RNA Interference Screening for Genes Regulating Drosophila Muscle Morphogenesis

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Abstract

RNA interference (RNAi) is the method of choice to systematically test for gene function in an intact organism. The model organism *Drosophila* has the advantage that RNAi is cell autonomous, meaning it does not spread from one cell to the next. Hence, RNAi can be performed in a tissue-specific manner by expressing short or long inverted repeat constructs (hairpins) designed to target mRNAs from one specific target gene. This achieves tissue-specific knock-down of a target gene of choice. Here, we detail the methodology to test gene function in *Drosophila* muscle tissue by expressing hairpins in a muscle specific manner using the GAL4-UAS system. We further discuss the systematic RNAi resource collections available which also permit large scale screens in a muscle specific manner. The full power of such screens is revealed by combination of high-throughput assays followed by detailed morphological assays. Together, this chapter should be a practical guide to enable the reader to either test a few candidate genes, or large gene sets for particular functions in *Drosophila* muscle tissue and provide first insights into the biological process the gene might be important for in muscle.

Keywords: *Drosophila*; muscle; sarcomere; RNAi; GAL4-UAS; genetic screens; off-target effects;

Running head: Muscle-specific RNAi in *Drosophila*

1 Introduction

RNA interference (RNAi) offers a simple method to test for gene function in a developing organism by knocking down target mRNAs [1-3]. In contrast to the situation in *C. elegans*, the RNAi machinery acts cell autonomously in *Drosophila* [4]. This enables the researcher to perform tissue-specific knock-down of chosen target mRNAs using the inducible GAL4-UAS system. The availability of transgenic genome-wide UAS inverted repeat collections (UAS-RNAi, UAS-IR, UAS-hairpin) in *Drosophila* [5,6] allows to test the function of almost any gene. The combination of these UAS-IR fly lines with the wide variety of GAL4 lines available in *Drosophila* facilitates tissue or cell-type specific knock-down at different developmental stages [3,7].

Here, we provide all practical details to knock-down mRNAs in developing and adult *Drosophila* muscles. Depending on the selected GAL4 line this can be done either in all body muscles or in specific muscle subgroups only. The addition of the GAL4 repressor GAL80-temperature-sensitive (GAL80-ts) allows an even more precise timing of the RNA knock-down onset during development [8]. We give practical tips how to most effectively plan and perform large scale screens in muscle, similar to those done successfully before [9-13], in particular how to effectively test for gene function by a combination of high-throughput assays and detailed but more time consuming muscle and sarcomere morphology and function assays. Together, this should enable the reader to quickly test any given gene for a function during *Drosophila* muscle development and also provide a starting point to identify the biological process the gene might be important for during muscle development.

2 Materials

Apart from the standard fly husbandry (fly food in small tubes and large bottles, stereomicroscope with CO_2 to sort flies, temperature and humidity controlled incubators to grow flies, brushes) we describe all the specific reagents and materials below to test gene function in *Drosophila* muscle by RNAi.

2.1 Fly strains and large fly collections

1. Large UAS-RNAi fly line collections (UAS-RNAi, UAS-IR) exist at three different stock centers, the Vienna *Drosophila* Resource Center (VDRC) in Vienna (https://stockcenter.vdrc.at), the Bloomington *Drosophila* Stock Center (BDSC) in Bloomington, Indiana (https://bdsc.indiana.edu/) and the NIG collection in Kyoto (https://shigen.nig.ac.jp/fly/nigfly/). Stock centers require the users to register with a laboratory account and then ship stocks to any location in the world for a small processing fee. The VDRC equips each fly vial with a barcode, which can simplify fly handling in large-scale screens. For a comprehensive comparison of the RNAi libraries in terms of library preparation, coverage, logistics and pricing see [3].

The Vienna and Kyoto collections contain mainly long inverted repeats (300 - 400 base pairs), whereas the TRiP collection found in Bloomington contains a large amount of short hairpins, based on the endogenous miR-1 scaffold [6]. The UAS-IR constructs of the TRiP collection are found in different UAS backbone vectors (Valium). Generally, the UAS-t based vectors (Valium 10 and Valium 20) do work better than the UAS-p based ones (Valium 22) for zygotic knock-down in muscle.

