Culture of *Drosophila* primary cells dissociated from gastrula embryos and their use in RNAi screening

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We provide a detailed protocol for the mass culturing of primary cells dissociated from *Drosophila* embryos. The advantage of this protocol over others is that we have optimized it for a robust large-scale performance that is suitable for screening. More importantly, we further present conditions to treat these cells with double stranded (ds) RNAs for gene knockdown. Efficient RNAi in *Drosophila* primary cells is accomplished by simply bathing the cells in dsRNA-containing culture medium. This method provides the basis for functional genomic screens in differentiated cells, such as neurons and muscles, using RNAi or small molecules. The entire protocol takes ~14 d, whereas the preparation of primary cells from *Drosophila* embryos only requires 2–4 h.

INTRODUCTION

Publication of the *Drosophila* genome has provided an unprecedented resource for functional genomic studies[1]. This, in combination with RNA-interference (RNAi) screening, has greatly facilitated the discovery of genes involved in various cellular or biological processes, which are not easily assayed by other means[2]. *Drosophila* cells have been extensively used for RNAi screens, as they allow development of cell-based assays and have several advantages over mammalian cells (see the comparison between *Drosophila* and mammalian cells in **Table 1**). Until recently, systematic RNAi screens have been limited to *Drosophila* established cell lines[3], such as the S2, Kc and clone 8. However, these cells have some disadvantages, as their origin and how they became immortal are most often unclear (see the comparison between *Drosophila* cell lines and primary cells in **Table 2**). Here, we report a protocol for RNAi in *Drosophila* primary cells (**Fig. 1**), which we have used to conduct both a large-scale RNAi screen for genes required for muscle assembly and maintenance of integrity[4,5], and a genome-wide RNAi screen for neural outgrowth genes[6]. As the differentiation of primary cells mimics normal differentiation *in vivo*[5], the data obtained from primary cells do not appear to suffer from the caveats associated with those generated from established cell lines. More importantly, RNAi in primary cells allows functional studies of genes in differentiated cells, whose features may not be easily recapitulated in cultured cells.

Overview of culturing of primary cells and comparison of this protocol with other methods

Here we describe a protocol for the mass culturing of primary cells suitable for large-scale screenings. In brief, as shown in **Figure 1**, a large number of flies are obtained by growing them in large insect cups, and used for setting up fly population cages for embryo collections. These young and healthy flies are maintained in the fly population cages by being fed with autoclaved yeast paste. A large amount of staged embryos are collected, sterilized and then physically dissociated into a single cell suspension using a glass homogenizer. Mechanical dissociation techniques have been widely adopted for the preparation of primary cells from *Drosophila* embryos, as *Drosophila* embryonic cells very poorly tolerate enzymatic treatments (with trypsin or collagenase)—the procedure typically used for cell dispersion from vertebrate tissues. Two approaches have been mainly used[7]: a homogenization method for the mass culturing of primary cells, and the culturing of primary cells made from a single embryo. The main advantage of the mass culturing procedure we describe here, compared with other mass culture protocols[8], is that we have optimized it for a large-scale performance better suited for screening. In addition, we have simplified the sterilization steps by using killed yeast paste to feed flies, which significantly minimizes yeast contamination of the primary culture—a problem often seen when live yeast is used. In the case of culturing primary cells made from single embryos, the whole contents of single embryos are drawn into a glass micropipette and expelled underneath a drop of culture media[9,10]. The advantages of this procedure are that it requires few embryos and it allows the culture of primary cells with a known genotype. Therefore, it is particularly useful for genetic analyses of mutant cell differentiation[11]. In this case, homozygous lethal mutations can be balanced over a green fluorescent protein (GFP) labeled balancer chromosome, so that the staged non-GFP mutant and GFP-positive heterozygous embryos can be selected using a fluorescence microscope (J.B.'s unpublished results).

TABLE 1 | Comparison of *Drosophila* and mammalian cells for RNAi

Drosophila cells	Mammalian cells
Use long dsRNAs that are cost-effective and easy to design	Use siRNAs that are costly and relatively difficult to design
Delivery of dsRNAs using a simple bathing method	Delivery of siRNAs using transfection or electroporation
Genes have less functional redundancy	Genes have more functional redundancy

dsRNA, double-stranded RNA; RNAi, RNA interference; siRNA, small interference RNA.

TABLE 2 | Comparison of *Drosophila* cultured cells and primary cells for functional study

***Drosophila* established cell lines**	***Drosophila* primary cells**
Immortal	Most, if not all, cells are mortal, and undergo final differentiation
Easy to obtain	Must be prepared *de novo* for every experiment
Cell origins are unknown	Cell origins are clear; developmental programs of most, if not all, cells can be recapitulated in primary culture
Single cell resolution	Single cell resolution
Amenable for RNA interference (RNAi) screening	Amenable for RNAi screening
RNAi efficiency depends on the protein level reduction due to protein turnover and serial dilution by cell division	RNAi efficiency depends on when the protein is expressed and when it is required in the course of cell differentiation

Applications of mass culturing of primary cells and primary-cell RNAi methods

The method described here for the mass culturing of primary cells, which is amenable to large scale *in vitro* molecular and biochemical analyses[12,13], is a useful starting point for the isolation of single cell types. In addition, this method allows further functional investigation of primary cells using many other kinds of more precise experimentation, such as recording techniques[5,10]. With some modifications of culture conditions, this method can be used for the establishment of stable cell lines[14]. The ability to carry out effective RNAi perturbation, together with these other methods, will allow a variety of questions to be addressed in *Drosophila* primary cells. It is important to note that expression of either wild-type or mutant proteins relevant to human diseases will lead to the development of cell-based assays to model human diseases that can then be used for RNAi and small molecule screens.

In principle, any fly genotype can be used for the preparation of primary-cell cultures, including: (1) cells prepared from homozygous viable and fertile mutant stocks; (2) cells prepared from embryos selected either manually or using a GFP selection and embryo sorter that are homozygous for a zygotic lethal mutation[15]; and (3) cells prepared from embryos expressing specific *Gal4, UAS*-gene X genotypes[16].

