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i. Abstract

RNA interference (RNAi) is a conserved sequence-specific gene silencing mechanism that is induced by double-stranded RNA (dsRNA). The development of methods that allow internalization of dsRNA and concomitant silencing of the desired gene has not stopped since the first demonstration of RNAi in *C. elegans*. In this chapter we describe how to introduce exogenous dsRNA into adult *Drosophila* in order to interfere with endogenous or viral gene expression.

ii. Key Words

Double-stranded RNA (dsRNA); dsRNA uptake; RNA interference; viruses; insects.

1. Introduction

Double stranded RNA (dsRNA) can trigger sequence-specific gene inactivation through RNA interference (RNAi) mechanisms in cells, as first demonstrated after injection into the worm *Caenorhabditis elegans* (***1***). This post-/co-transcriptional silencing effect was soon discovered in many diverse organisms ranging from plants (***2***) to fruit flies (***3***). If introduced into cells, dsRNA precursors (that can vary in length and origin) are diced into shorter dsRNAs (called small interfering RNAs or siRNAs) which serve as the guides for translational inhibition (reviewed in (***4***)).

Drosophila biology has greatly benefited from the use of RNAi. dsRNA injection into embryos before cellularization stage has been used as a tool to study developmental aspects or gene function (***3***). However, many genes cannot be silenced during development without having a lethal effect, and syncytial stage in embryos does not permit the targeting of specific cells. Injection of dsRNA into the haemolymph of *Drosophila* larvae has also been attempted but triggering RNAi in most tissues, except for haemocytes, seems impossible (***5***). In adult *Drosophila*, injecting dsRNA can induce a specific knockdown, allowing an *in vivo* functional analysis of the targeted genes (***6***, ***7***, ***8***). *In vitro* evidence shows that *Drosophila* S2 cells (derived from embryonic precursors of haemocytes) can autonomously take up long dsRNA (~200-500bp) from exogenous medium (***9***, ***10***), reminiscent of the environmental RNAi shown in *C. elegans* (reviewed in (***11***)).

In an immunological context, it was established that the core components of the RNAi machinery and the siRNA pathway are essential for antiviral defense (***12***, ***13***, ***14***). Interestingly, an effective dsRNA uptake mechanism is essential to generate a systemic antiviral state from a locally initiated infection, showing that, upon viral infection, dsRNAs can spread throughout the whole adult *Drosophila* (***15***). Taken together, these data foster the following model of immunity: during lysis, infected cells release viral dsRNAs, which can be taken up at a distance by haemocytes and perhaps other adult tissues in the flies in order to generate a systemic antiviral state. A mechanism for dsRNA uptake has been shown in other insects, like the mosquito *Aedes aegypti* (***16***).

In this chapter, we describe how to prepare and inject dsRNA in adult *Drosophila*. We have used this protocol to initiate a strong antiviral response in the context of a viral infection (***15***) but in our hands

this same protocol has successfully been used to silence endogenous gene expression.

2. Material

2.1. Fly stocks

Flies are grown on a standard yeast/agar medium at 25°C on a 12-hour light/dark cycle. All adult flies used for injection must be at least 5 days old (*see* **Note 1**).

2.2. Total RNA isolation

1. TRIzol Reagent (Invitrogen, Carlsbad, CA)
2. Chloroform
3. Isopropyl alcohol
4. Nuclease-free 75% Ethanol conserved at –20°C
5. Nuclease-free water
6. RNaseZap RNase decontamination solution (Ambion, Austin, TX)
7. Pellet pestle (# Z359971-1EA) (Sigma, St. Louis, MO)

2.3. Reverse Transcription

1. SuperScript II Reverse Transcriptase (200 U/μL) (Invitrogen, Carlsbad, CA)
2. First-Strand Buffer (5X): 250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$
3. 10 mM (each) dNTPs Mix (dATP, dCTP, dGTP, dTTP)
4. 100 mM DTT (dithiothreitol)
5. RNase OUT Ribonuclease Inhibitor (40 U/μL) (Invitrogen, Carlsbad, CA)
6. Random Hexamers (50 ng/μL)
7. Nuclease-free water
8. Nanodrop UV spectrophotometer