In our experience, it is not clear a priori if a short or a long hairpin does result in more efficient knock-down of the target mRNAs. Thus, we recommend to test all available hairpins for a given gene, if the function of this gene should be investigated.

2. Large scale fly line GAL4 collections are also available at the BDSC and the VDRC. Various muscle-specific GAL4 lines are available from these sources, however some other useful GAL4 lines for muscle-specific RNAi must be requested from specific laboratories. Table 1 provides a comprehensive list of useful muscle-specific GAL4 lines, including their expression characteristics and sources from where to potentially acquire them.

3. Transgenic or knock-in fluorescent fly marker strains and their sources as listed in Table 2. These lines are useful markers to visualize muscle components when assaying gene function after RNAi knock-down either using live imaging or also after fixation as antibodies against the tags can easily be used for visualization of the protein.

2.2 Reagents for functional muscle tests

1. Flight test: One meter long Plexiglas tube with a diameter of 15 cm, with 5 indicated subdivisions (20 cm each), water at the bottom in a Petri dish and a funnel inserted at the top of the cylinder to drop down flies [14];

2. Spontaneous muscle contractions during development: a standard spinning disc or scanning confocal microscope is required for imaging;

2.3 Reagents for muscle morphology assays

1. Live muscle protein markers listed in Table 2;

2. Antibodies against muscle and sarcomere proteins listed in Table 3;

3. PBS and PBT (PBS with 0.3% Triton-X);

4. Normal goat serum (NGS);

5. Relaxing solution (20 mM phosphate buffer, pH 7.0; 5 mM $MgCl_2$; 5 mM EGTA, 5 mM ATP; 0.3% Triton X-100) (needs to be freshly prepared);

6. Fixing solution (4% Paraformaldehyde (PFA) in PBS with 0.3% Triton X-100 or 4% PFA in relaxing solution);

7. Mounting medium for fixed samples (Vectashield, optionally with DAPI);

8. 50% glycerol for mounting larvae and live samples;

9. Microscope slides and cover slips;

10. Dissection materials: sharp scissors (Fine Science Tools, No. 15000-02), blunt forceps, sharp forceps (Dumont #5, Fine Science Tools), glass embryo plates with lids, silicon-filled Petri dish, insect pins, double-sided tape, microtome blade (PFM C35, No. 207500003), transparent nail polish;

11. Custom-made imaging slide with a groove in the middle to fit the pupae;

12. Barcode reader and printer (e.g. Zebra TLP2824);

13. A standard point scanning confocal microscope for imaging;

3 Methods

3.1 Planning of experiments – testing positive and negative controls

It is well known that RNAi can result in unwanted unspecific effects, mainly knock-down of other genes than the anticipated gene. These off-target effects strongly depend on the GAL4 line, the temperature and the assay conditions used. Thus, it is recommended to choose a set of negative control genes, which have no role in muscle [10] to estimate how many of these would score positive in the assay chosen. This can be used to calculate the false positive rate (number of negative control genes scoring positive divided by all negative control genes tested). If the false positive rate is too large, the assay conditions need to be adjusted.

The false negative rate can be estimated by testing a set of positive control genes, which are known to play a role in the tested process (number of positive control genes scoring negative divided by all positive control genes tested). In particular for a large scale screen these numbers are important to calculate the false discovery rate (false positive rate divided by total hit rate) [3]. If the false negative rate is high, the co-expression of UAS-Dcr2 can be used to enhance RNAi activity [5]. However, this usually also increases the false positive rate. Together, these gene test sets can be used to optimally adjust RNAi knock-down efficiencies to the used GAL4 line and the particular process studied.

3.2 Fly crosses for muscle-specific RNAi

All fly crosses should be performed in an incubator at defined temperature and humidity (about 70%). Note that GAL4 activity is higher at higher temperature. Thus, raising the

temperature from the standard 25ºC to 27ºC or even 29ºC will increase the level of knock-down.