Previous studies have shown that, based on their morphology, there are at least five distinct types of cells in *Drosophila* primary cultures prepared from embryos: the neuron, the muscle, the epithelial (fat-body cells), a macrophage-like (hemocytes) and a fibroblastic cell type (including chitin-secreting, tracheal and imaginal disc cells)[17,18]. It is reported that about 15% of cells dissociated from gastrulating embryos are myogenic[5], and about 35% are neuronal cells[19]. Owing to their abundance and rapid differentiation, as well as their easy visual identification, both neurons and muscles are among the most characterized cell types developing in primary cultures, whereas other cell types are relatively rare in the mature culture[7]. In this protocol, we particularly focus on the characteristics of neuronal and muscle cells, and the effects of RNAi, although all the previously described cell types were also observed in the primary cultures (data not shown). It is not completely clear at this point whether all other cell types actually take up dsRNAs through bathing. However, we believe that at least some of these cell types may be amenable to the RNAi treatment. For example, we have uncovered noncell autonomous muscle phenotypes for the gene *tiggrin* (*Tig*) (J.B.'s unpublished observations). *Tig* encodes an extracellular matrix protein that functions as a ligand for *Drosophila* αPS2βPS integrins, and is expressed in hemocytes and fat-body cells, but not in muscle cells[20]. Therefore, the protocol described here may also be very useful for designing RNAi and drug screens in other cell types besides neurons and muscles. As primary cultures are a mixed cell population, they are best used for designing image-based assays.

Limitations of primary-cell RNAi

RNA interference in primary cells is best suited for the study of processes occurring during late embryogenesis and beyond, such as muscle assembly and maintenance of integrity[4,5], and neural outgrowth[6]. As dsRNAs are applied to midgastrula stage cells, some early differentiation processes may occur too quickly in the culture for RNAi to have an effect. For example, the process of myoblast fusion cannot be dissected by primary cell RNAi, as it takes place within 2 h after plating the cells[5]. When using primary cells for RNAi screens, it is important to keep in mind that most primary

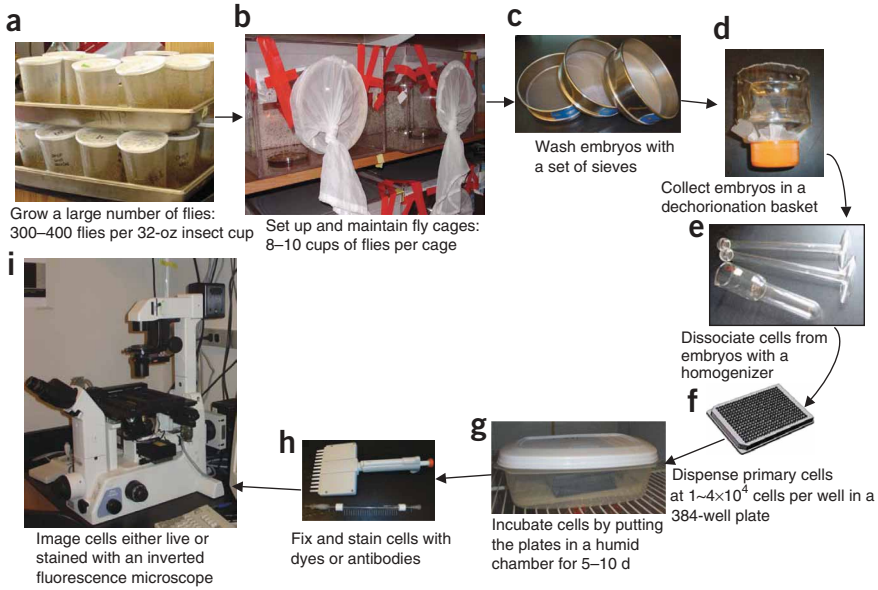

Figure 1 | Outline of the experimental part of the protocol is schematized with the typical equipment used. 32-oz insect cups (**a**); population cages (**b**); a set of sieves for washing embryos (**c**); a dechorionation basket (**d**); a set of 100-ml homogenizer (**e**); a 384-well plate (**f**); a humid chamber (**g**); a multichannel pipettor and an aspiration wand (**h**); and a Nikon TE300 microscope (**i**).

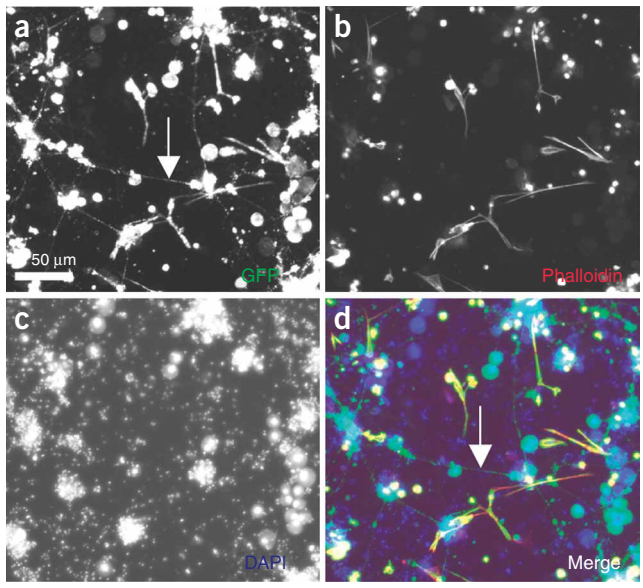

Figure 2 | Primary cell cultures derived from Drosophila embryonic cells contain a mixture of cell populations including muscles and neurons. Primary cells were isolated from the embryos carrying *Dmef2–Gal4, D42–Gal4, UAS–mito-GFP* transgenes, in which *Dmef2–Gal4* and *D42–Gal4* drive expression of *mito-GFP* in muscles and motor neurons, respectively. Both primary muscles and neurons can be seen by GFP (**a**). The white arrow in (**a**) points to the neuronal extensions. A branch-like muscle structure is visualized by phalloidin staining of actin (**b**). The nuclei of all the cells in the culture can be detected by DAPI (4′,6-diamidino-2-phenylindole) staining (**c**). In the merged image (**d**), muscles are stained red, and staining of green positive, but red negative shows neurons and their extensions (white arrow), and DAPI staining is in blue. Clearly, other cells besides neurons and muscles also are present in the culture, as revealed by DAPI staining alone in (**c**). Images were collected with an automated Nikon TE300 (Universal Imaging, Downingtown, Pennsylvania, USA). Scale bar: 50 μm.

cells usually undergo either no or very limited cell divisions in the culture, once they are dissociated from gastrula embryos. Thus, primary cell RNAi will most likely be more effective on genes that are turned on and/or used later in the course of cell differentiation and homeostasis.

Experimental design

Mass culture of primary cells. The success of primary cell RNAi experiments relies on obtaining good primary cultures, judged by how well the cells-of-interest are differentiated in culture. For example, primary muscles often have a branch-like morphology (**Fig. 2**), and usually spontaneously move in the culture[5], whereas differentiated primary neurons form clusters with their cell bodies, and connect with other clusters with their axon cables (**Fig. 3**)[6]. The developmental pattern of primary cultures of *Drosophila* cells can vary greatly[18], possibly because of the following multiple variables: the fly conditions, the degree of dissociation and possible damage caused to the cells, the initial cell density, the quality of the serum used for supplementing the medium and the substrate that the primary cells are grown on.