2.4. Polymerase Chain Reaction

1. Thermocycler
2. Thermophilic DNA Polymerase (*Thermus aquaticus*) (5 U/μL)

3. PCR Buffer (10X): 200 mM Tris-HCl, pH 8.4, 500 mM KCl

4. 10 mM (each) dNTPs Mix (dATP, dCTP, dGTP, dTTP)

5. 25 mM $MgCl_2$

6. Nuclease-free water

2.5. dsRNA production

1. T7 RNA Polymerase Buffer (5X): 400 mM Hepes-KOH, pH 7.5, 120 mM $MgCl_2$, 10 mM Spermidine (Sigma, St. Louis, MO), 200 mM DTT (Sigma, St. Louis, MO) (*see* **Notes 2, 3**). Store in aliquots at –20°C for several months.

2. T7 RNA Polymerase (20 U/μL) (Ambion, Austin, CA)

3. RNase OUT Ribonuclease Inhibitor (40 U/μL) (Invitrogen, Carlsbad, CA)

4. 100 mM (each) nucleoside triphosphate (ATP, CTP, GTP, UTP) (Fermentas, Burlington, ON)

5. 5 M Ammonium acetate (Ambion, Austin, TX)

6. Phenol/Chloroform/Isoamylic alcohol (25/24/1), pH 6.6 (Ambion, Austin, TX)

7. Isopropyl alcohol

8. Nuclease-free 75% ethanol

9. 1 M Tris-HCl, pH 7.0 (Ambion, Austin, TX)

10. Nuclease-free water

11. Nanodrop UV spectrophotometer

2.6. Microinjection needles fabrication

1. P-97 Flaming/Brown micropipette puller (Sutter Instrument Company, Novato, CA)

2. Borosilicate glass tubes (# 3-000-203-G/X: outside diameter: 1.14 mm, inside diameter: 0.53 mm, overall length: 3.5" (Drummond Scientific Company, Broomall, PA) or # BF100-50-10: outside diameter: 1.00 mm, inside diameter: 0.50 mm, overall length: 10 cm (Sutter Instrument Company, Novato, CA)

3. Mineral oil

4. Pasteur pipette

2.7. Injection

1. Automatic nanoinjector Nanoject II variable volume (Drummond Scientific Company, Broomall, PA). The Nanoject II is set up to inject 50.6 nL by moving positions of the dip switches up (U) or down (D) as DDUD from position 1 to 4 respectively.

2. Stereozoom microscope

3. Cold-light reflector lamp

4. CO_2-anaesthetizing system (Genesee Scientific, San Diego, CA)

3. Methods

3.1. Template selection

In vitro dsRNA synthesis can be made from various DNA templates generated by PCR. Either genomic DNA or cDNA can be used to amplify the gene region targeted by the silencing assay. Keep in mind that only small interfering RNAs (generated from the dsRNA sequence by the RNAi machinery) that match with transcript sequences will generate a knockdown (*see* **Note 4**). For this reason, it is preferable to proceed on cDNA templates (to avoid intronic DNA contaminations) obtained from flies expressing the gene of interest. Alternatively it is possible to design primers within a single exon if genomic DNA is to be used as matrix. The complete protocol from RNA isolation to dsRNA production is described in **Sections 3.3 to 3.6**.

3.2. Primers design

MIT's Primer3 program (http://frodo.wi.mit.edu/primer3/) is suitable to design oligonucleotides, for amplification of the target sequence. Optimal oligo size is 22 nt (±2) length for a 60°C annealing temperature. At the 5'-end of each oligonucleotide, add a 23 nt T7 promoter sequence (5' - TAATACGACTCACTATA**G**GGAGA - 3') [the G in bold is the first base incorporated into RNA during transcription]. Order primers in salt-free water at 100 µM. dsRNA products ranging from 200 to 1,500 bp have been shown to work in RNAi but ~300-600 bp are usually used for an efficient uptake and silencing. Also remember to check that the total dsRNA product sequence does not contain ≥ 19-mer matching other gene sequences to prevent non-specific knockdown of gene expression (off-target effect).