1. Collect virgin females from a chosen muscle-specific GAL4 line (see Table 1), which in addition may contain a fluorescently-tagged marker protein if in addition microscope based evaluation is planned or UAS-Dcr2 if RNAi efficiency should be enhanced (*see* **Notes 1, 2**).

2. Cross the collected virgin females with males from the UAS-IR line against the gene of interest. Use 3 to 6 virgin females per small tube and about 20 virgin females per large bottle with a similar amount of males.

3. Incubate in the incubator until the desired age and then perform the desired assay at larval, pupal or adult stages (*see* **Note 3**).

3.3 High throughput assays after RNAi based gene knock-down: lethality, locomotion and flight tests

If a gene is essential for muscle function, an efficient knock-down in muscle results in lethality. If the gene is only required for flight muscle function, its knock-down results in flightlessness. These simple assays can be easily used to test the function of many hundred genes in muscle [10].

1. Perform an RNAi cross as described in 3.2. Use barcodes if a high-throughput screen is done.

2. Assay after 10 days at 27ºC or after 14 days at 25ºC if adult progeny carrying GAL4 and UAS-IR construct are present (viable) or not (*see* **Note 4**). If not, check the vial for dead larvae or pupae to determine the lethality stage.

If a lethality stage was identified, low-throughput assays (see 3.4 and 3.5) can be used to investigate the muscle phenotype of the respective stage in more detail (*see* **Note 3**).

3. Locomotion assay: if progeny of the cross are viable collect 30 males (*see* **Note 4**) and incubate them for another 7 days at 27ºC or 25ºC. To roughly estimate locomotion, bang the flies down in the vial and estimate the speed of climbing compared to wild-type. Obvious locomotion defects can be easily identified by this high-throughput assay.

4. Flight test: use the same flies from 3 and drop them into the flight cylinder through the funnel at the top. Score the zone in which the flies are landing. Wild-type flies will land in the upper 2 zones of the cylinder. Flightless animals will drop into the water at the bottom of the tube (*see* **Note 5**). If more than 70% of the flies tested end in the water the flight muscle have a major functional defect and should be investigated in more detail by antibody stainings.

5. If a high-throughput screen is done all results should be collected using an electronic database, such as Filemaker or Microsoft Access. The use of barcodes and barcode scanners ensures the error-free entry of the fly line data.

6. Re-test the line blindly if it scored positive in the high-throughput assay by feeding it again into the screen (point 1).

7. If an RNAi line scores reproducibly positive, additional independent RNAi lines targeting the same gene should be tested in the same assay. Ideally, at least 2 independent lines should score positive before starting a detailed phenotypic analysis (see 3.4 and 3.5). This quality check efficiently eliminates unspecific off-target hits (*see* **Note 6**).

3.4 Low throughput fixed assays after RNAi based gene knock-down: whole mount, muscle dissections and antibody stainings

3.4.1 Larval muscles: The larval muscle morphology can be visualized in whole mount larvae with a fast heat fixation protocol [10]. This requires having a fluorescent muscle marker in the genetic background (see Table 2) such as ZASP66-GFP (Protein-GFP trap), labelling Z-discs, or *Mhc*-Tau-GFP, labelling all the larval body wall muscles. Depending on the lethality stage use blunt forceps to collect larvae after 2-5 days at 27°C (L2 and L3 larvae are easier to handle) and immobilise them by placing them into hot water (65°C) for a few seconds and then mount them in 50% glycerol on a glass slide. Since the larvae are thick use coverslips as spacers between the slide and the coverslip located on the larvae. The larvae can be imaged using a low magnification objective (10x or 20x) of a fluorescent microscope or with a point scanning confocal at high resolution. More detailed muscle morphology and also neuromuscular junctions can be visualized by preparing larval filets and performing antibody stainings using either sarcomeric antibodies such Mhc, α-Actinin or postsynaptic density antibodies such as Dlg1 [15-17].