Flies used for egg laying. Flies used for egg laying should be young (within a week old) and healthy (free of viral or bacterial infection). Primary-cell cultures derived from unfertilized or infected embryos are useless, as primary cells cannot differentiate well and usually survive for a couple of days and eventually die.

Embryo collection. To obtain a large amount of synchronized embryos, it is essential to let the flies do a 1-h prelay with fresh plates coated with yeast paste. Female flies fed with old food for a long period of time, such as overnight, tend to hold their eggs, which then initiate development inside the flies before being laid. Thus, prelay collections usually contain eggs at various developmental stages, and should be discarded. Fresh plates streaked with yeast can then be used to collect embryos for primary cell preparation. This second collection will contain embryos with relatively synchronized stages with regard to development. For the collection plates, it is recommended to cover the plates with a minimal amount of yeast paste (e.g., just sufficient to entice the flies to lay eggs), otherwise, too much yeast may be difficult to eliminate during the rest of the preparation. In addition, we usually maintain flies used for egg collections in a room with a light 12-h cycle and a dark 12-h cycle to facilitate collections of synchronous embryos. As flies tend to lay most of their eggs just after the lights go out, we usually carry out the plate changes just before the lights are turned off.

Homogenization. To avoid excessive homogenization and possible damage to the cells, it is important not to overload the homogenizer with too many embryos. The ratio between the

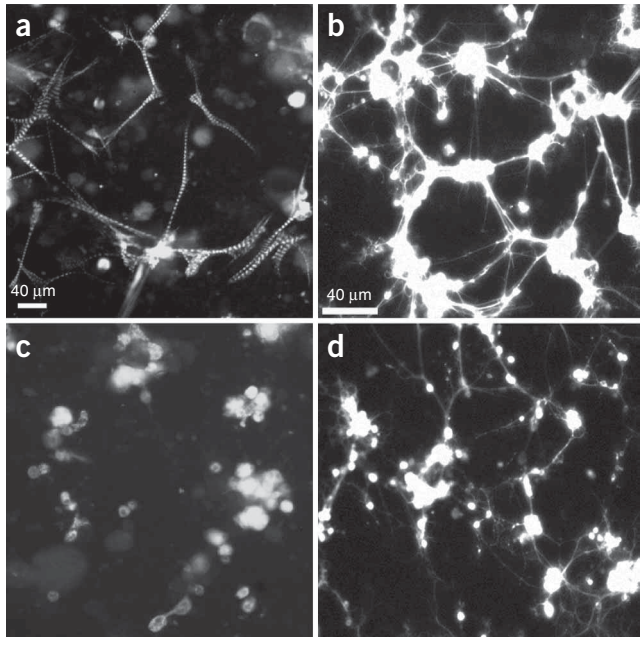

Figure 3 | Examples of RNAi phenotypes in primary muscle and neural cells. Wild-type primary muscle cells were labeled with myosin heavy chain–green fluorescent protein (MHC–GFP) derived from Wee-P26 embryos and treated with control *lacZ* dsRNAs (**a**) primary neurons and glia were labeled with mCD8-GFP, driven by *elav-Gal4* and *gcm-Gal4* promoter lines and treated with control dsRNAs (**b**). Primary muscle cells were treated with *CG14991* dsRNAs (**c**) and primary neurons were treated with *alphaCOP* dsRNAs (d). The cultures were treated with dsRNAs at 18 °C for 8 days. The images show GFP fluorescence. The muscle and the neuron images were collected with an automated Nikon TE300 (Universal Imaging) and a Discovery1 automated wide-field microscope (Universal Imaging), respectively. Amplicons used for making dsRNAs targeting CG14991 and alphaCop are DRSC08450 and DRSC08706, respectively (http://flyrnai.org). Scale bars: 40 μm. Scale bar in (**a**) is for (**a**) and (**c**); in (**b**) for (**b**) and (**d**).

volume of embryos and that of media should not be greater than 0.5 ml of embryos per 40 ml of media (or ~100–200 embryos per ml of media). Overloading the homogenizer with too many embryos will cause an increase in the amount of debris and the number of dead cells.

Culture media. We use Shields and Sang M3 medium for primary cell culture. Other insect culture media, such as D-22, Grace's and Schneider's, also can be used to support primary cell culture. However, we found that M3 medium works best for primary cells to survive through the serum-starvation step for RNAi treatment. The pH of the media needs to be adjusted to ~6.8–6.9 to be optimal for primary-cell culture[18].

Insulin. Insulin is used as a supplement in the culture medium to support and stimulate primary-cell growth. We have found that too much insulin can lead to primary-cell overgrowth (K.S.'s unpublished observation). Therefore, we used insulin at a concentration tenfold lower than that used for cultured cells, such as clone 8.

Serum. Serum supplements are essential factors for primary-cell differentiation. We used heat-inactivated (HI) serum for primary-cell culture, which is purchased from JRH Biosciences, Inc. (Lenexa, Kansas, USA), and supplied in a noninactivated form. To avoid destroying important factors essential for the differentiation of primary cells, we reduced the length of time for heat inactivation (25 min instead of 30 min). It is also possible to buy HI serum from Sigma-Aldrich (St. Louis, Missouri, USA), but we do not have extensive experience with this reagent. In addition, noninactivated serum can be used directly for primary-cell culture. It may have some advantages, as a slight lytic activity present in the non-inactivated serum may help the cultures by promoting separation, flattening out and multiplication of some of the cell types. However, excessive lytic activity may destroy cells, therefore, the complete medium containing serum should be kept at room temperature (~20–25 °C) for at least 3 d before use to reduce the lytic activity from a sometimes-excessive initial level[10,18]. No matter what form of serum is chosen for use, it is extremely important to test different serum lots to identify those that allow good differentiation of primary cells.

Cell density. Cells should be plated at an appropriate density. To begin with, test a range of $1–5\times10^6$ cells ml^{-1} and plate out at $1.7–2.5\times10^5$ cells cm^{-2}. This plating density is important as differentiation of primary cells including neurons and muscles will be compromised when they are seeded at too high a density.

Substrate. There are mixed cell populations in the primary-cell culture. In general, both neuronal cells and muscles can differentiate well on plastic culture-treated 384-well plates (**Figs. 2** and **3**). However, although neurons can differentiate well on the coverglass (without coating), muscle cells cannot. In contrast, muscle cells are more prominent in cultures grown on human vitronectin-coated coverglass (**Fig. 4**).