3.3. Total RNA isolation

TRIzol Reagent combines acidic phenol and guanidinium thiocyanate in order to lyse cells, inactivate RNases, and remove lipids. Manipulate this toxic solution with gloves and in a chemical fume hood. Remember to work in an RNase-free area (cleaned with RNase Zap) and keep samples on ice as much as possible.

1. Harvest three to five flies in a clear polypropylene tube and euthanize them by flash freezing in liquid nitrogen or in a dry ice/ethanol mixture (*see* **Note 5**).

2. Add 200 µL TRIzol Reagent and process the flies with a pellet pestle until a homogenized tissue sample is obtained. Complete with 800 µL TRIzol Reagent and incubate 5 min at room temperature.

3. Proceed to the phase separation by adding 200 µL chloroform, vortex for 1 min, and centrifuge at 12,000 x g for 15 min at 4°C (*see* **Note 6**).

4. To precipitate RNA, transfer the aqueous phase to a new pre-chilled tube and add 400 µL isopropyl alcohol before centrifuging at 12,000 x g for 15 min at 4°C (*see* **Note 7**).

5. Remove the supernatant and wash the RNA pellet with 500 µL of chilled 75% ethanol. Mix by vortexing and centrifuge at 7,500 x g for 5 min at 4°C.

6. Air dry the RNA pellet by leaving the sample open under the extractor hood (*see* **Note 8**) and dissolve it by pipetting up and down in 40 to 80 µL nuclease-free water (*see* **Note 9**).

3.4. Reverse Transcription

1. Prepare RNA at a concentration of 1 µg/µL (*see* **Note 10**).

2. In a PCR tube, mix:

    a. 1 µL Random Hexamers

    b. 1 µL 10 mM dNTPs Mix

    c. 1 µL RNA sample (or nuclease-free water as a negative control)

    d. 9 µL nuclease-free water

3. Heat 5 min at 65°C, and quick chill on ice (*see* **Note 11**).

4. Spin down and add:

    a. 4 µL 5X First-Strand Buffer

    b. 2 µL 100 mM DTT

    c. 1 µL 40 U/µL RNase OUT

5. Mix gently, spin briefly down, and incubate 2 min at 25°C before adding 1 μL of 200 U/μL SuperScript II Reverse Transcriptase.

6. Incubate at 42°C for 50 min to allow elongation and then inactivate the reaction by heating at 70°C for 15 min.

7. Aliquot and store cDNA at –20°C if necessary.

3.5. Polymerase Chain Reaction

Once cDNA from desired transcriptome is obtained, perform PCR with synthesized primers.

1. For a single PCR reaction, mix:

    a. 2.5 μL 10X PCR Buffer

    b. 2 μL 25 mM $MgCl_2$

    c. 0.5 μL 10 mM dNTPs Mix

    d. 16.75 μL nuclease-free water

    e. 0.25 μL 1.25 U/μL thermophilic DNA Polymerase

2. Transfer 22 μL of PCR Mix in a PCR tube, and add:

    a. 1 μL 10 μM forward primer

    b. 1 μL 10 μM reverse primer

    c. 1 μL cDNA (or nuclease-free water as a negative control)

