: mRpL19, mitochondrial large ribosomal subunit protein 19; TFAM, mitochondrial transcription factor A; mtDNA helicase; mtRNA polymerase; mtTFB1, mitochondrial transcription factor B1; G-ATPase, G subunit of mitochondrial adenosine triphosphate synthase.