Primary cell RNAi. *dsRNA design.* Primers for dsRNA synthesis are designed to minimize off-target effects by following the protocols described at http://flyrnai.org/ or by Ramadan *et al.*[21]. Gene specific primers tailed with the T7 polymerase promoter sequence are used for PCR amplification of DNA templates for dsRNAs. The dsRNAs are then generated through *in vitro* transcription (IVT) of PCR templates using T7 polymerases. The size of dsRNAs can range from 200 to 900 nt or longer[21]. dsRNAs targeting *Drosophila* exogenic genes such as *lacZ* or *gfp* are usually used for wild-type or negative controls to monitor general dsRNA toxicity to the cells. Positive control dsRNAs should be chosen based on the specific assay, such that the targeting genes are known to be involved in the process analyzed. For example, dsRNAs targeting *myosin heavy chain* (*MHC*), the gene that encodes one of the muscle myosin components and is required for myofibril assembly, is used as a positive control for the experiments examining the effects of RNAi on muscle cell morphology.

Treatment of primary cells with dsRNAs. Like stable cultured cells, such as S2 cells, *Drosophila* primary muscle and neuronal cells are amenable to the RNAi treatment using a simple bathing method. To facilitate effective cellular uptake of dsRNAs from the medium, it is essential to starve the cells with serum-free medium-containing dsRNAs[22]. A serum-starvation period of 30 min is sufficient for effective RNAi to take place in stable cultured cells, whereas prolonged serum starvation (over 1 h) leads to cell death. In contrast, RNAi in primary muscle and neuronal cells requires at least 6 h of serum starvation. This may be because the primary cells newly dissociated from embryos may have undergone mechanical trauma or shock, and require time to recover and resume their

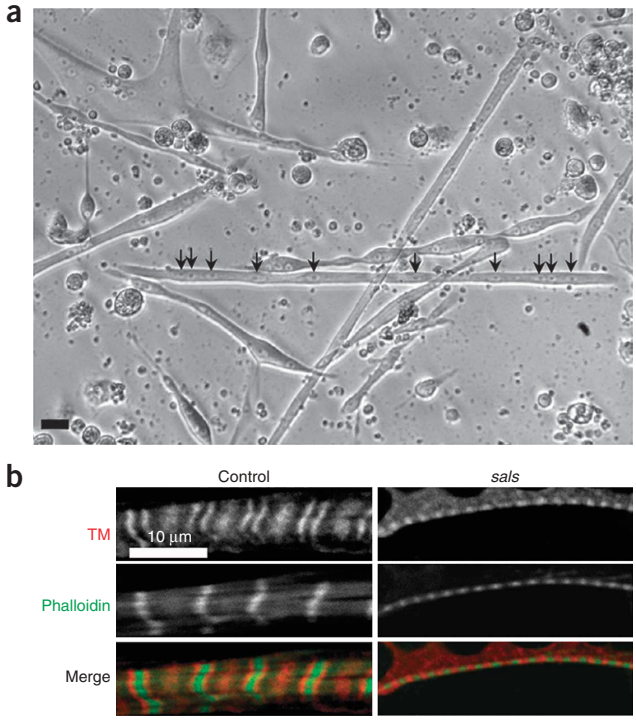

Figure 4 | Images of primary cells cultured on the human vitronectin-coated coverglass slides. A phase-contrast micrograph of primary cells showing that multinucleated primary muscles are prominent in the culture (**a**). Black arrows point to each individual nucleus in the muscle with ten nuclei. (**b**) Confocal micrographs of primary muscles immunofluorescently stained with anti-tropomyosin (TM) (red in the merge) and with phalloidin for actin (green in the merge). The panels on the left are the wild-type control muscle and those on the right are the *sals(CG31374)* RNAi muscle with shortened sarcomeres[4]. Images were collected using a Leica TCS SP2 AOBS confocal microscope. Amplicon used for making dsRNAs targeting *sals* is DRSC14925 (http://flyrnai.org). Scale bar: 10 μm.

TABLE 3 | Commercially available antibodies for labeling primary neurons and muscles

Antibody	Specificity	Dilution	Vendor	Catalogue/clone number
Goat anti-Horseradish peroxidase (HRP): Fluorescein isothiocyanate (FITC)	Pan neuronal, unknown cell surface protein(s)	1:100	Jackson ImmunoResearch	123-095-021
Goat anti-HRP FITC	Pan neuronal, unknown cell surface protein(s)	1:200	ICN Biomedicals	855977
Anti-HRP rhodamine	Pan neuronal, unknown cell surface protein(s)	1:300	Jackson ImmunoResearch	323-295-021
Rat anti-elav	Pan neuronal, nuclei	1:100	Developmental Studies Hybridoma Bank (DSHB)	7E8A10
Mouse anti-neuroglian	Motor and sensory neurons	1:10	DSHB	BP104
Mouse anti-fasciclin II	Motor neurons	1:5	DSHB	1D4
Mouse anti-futsch	Sensory neurons	1:5–1:10	DSHB	22C10
Mouse anti-tubulin	Microtubules	1:1,000	Sigma	
Mouse anti-synaptotagmin	Synapses	1:10	DSHB	3H2 2D7
Rabbit anti-tyrosine hydroxylase	Dopaminergic neurons	1:1,000	Pel-Freez	P40101-0
Rabbit anti-Gamma-Aminobutyric Acid (GABA)	GABAergic neurons	1:1,000	Sigma	A2052
Rabbit anti-serotonin	Serotonergic neurons	1:2,000	Sigma	S5545
Rat anti-tropomyosin	Muscle thick filament component	1:500	Babraham Institute	BT-GB-141P
Rat anti-myosin heavy chain (MHC)	Muscle myosin	1:500	Babraham Institute	BT-GB-147P
Rat anti-projectin	Myofibril component	1:500	Babraham Institute	BT-GB-150P

normal activities after being plated in the culture, therefore making them more resistant to serum starvation. Differentiation of both primary muscle and neuronal cells is not dramatically affected even after 24 h of serum starvation (J.B.'s unpublished results). However, primary muscle-cell differentiation can be significantly disrupted, whereas primary neurons still differentiate well after a 48-h serum-starvation period (K.S.'s unpublished results).