3. Gently mix, spin down briefly and run in thermocyler (33 cycles: 94°C 30s, 60°C 30s, 72°C 60s) (*see* **Note 12**).

4. Check the result on a 1% agarose gel.

3.6. dsRNA production

1. In a polypropylene tube, prepare a T7 Mix:

    a. 20 μL 5X T7 RNA Polymerase Buffer

    b. 5 μL of each 100 mM nucleoside triphosphate (ATP, CTP, GTP, UTP)

    c. 2 μL 40 U/μL RNase OUT

d. 1 μL 20 U/μL T7 RNA Polymerase

2. Per one T7 *in vitro* transcription reaction, in a 1.5 mL polypropylene tube, mix:

a. 1 μg PCR product

b. 43 μL T7 Mix preparation

c. up to 100 μL with nuclease-free water

3. Incubate at 37°C for 16 hrs or overnight (in a dry bath incubator or in a bacterial incubator to avoid condensation).

4. After *in vitro* transcription, add 340 μL nuclease-free water, 60 μL 5 M Ammonium acetate (samples can be stored at –20°C at this stage if needed), and 500 μL Phenol/Chloroform/Isoamylic alcohol stored at 4°C.

5. Mix by vortexing 15 s, and centrifuge at 16,000 x g (maximum speed in a microcentrifuge) for 5 min at 4°C

6. Transfer aqueous phase in a new tube, add 1 volume (~500 μL) of isopropyl alcohol, mix by inversion and incubate at least 1 hr at –20°C.

7. Centrifuge at 16,000 x g for 15 min at 4°C, wash the pellet with 800 μL of 75% ethanol, and centrifuge again as previously.

8. Air dry the RNA pellet and dissolve it in nuclease-free water or 10 mM Tris-HCl.

9. Measure RNA concentration, and adjust it at 3 μg/μL (samples can be stored at –20°C at this stage until annealing).

10. Incubate the RNA sample for 15 min at 65°C in a dry bath incubator, then remove the heating block, or switch off the incubator, and let it cool down to room temperature for an efficient annealing.

2. Check on a 1% agarose gel (**Fig. 1**), and store in 20 μL aliquots at –20°C or –80°C.

3.7. Preparation of Needles for Microinjection

1. In order to make < 1 μm needles from borosilicate tubes, the micropipette puller machine program is set up by the following parameters: heat: 515, pull: 60, velocity: 60, time: 250, pressure: 500 (*see* **Note 13**).

2. Once needles are pulled, back-fill microinjection needles with mineral oil using a Pasteur pipette previously thinned (*see* **Note 14**). Take care to remove any air bubbles inside.

3. Needles can be stored at room temperature for several months.

4. Just before use, delicately break the tip of the needle with thin tweezers, taking care of making the tip thin enough to avoid major injury to flies, but thick enough to ensure full delivery of the solution (*see* **Note 15**). Empty almost all of the mineral oil from the needle, and fill it with the solution to inject.

3.8. Injection

1. Flies must be CO_2-anaesthetized and positioned for intra-thoracical injection (*see* **Note 16**). Needles are applied between the supraalar bristles (SA1, SA2) and the presutural bristle (PS), in the intrascutal suture level (**Fig. 2**). Note that introduction of the needle in this region should not encounter any resistance, as if you are introducing the needle in a hole (*see* **Note 17**).

2. 50 nL of an appropriate dsRNA solution (3 μg/μL) or a buffer solution as a control (10 mM Tris-HCl, pH 7.5) are injected using the nanoinjector.

3. After injection, let flies recover in appropriate vials in horizontal position (to avoid flies to get stuck to the medium), and verify the appearance of melanization spots at the injection site (*see* **Note 18**).

4. Track the silencing of your targeted gene by a method of choice. Efficiency of silencing will be dependent on the dsRNA preparation and time-dependent on your target (mRNA stability, protein turnover).

4. Notes

1. Younger flies are unable to take up dsRNA as also shown in (*7*).

2. This homemade buffer has been optimized over commercial buffers for better results yields.

3. It is important to use KOH in order to buffer HEPES and not NaOH. Spermidine stimulates T7 RNA polymerase activity, DTT works as a reducing agent to avoid disulfide bonds. DTT is not easily dissolved so it may be necessary to heat at 55°C the solution until crystals disappear. Adjust concentration of reagents in nuclease-free water. Dissolve DTT and spermidine in water.