3.4.2 Pupal or adult muscles: The histological dissection followed by antibody stainings for pupal or adult flight and abdominal muscles have been described in detail elsewhere [17]. Here we briefly describe how to dissect and stain indirect flight muscles at pupal and adult stages. These assays can be used for example to visualise the actin cytoskeleton of sarcomeres (using phalloidin), the nuclei (using DAPI) together with other proteins of interests using antibodies listed in Table 3. These assays allow analysis of not only gross developmental defects such as muscle atrophy or attachment defects but also detailed

myofibril and sarcomere quantification to identify putative defects after RNAi mediated knock-down (*see* **Note 7**).

Dissections of flight muscles at pupal stages (works best from 8 hr to 50 hr APF):

1. Set up the RNAi cross (see 3.2).

2. To stage pupae of the correct age, collect young white pre-pupae (0-1 h old) with a wet brush and place them on a slide with a stripe of double-sided tape on it. Put the slide into a Petri dish that contains a wet filter paper to avoid drying of pupae.

3. Incubate the pupae until the desired stage.

4. Under the dissection microscope use blunt forceps to remove all the parts of the pupal case that is not facing the slide. Since the pupae are placed on a double-sided tape the bottom of the pupal case will remain sticking to the slide. Using a wet brush gently lift the pupa and transfer to a silicon-filled Petri dish filled with ice-cold PBS. Until all the pupae from the same genotype are collected keep this dish on ice. This should not take more than 15 minutes to decrease developmental variation among different pupae.

5. Orient the pupae with the ventral side facing up. To fix the position of the pupa use 2 fine insect pins and push them through the posterior part of the abdomen until the pins are inserted into the silicon.

6. Since the flight muscles are located on the dorsal side of the thorax ventral part needs to be removed. Using fine scissors make a small hole in the head of the pupa. Using this hole as an entry point for the scissors cut the pupa on both sides laterally until the middle of the abdomen. Then remove the ventral side by cutting it on the posterior side.

7. On the remaining dorsal part there are other tissues such as gut, trachea and fat. Using blunt forceps remove these tissues. Avoid touching the muscles underneath.

8. Using fine scissors cut the thorax into two along the dorsal midline until the position insect needles were fixed. Then cut each thorax half at the posterior end and transfer into a glass embryo dish filled with ice-cold PBS.

9. Once all the thorax halves from all the pupae are collected remove PBS and add cold 4% PFA in PBT (200 µl suffice to cover all the samples). Fix for 15 min at room temperature. Remove the fixative and add PBT.

10. Once the tissue is fixed standard staining procedures can be used (see Note 8). In all subsequent steps use PBT as the medium to improve antibody penetration. In general, block the tissues for 1 h at room temperature using NGS diluted 1/30 in PBT, incubate in the primary antibody overnight at 4 ºC and in the secondary antibody for 2 hr at room temperature. In between the antibody steps and after the secondary antibody incubation wash minimum 3 times, 10 min each, with PBT. To visualize actin cytoskeleton use Rhodamine-phalloidin (1/500 in PBT) added to the secondary antibody solution. Mount half thoraces using Vectashield (optionally with DAPI to visualize nuclei) as the mounting medium. Immobilize the coverslip using a transparent nail polish.

11. Image the muscles using a scanning confocal microscope.

Dissections for adult flight muscles:

1. On the fly pad with CO_2 supply anesthetise flies and use fine scissors to remove the wings, legs, abdomen and head.

2. Transfer the thorax to a glass embryo dish filled with ice-cold PBS. After all the thoraces from the same genotype are collected in the embryo dish remove the PBS and add 4% PFA in relaxing solution (*see* **Note 9**). Fix for 15 min at room temperature. Remove the fixing solution and wash 2 times with relaxing solution.

3. To visualize the dorsal-longitudinal flight muscles (DLMs), the thorax needs to be cut sagittally. Thus place the thorax on a slide with a stripe of double-sided tape on it. The anterior most part of the thorax should be facing the slide. Using a microtome blade cut the thorax in sagittal plane, ideally in one movement to avoid disrupting the myofibrils with the movement of the blade. Transfer the half thoraces into a glass embryo dish and proceed with standard staining procedure, as described for early pupa (*see* **Note 8**).