Phenotypic analysis. Phenotypic analysis should be done after 5–7 d of culture at 25 °C or 8–11 d at 18 °C. At these times, the primary cultures have become stable, and both primary muscle and neuronal cells have differentiated and assumed their stereotypic morphology. As shown in **Figure 2**, both primary neurons and muscles can be observed with GFP in the primary culture derived from embryonic cells carrying *Dmef2–Gal4*, *D42–Gal4*, *UAS–mito-GFP* transgenes, as *Dmef2–Gal4* and *D42–Gal4* drive the expression of GFP targeted to the mitochondria in muscles and neurons, respectively. Primary muscles, which also can be visualized by a strong phalloidin staining, exhibited a branch-like morphology, whereas primary neuron clusters extend microtubule-rich axons toward each other. As we do not change the culture media, primary-cell cultures either deteriorate or undergo significant morphological changes after being maintained at 25 °C for more than 10 d. As there is no sorting of cells in the primary-culture protocol that we describe, the cells-of-interest (e.g., neurons and muscles or other cell types) need to be labeled in order to be identified in the mixed cell population. This can be easily done using GFP, which can be expressed specifically in the tissue of interest using the Gal4–UAS method[6] as shown in **Figure 2**, or using a GFP-transgene[5]. For example, primary muscles can be visualized with the expression of MHC–GFP fusion protein (**Fig. 3**). An alternative approach is to detect the cells of interest by immunofluorescence staining using dyes or antibodies targeting specific cell types[4,5] (see **Table 3** for the list of commercially available antibodies and dyes used for staining primary neurons and muscles). For example, all primary neurons are stained positively for HRP and Elav, and the subsets of motor and sensory neurons express Fasciclin II and Futsch, respectively. Primary muscles can be visualized by staining with antibodies targeting either tropomyosin, MHC or projectin. Live-cell imaging allows an observation of cell morphology or other structures, such as neuronal extensions, in a live culture without being disrupted or altered by the fixation process. Therefore, the time when the cultures are imaged is very important for live-cell imaging, as they may look different from one day to the other. This is in contrast to fixed cells, which have been preserved by the fixative, thus allowing a more flexible schedule, as they can be stored for at least a month after fixation. To prevent loss of cells during solution change, a modified aspiration wand is used (see Equipment Setup). For the assay based on cell morphology, changes in morphology between control and treated cells are used to score primary cell RNAi phenotypes (**Figs. 3** and **4b**).

384-well plates are used to grow primary cells for screening or imaging analysis at a lower magnification, as their plastic feature is not suited for the imaging analysis at a magnification of objective lens higher than ×40. Images with a much higher magnification and better resolution obtained using a confocal microscope can be achieved by growing primary cells on a coverglass chamber slide, followed by imaging either live or stained cells. This is particularly useful when the detailed analysis of primary-cell RNAi phenotypes is required (see example in **Fig. 4**). The area for growing cells in each well of 8-well chamber slides is about tenfold that of the area of each well of 384-well plates. Accordingly, the number of cells required for growing on 8-well chamber slides and the amount of media used for culture, as well as the reagents used for subsequent fixation and staining steps, should be scaled up by a factor of 10 for each well. In addition, the regular culture medium, such as Shield and Sang M3, should be better replaced with the fly physiological saline buffer, such as HL6, to reduce autofluorescence emitted from culture media[23].

MATERIALS

REAGENTS

- Shields and Sang M3 media (Sigma, cat. no. S3652) ▲ CRITICAL We have tried other insect media, and have found that M3 works best for allowing cells to survive the serum-starvation treatment.
- Fetal bovine serum (FBS) (JRH Biosciences, cat. no. 12103-500M)
- Penicillin/Streptomycin (Pen/Strep), 100× solution (Invitrogen, cat. no. 15070-063)
- Insulin from bovine pancreas (Sigma, cat. no. I-6634)
- HCl (Sigma, cat. no. H-1758) ! CAUTION HCl is toxic when inhaled, causes burns is irritating to the respiratory system and should be handled with eye and skin protection and contained within a fume hood.
- NaOH (Sigma, cat. no. S2770) ! CAUTION Corrosive and causes burns. Avoid skin contact.
- Yeast-dry, active and granular (Labscientific, cat. no. FLY-8040-5)
- Sodium hypochlorite solutions 1%–5% (wt/vol), Bleach solution (LabChem, cat. no. LC24640) ! CAUTION Corrosive and irritating, causes burns.
- Tegosept (*Drosophila* anti fungal agent) (p-hydroxybenzoic acid methyl ester, methyl 4-hydroxybenzoate, Nipagine) (Genesee Scientific, cat. no. 20-259)
- Ethyl acetate (Sigma, cat. no. 34972)
- Molasses (available from any grocery store)
- Agar (Genesee Scientific, cat. no. 66-101)
- 0.4% (wt/vol) trypan blue solution 100 ml (Sigma, cat. no. T8154)
- Phosphate buffered saline (PBS) packets (Sigma, cat. no. P3813-10PAK)
- Formaldehyde (methanol free) 16% (vol/vol) ultrapure (Polysciences, cat. no. 18814-20) ! CAUTION Formaledhyde is a potent carcinogen and should be handled only with skin and eye protection and contained within a fume hood.
- Triton X-100 (Sigma, cat. no. 9002-93-1)
- Bovine serum albumin (BSA) (Sigma, cat. no. A3912)
- Sodium azide (NaN_3) (Sigma, cat. no. 71289) ! CAUTION Sodium azide is hazardous and combustible and should be handled with skin and eye protection and contained with a fume hood.
- Human vitronectin (Chemicon, cat. no. CC080)
- Reagents for making dsRNAs (Please refer to Ramadan *et al.*[21])
- Alexa fluor conjugated dyes and secondary antibodies (Molecular Probes)
- Vectashield mounting medium (Vector Laboratories, cat. no. H-1000)

EQUIPMENT

- 32-oz insect cups (Superior Enterprise, cat. no. IN-32)
- Large embryo-collection cages (Genesee Scientific, cat. no. 59-101)
- Petri dishes size 100 × 15 mm (Genesee Scientific, cat. no. 32-107)
- Petri dishes size 150 × 15 mm (Genesee Scientific, cat. no. 32-106)
- Fly population cages: the cage made of Plexiglas (0.25-inch thickness) has a 12 × 18-inch opening covered with Nytex netting on the top and an 8-inch circular hole on one side, to which a sleeve is attached for entry. The sleeve is made of fabric with elastic bands at one end (**Fig. 1**)
- Dechorionation baskets: homemade see EQUIPMENT SETUP
- # 25 U.S. standard testing sieve (0.71-mm opening size) (VWR, cat. no. 57334-454)
- # 45 U.S. standard testing sieve (0.355-mm opening size) (VWR, cat. no. 57344-462)
- # 120 U.S. standard testing sieve (0.125-mm opening size) (VWR, cat. no. 57344-474)
- Dounce Tissue Grinder, Wheaton 7 ml (VWR, cat. no. 62400-620; clearance of 0.005–0.0035 inches)
- Dounce Tissue Grinder, Kontes 40 ml (VWR, cat. no. KT885300-0040)
- Dounce Tissue Grinder, Kontes 100 ml (VWR, cat. no. KT885300-0100, large pestle clearance mm: 0.254–0.508, small pestle clearance mm: 0.013–0.140)
- Paintbrushes (available from any art store)
- Nitex mesh (Sefar Filtration, cat. no. 03-310/45)
- Costar, 384-well, black, clear-bottom plates, TC treated (VWR, cat. no. 29444-078) (culture area: 0.06 cm^2 per well; well capacity 120 µl)
- Lab-Tek II chambered coverglass, 8 well (Nunc, cat. no. 155409) (Fisher Scientific, cat. no. 171080) (culture area 0.7 cm^2 per well, working volume 0.2–0.5 ml)
- Aspiration wand 24 channel (V&P Scientific, cat. no. VP186L)
- Aspiration wand 16 channel (V&P Scientific, cat. no. VP189L)
- Multichannel pipettor Thermo Labsystems, 5–50 ml (VWR, cat. no. 53515-028)
- Hemocytometer (VWR, cat. no. 15170-172)
- 50 ml centrifuge tube/cap (VWR, cat. no. 21008-730)
- 15 ml centrifuge tube/cup (Fisher Applied Scientific, cat. no. 05-538-53D)
- 100-micron cell strainer (VWR, cat. no. 21008-950)
- Aluminum tape, 384-well (VWR, cat. no. 29445-082)
- Multipurpose centrifuge 5804 (Eppendorf)