4. Coding sequences but also 3'untranslated regions work as excellent targets.

5. Manipulating liquid nitrogen needs precaution, you should prefer prepare a "slurry" mixture by carefully adding pure ethanol on dry ice. The fact that tubes can stand right in the mixture provides a better contact with the slurry and so a better cold convection to your samples.

6. After centrifugation, you can observe three phases: a lower red phenol/chloroform phase containing proteins and lipids, a viscous white interphase containing DNA, and an upper colorless aqueous phase containing RNA.

7. In order to improve RNA purity, it is preferable to sacrifice a little amount of the aqueous phase close to the interface.

8. Do not let the RNA sample dry completely. Once water added, you can heat the sample at 60°C for 10 min on a dry bath to help dissolution.

9. RNA can be stored in nuclease-free distilled water at –20°C or –80°C. There is no consensus on RNA storage, but in water, in a Tris-EDTA buffer (10 mM Tris, pH 7.0, 1 mM EDTA), or in 1 mM sodium citrate (pH 6.4), RNA is stable at least one year at –80°C. RNA can also be stored in pure deionised formamide at –70°C, or in ethanol from the precipitation step at –20°C.

10. A 20 μL reaction volume can be used for 1 ng to 5 μg of total RNA.

11. The heat step allows the secondary structure denaturation.

12. As a standard running condition, you can use: a) 94°C for 5 min, b) 94°C for 30 sec, c) 60°C for 30 sec, d) 72°C for 1 min, e) steps b-d 33 cycles, f) 72°C for 5 min, g) holding at 4°C.

13. For more technical information about the parameters, download the supplier's pipette cookbook: http://www.sutter.com/products/product_sheets/p97.html.

14. In order to make Pasteur pipette thinner, light a Bunsen burner, hold the pipette at each extremity and heat the thinnest part of it on the flame for few seconds, turning continuously the pipette until the thin part becomes red. When it starts to melt, remove quickly from the flame and pull constantly until ductility is lost. Then, cut the thinner Pasteur pipette at an appropriate distance to fill the micropipette. Remind to not wear gloves with Bunsen burner.

15. In a nutshell, proceed with care, and practice a lot!

16. To improve fly survival, it is best not to leave flies more than half an hour on the CO_2-anaesthetizing pad. We suggest injecting flies in small groups, knowing that a good experimenter can inject up to a hundred flies per hour.

17. Injection can be performed intra–abdominally **(*6*)**, but in our hands lethality after injection is higher.

18. Melanization spots appear a few minutes after injury, but for convenience and to prevent repeated anaesthesia at the same day, you should check the day after. For an overview of melanization, see **ref. (*17*)**.

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Figures

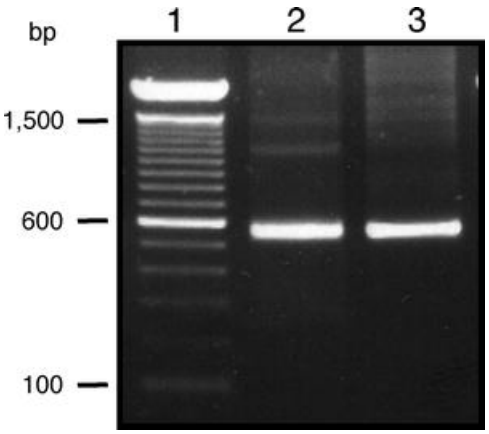

Figure 1: Checking for annealing of dsRNA.

After *in vitro* transcription a 1/10th dilution of the obtained dsRNA was subjected to electrophoresis in a 1% agarose gel, stained with ethidium bromide. Lane 1: molecular weight marker. Lanes 2 and 3: dsRNA after annealing. Note the presence of a sharp band at the desired size (around 500 bp).