3.5 Low throughput live assays after RNAi based gene knock-down: myoblast fusion, myotube attachment, myofibrillogenesis and muscle twitching

A detailed video protocol how to image developing adult flight or abdominal muscles at pupal stages has recently been published [18].

1. Set up RNAi cross (see 3.2) using the appropriate live marker in addition to the GAL4 line and the UAS-IR, e.g. Mef-GAL4, UAS-GFP-Gma to image myoblast fusion or myotube attachment, Mhc[weeP26]-GFP to image myofibrillogenesis or Talin-GFP to image spontaneous muscle twitching.

2. To stage pupae of the correct age, collect young white pre-pupae (0-1 h old) with a wet brush and place them on a slide. Put the slide into a Petri dish that contains a wet filter paper to avoid drying of pupae.

3. Incubate the pupae at 27 ºC until the desired age, e.g. 12 hr for imaging myoblast fusion, 18 hr to image myotube-tendon attachment or 30 hr to image myofibrillogenesis or 30 hr to 48 hr to quantify spontaneous muscle twitching.

4. Use sharp forceps to open an observation window by cutting away parts of the pupal case on the dorsal side above the thorax or above the abdomen depending on which muscles should be observed (see [18] for a video).

5. Place the pupae on a custom-made slide with a deeper groove that fits a large part of the pupae, with the observation window facing up (*see* **Note 10**). Place a coverslip on each side of the pupae as a space holder. Put a drop of 50% glycerol on a third coverslip for each pupa mounted, respecting the correct spacing of all pupae used. Place this coverslip on the pupae to cover the observation window of each pupa with the glycerol to avoid drying out. Fix the coverslip with sticky tape [18].

6. Depending on which structure should be observed this preparation is suitable for fast imaging, including laser micro-lesions followed by imaging with a spinning disc confocal [19], long-term imaging over several hours observing myoblast fusion or attachment, or high resolution imaging observing myofibrillogenesis using a two-photon microscope [19,20]. It can also be used to quantify spontaneous muscle contractions using a regular scanning confocal microscope with a fast scanning option [21]

4 Notes

1. In the GAL4 stock, using a Y-heat-shock-hid virginizer chromosome facilitates virgin collection [22].

2. If an RNAi library with long dsRNAs are used (VDRC, NIG-FLY, and first generation of TRiP libraries), using Dicer-2 together with the GAL4 driver increases knock-down efficiency. However this also increases the risk of off-targets, which needs to be controlled carefully.

3. If RNAi results in lethality at early stages, however later developmental stages are of interest, use *Tub*-Gal80-ts to overcome the early lethality (TARGET) [8]. Alternatively to overcome early lethality fly crosses can initially be kept at low temperatures and then shifted to higher temperatures during late larval or pupal stages.

4. Since RNAi works more efficiently in males [5] use males for scoring.

5. If flight tests are used for a high throughput screen, it is practical not to place a water-filled Petri dish at the bottom but simply count the flies in the lower zone. This enables the simple cleaning of the flight tube with a vacuum cleaner before the next flight assay.

6. An even better specificity test than testing a second independent RNAi line is to rescue the RNAi phenotype by either expressing an RNAi insensitive construct from a related fly species [23] or to over-express the target gene using a UAS construct. This often results in inefficient knock-down and thus rescue of the phenotype.

7. The ImageJ plugin MyofibrilJ (https://imagej.net/MyofibrilJ) can be used for detailed analysis of sarcomere phenotypes as it allows measuring sarcomere length and width automatically using Phalloidin labelled-muscles [21].

8. After fixation, half thorax samples can be kept in PBT at 4°C for a few days until the staining steps will be performed.

9. For the mature muscles, which can contract, it is important to use relaxing solution instead of the PBT to make sure that the muscles are in the relaxed state.

10. Turn the pupae carefully that the observed structures are closest to the surface. The deeper the muscles are located, the less fluorescent light can be detected.

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