REAGENT SETUP

Heat inactivating serum Heat-inactivate the serum purchased from JRH Biosciences following the instructions provided by Cell Culture Techniques, Technical Support from JRH Biosciences. To preserve the important factors required for muscle differentiation, heat-inactivate the serum for 25 min instead of 30 min at 56 °C. Unused portions of serum can be refrozen at −20 °C for later use. Frozen HI serum can be used for up to 6 months.
▲ CRITICAL Always test the differentiation capacity of serum of different lots in *Drosophila* primary cells before deciding which one to use.

1000 × Insulin in M3 media

1. Empty a vial of insulin (50 mg, >27 U mg^{-1}) into a 200-ml beaker.
2. Wash the vial with 10 ml of 0.01 NHCl and pour the wash solution onto the insulin powder. ! CAUTION HCl is toxic when inhaled, causes burns, is irritating to the respiratory system and should be handled in the fume hood.
3. Add 90 ml of M3 media slowly into the beaker to dissolve the insulin.
4. Filter in a hood with a 0.2 µm Millipore filter and pour into a sterile bottle.
5. Dispense into 5-ml aliquots into sterile, disposable 15-ml centrifuge tubes.
6. Label with the date and store frozen at −20 °C. Frozen insulin aliquots can be used for at least 2 years.
7. Use 100 µl 100 ml^{-1} media to give a final concentration of 500 ng ml^{-1} (or ~10 mU ml^{-1}). ▲ CRITICAL The insulin concentration can be reduced if the primary cells overgrow. Insulin solution kept at 2–8 °C is stable for up to 1 year. Avoid freezing and thawing of insulin solutions, which will lead to stability problems or aggregation.

Culture media **Serum-free medium:** Shields and Sang M3 medium, Pen/Strep (10 U ml^{-1} penicillin, 10 µg ml^{-1} streptomycin) and insulin (500 ng ml^{-1}).
Serum-containing medium: Shields and Sang M3 medium, pH6.8–6.9, Pen/Strep (10 U ml^{-1} penicillin, 10 µg ml^{-1} streptomycin), insulin (500 ng ml^{-1}) and 10% FCS (vol/vol).
▲ CRITICAL Check the pH of medium, and adjust it to pH6.8–6.9 with either HCl or NaOH, then filter the medium to sterilize before adding antibiotics, insulin and serum. Once the medium has been tested to be able to support good differentiation, it should be aliquoted and stored at 2–8 °C (for up to 3 months) or frozen at −20 °C (for up to 6 months).

Egg-collecting plates Prepare the following mixture: water 2,500 ml, molasses 360 ml and agar 88 g. Autoclave for 1 h. Slowly add 0.5 ml of Tegosept (10% (wt/vol) methyl p-hydrobenzoate in 95% ethanol) and 20 ml of ethyl acetate. Mix well and pour into petri dishes. This recipe yields ~200 150 × 15-mm dishes. The plates can be stored at 4 °C for up to 6 months. Longer storage may cause molasses agar gel to shrink and detach from the plates.

Killed yeast paste Grind yeast granules as finely as possible with a mortar and pestle or coffee grinder. Place in a beaker and autoclave. Add enough water (sterile hot water works best) to make a paste with the consistency of toothpaste.
▲ CRITICAL To avoid introducing yeast contamination to cultures, do not use live yeast to feed flies that will be used for laying embryos for primary-cell preparation.

Autoclave the following using dry cycle (exposure time: 30 min; temperature: 121 °C; and drying time: 30 min):
2 beakers (opening is covered with foil)
Dounce homogenizers (tubes and pestles) (wrapped with foil)
Paintbrushes (wrapped with foil)
Paper towels (cut into smaller size for convenience and put in a large beaker and cover it with foil)

HL6 physiological solution as described by Macleod *et al.* (2003)[23].
Fixation buffer (4% formaldehyde in PBS (vol/vol))
PBST (PBS and 0.1% Triton X-100 (vol/vol))
PBSTB (PBS, 0.1% Triton X-100 (vol/vol) and 1% BSA (wt/vol))
PBSTN (PBS, 0.1% Triton X-100 (vol/vol) and 0.02% NaN3 (wt/vol))
! CAUTION Sodium azide is hazardous and combustible and should be handled with skin and eye protection and contained within a fume hood.

EQUIPMENT SETUP

Dechorionation baskets Cut the bottom off a 50-ml conical tube or a 250-ml plastic bottle. Then cut the top from the tube cap or bottle cap to make a retaining ring. Place a Nitex mesh over the tube or bottle opening and screw on the modified cap (**Fig. 1**).

Instruments to handle liquid aspiration see Ramadan *et al.* (2007)[21].

Coat coverglass chamber slide with human vitronectin

1. In the tissue culture hood, dilute human vitronectin in PBS to a final concentration of 10 ng ml^{-1}. Add 100 µl to each well of a coverglass 8-well chamber slide.
2. Incubate the slide for 2 h at room temperature (~20–25 °C) or overnight at 4 °C.
3. Just before plating cells, remove the vitronectin solution from the well.

The vitronectin solution can be kept at 4 °C and reused at least twice for coating. Vitronectin-coated coverglass chamber slides may be stored at 4 °C for up to 1 week.