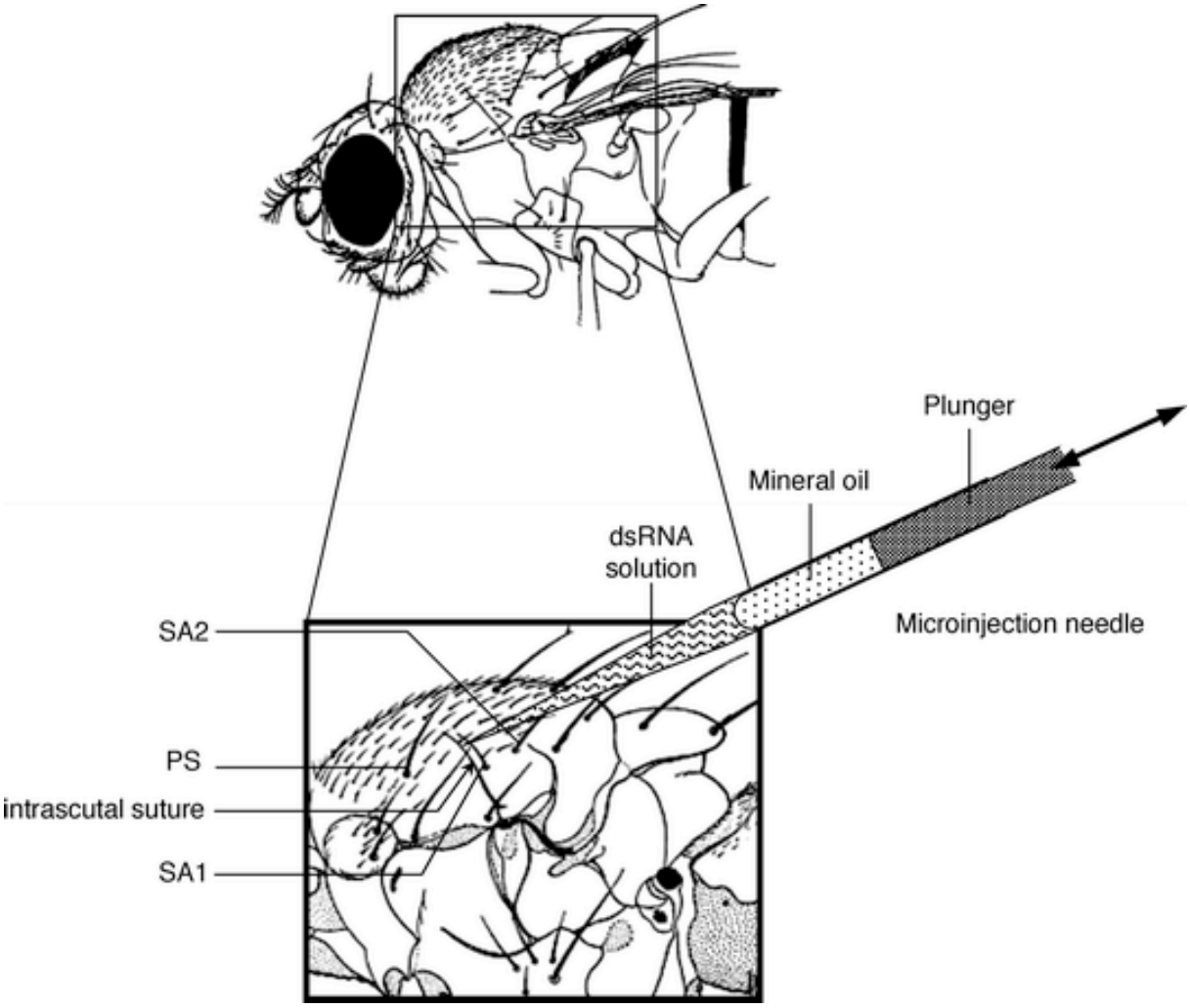

Figure 2: Scheme from intra-thoracic injection in adult *Drosophila*

Needles are gently introduced between the supraalar bristles (SA1, SA2) and the presutural bristle (PS), in the intrascutal suture level (see insight). Reproduced from Biology of Drosophila (Chapter: The external morphology of the adult, p. 402) with permission from Cold Spring Harbor Laboratory Press.