Humid chamber To prevent evaporation in the wells of the plates during incubation, the culture plates are kept in a humid chamber. To set up a humid chamber, line the bottom of a plastic container with a wet paper. To prevent condensation on the lids of culture plates, the lid of the container should be loosely closed before it is put in a temperature-controlled incubator or environment.

PROCEDURE

Preparation of fly cages ● TIMING variable, minimum 2 weeks

1| Grow *Drosophila* (wild-type strains such as *Oregon-R* or any genotype) in convenient containers such as 32-oz insect cups. For the basic methods for the laboratory culture of *Drosophila melanogaster*, please refer to the book edited by Sullivan *et al.*[24].
? TROUBLESHOOTING

2| Transfer newly enclosed flies (~3,000) to large embryo-collection cages or fly population cages.

3| Move the cages into a room with a temperature of ~25 °C and humidity ~65% in a light 12-h and a dark 12-h cycle to facilitate the collection of synchronous embryos.

4| Maintain the flies in the cages by feeding them killed yeast paste streaked on molasses plates. Change the molasses plates (with killed yeast paste) once a day for 2–3 d.
? TROUBLESHOOTING

Collection of synchronous embryos ● TIMING 6–8 h

5| On the day for primary-cell preparation, carry out a 1-h pre lay by feeding the flies with fresh plates coated with killed yeast paste.

6| Just before the 12-h dark cycle, replace the pre lay plates with fresh molasses plates streaked with a minimal amount of yeast paste. Discard the pre lay collection, which contains asynchronous older embryos.

7| Collect embryos for a period of 1–2 h.

8| Remove the plates containing the relatively synchronous population of embryos and store at 25 °C for 5–6 h. Embryos will be at the advanced gastrula stage at this time.

Collection and washing of embryos ● TIMING 10 min

9| Loosen the embryos from the plates with a wet paintbrush, and rinse thoroughly with tepid tap water through a stacked set of sieves, so that the embryos will be collected in the finest mesh sieve at the bottom. Rinse for a few minutes to remove as much yeast and fly debris as possible.

10| Tip the sieves so that the embryos accumulate at one side, and gently wash them off from the sieve into a dechorionation basket.

11| Move to the tissue culture hood area without letting the embryos dry out.

Homogenization of embryos and plating of cells ● TIMING 2–4 h

12| Sterilize and dechorionate the embryos by immersing them in 50% (vol/vol) bleach for 5–10 min. Rinse the embryos off the wall of the basket with 70% (vol/vol) ethanol. Alternatively, wash the bleach off embryos with distilled water before ethanol treatment.
! CAUTION Bleach is corrosive and irritating, and causes burns. It should be handled with care.
▲ CRITICAL STEP From this point onward embryos should be handled in sterile conditions and in the tissue culture hood.

13| Rinse the embryos thoroughly to remove the bleach and/or ethanol with autoclaved distilled water.
? TROUBLESHOOTING

14| During the dechorionation step, fill a Dounce homogenizer with the appropriate volume of room temperature M3 medium based on the amount of embryos.
▲ CRITICAL STEP The ratio between the volume of embryos and that of media should not be over 0.5-ml embryos:40-ml media (or ~100–200 embryos:ml).
? TROUBLESHOOTING

15| Place the rinsed embryos in a sterilized beaker with the amount of M3 media enough to submerge the embryos. Let the embryos soak for 2–5 min.

16| Disassemble the dechorionation basket and blot the Nitex mesh dry with sterilized paper towels.

17| Transfer the embryos into the homogenizer with a sterilized paintbrush.

18| Homogenize gently with short strokes using a loose pestle without reaching the bottom of the tube until all the embryos are suspended. Then gently, but firmly, homogenize with eight full strokes.
▲ **CRITICAL STEP** Do not twist the pestle as it moves up and down.

19| Transfer the homogenate into a sterile 50-ml conical centrifuge tube either by pouring or pipetting, and cap the tube.

20| Centrifuge for 10 min at 40*g*, room temperature to pellet the tissue debris, large cell clumps and vitelline membranes. Transfer the supernatant containing the cells and yolk to a clean tube and repeat the centrifugation step for 5 min.
? TROUBLESHOOTING

21| Carefully transfer the supernatant by pouring or pipetting into a clean tube and spin for 10 min at 360*g*, room temperature to pellet the cells. Discard the supernatant. Resuspend the cell pellet in 10 ml of culture medium (serum-free medium for RNAi experiments).

22| Remove 32 µl of the cell suspension and mix well with 8 µl of 0.4% (wt/vol) trypan blue solution. Stain for 5 min and count using a hemocytometer to estimate the cell density. Only count unstained viable cells. Meanwhile, repeat step 21 to pellet the cells.

Estimate the cell density using the following calculation: Cell density (CD) = (N1 + N2 + N3 + N4)/4 × 1.25 × 10^4 cells ml^{-1} where N1, N2, N3 and N4 are the cell numbers counted at the four corners of the hemocytometer.

23| Resuspend the cells to a desired concentration. To start, try a range of 1–5 × 10^6 cells ml^{-1} and plate out at 1.7 to 2.5 × 10^5 cells cm^{-2}. Thus, the volume of medium (V) added will be calculated as V = CD × 10 /(1–5) × 10^6 ml.
? TROUBLESHOOTING

Primary-cell RNAi for cells grown on a 384-well plate ● TIMING 6–10 d

24| Design primers for dsRNA synthesis and generate dsRNAs by following the protocols described at http://flyrnai.org/ or by Ramadan *et al.*[21].

25| Dispense 5 µl of dsRNAs into each well at a final concentration of 250 ng per well in a 384-well plate.

26| Use a multi channel pipette, dispense 10 µl (1–4 × 10^6 cells ml^{-1}) of primary cells per well in a serum-free M3 medium in a 384-well plate containing a different dsRNA in each well. Alternatively, cells can be cultured on 8-well glass chamber slides for confocal imaging, see **Box 1** for details.
? TROUBLESHOOTING

27| Centrifuge the plates for 1 min at 300*g*, room temperature. Culture the cells in serum-free medium at 25 °C for 22 h or at 18 °C for 1–2 d.

28| Add 30 µl of serum-containing culture medium to each well.

BOX 1 | CULTURE OF CELLS ON A COVERGLASS CHAMBER SLIDE FOR CONFOCAL IMAGING ● TIMING 10–14 D

1. Plate 100 µl of primary cells (1–4×10^6 cells ml^{-1}) in the well of a coverglass 8-well chamber slide (for primary muscle studies, coat the coverglass chamber slides with human vitronectin, see EQUIPMENT SETUP).
2. Scale up RNAi conditions for use with a coverglass 8-well chamber slide. Use tenfold the volumes that are used for 384-well plates (Steps 25–30). Aspirate the liquid from the well using a Pasteur pipette connected with a vacuum instead of using an aspiration wand.
3. For live imaging of primary cells with confocal microscopy, remove the media and replace with HL6 physiological solution immediately before imaging to reduce background fluorescence of culture media.
4. For imaging of fixed cells, fix and stain primary cells following the same conditions as those for the cells grown on a 384-well plate, but scale up the volumes by a factor of 10 (Steps 33–40).
5. Image cells using an inverted confocal microscope[4,6].

29| Centrifuge the plates for 1 min at 300*g*, room temperature.

30| Place the plates in a humid chamber and culture the primary cells for an additional 5–7 d at 25 °C.
? TROUBLESHOOTING

31| Image the cells either live[6] or after immunofluorescence staining[5] (Steps 32–40).

Primary cell staining and image acquisition for cells grown on a 384-well plate ● TIMING 2–4 d
32| Gently remove the media using an aspiration wand (see EQUIPMENT SETUP).
▲ CRITICAL STEP For each of the following steps, remove the liquid using an aspiration wand.

33| Fix the cells with 30 µl of fixation buffer for 10–20 min.
! CAUTION Fixation buffer contains formaldehyde, which is a potent carcinogen. Avoid exposure.

34| Wash the cells twice with 50 µl of PBST for 5–10 min each to remove the fixative and permeabilize the cell membranes.
■ PAUSE POINT Fixed cells may be stored in PBST at 4 °C for several days.

35| Stain the cells with a primary antibody in 20–50 µl of PBSTB overnight at 4 °C or 1–2 h at room temperature.

36| Wash the cells three times with 50 µl of PBST for 10 min each.

37| Stain the cells with a suitable Alexa fluor conjugated secondary antibody in 50 µl of PBSTB for 1 h at room temperature.

38| Wash the cells twice with 50 µl of PBST for 10 min each at room temperature.

39| Mount the cells in one drop (~20 µl) of Vectashield mounting medium. For large-scale experiments, the stained cells can be left in 50 µl of PBSTN.
! CAUTION PBSTN contains sodium azide, which is hazardous and combustible and should be handled with skin and eye protection and contained within a fume hood.

40| Acquire images using an inverted wide-field fluorescence microscope with a stage to hold plates.

● TIMING
Steps 1–4, Preparation of fly cages and culture: variable, minimum 2 weeks
Steps 5–8, Collection of synchronous embryos. Changing the molasses egg-laying plates only takes 5 min. But waiting time is about 6–8 h
Steps 9–11, Collection and washing of embryos: 10 min
Steps 12–23, Homogenization of embryos and plating of cells: 2–4 h
Steps 24–31, Primary cell RNAi for cells grown on a 384-well plate: ~6–10 d
Steps 32–40, Primary-cell staining and image acquisition for cells grown on a 384-well plate: 2–4 d
Box 1, Culture of cell on a coverglass chamber slide for confocal imaging: 10–14 d

? TROUBLESHOOTING
Troubleshooting advice can be found in Table 4.

TABLE 4 | Troubleshooting table.

Step	Problem	Reason	Solution
1	Cells fail to differentiate They remain round and die	Unhealthy flies, may be contaminated with virus or bacteria	Start with healthy flies free from bacteria or virus, which usually are present in the interface between the embryo vitelline membrane and chorion. To re-establish healthy stock, treat eggs from overnight collection a total of three times with bleach for 25 min each, and wash eggs with tap water extensively between each bleach treatment. Inoculate cleaned eggs into a fly bottle. Adults that come out from the bottle can be expanded and used for primary cell preparation

(continued)

TABLE 4 | Troubleshooting table (continued).

Step	Problem	Reason	Solution
4	Very few eggs laid	Cold egg collection plates used	Prewarm the plates in the incubator before putting them in the fly cage
13	Dead cells	Bleach, which is very toxic to cells, may not be completely washed off	Thoroughly rinse the eggs with water to completely remove the bleach
14	Too much debris in the culture	Wrong volume ratio between embryos and medium	Use fewer embryos in each dounce or switch to a larger dounce to increase the medium volume
20	Large cell clumps and vitelline membranes present in the culture	Embryos not sufficiently homogenized	Use more strokes to grind the embryos. Alternatively, pass the supernatant through a 100-μm cell strainer to get rid of large cell clumps and vitelline membranes
23	Cells are clumpy and do not differentiate	Cells are plated at a high density	Plate cells at a lower density
26	Cells are floating, and do not stick to the plates	Plates may not be suitable for primary cell culture	Switch to another lot of plates
30	Media evaporation, and/or condensation on the lid of the culture plates	Possible temperature fluctuation in the incubator	Make sure to keep the temperature constant. If using a humid chamber does not improve the situation, use an aluminum tape to seal the plate. Make sure each well is individually sealed

ANTICIPATED RESULTS

This protocol for mass culturing of primary cells is optimized for large-scale RNAi or drug screening. Fly population cages with healthy flies, once set up, can be maintained and used for 4–5 consecutive days, thus, 4–5 sets of egg collections may be obtained for primary-cell preparation. Based on our experiments, one cage of flies can produce enough primary cells for two 384-well plates (see **Fig. 5** for the relative scales). The whole length of time needed for primary cell-related screening usually is not determined by the time required for mass culturing of primary cells, but by the time used for imaging plates and its follow-up imaging analysis. The imaging methods and analysis tools used for dissecting the phenotypes of primary cells are similar to those for cultured cells[6,21], and should be customized for the assay.

After the treatment with dsRNAs and an extended period of culture, primary cells can demonstrate various phenotypes due to the depletion of target proteins. For example, wild-type control primary muscles usually show branch-like structure and striated sarcomeres (**Fig. 3a**), whereas primary muscles treated with dsRNAs targeting the gene CG14991 round up (**Fig. 3c**). CG14991 encodes fermintin I, the protein that may be required for integrin signaling. In the case of wild-type primary neurons, neuroblasts divide into clusters (neuromeres) containing ~16 daughter cells, and the microtubule-rich axons project from the neuromeres to neighboring neuromeres. Therefore, the axonal bundles of mature wild-type control cultures have relatively little branching and are well-fasciculated (**Fig. 3b**). In contrast, in cultures treated with dsRNAs targeting alphaCOP, a component involved in protein transport, cell division is impaired as indicated by smaller neuromere diameters and axonal outgrowth is greatly reduced as indicated by the lack of long projections and the lack of connectivity to adjacent neuromeres[6] (**Fig. 3d**).

Images with a higher resolution can be achieved by culturing primary cells on a coverglass chamber slide either uncoated[6] or coated with human vitronectin[4] (**Fig. 4a**), followed by a confocal microscopic analysis at a higher

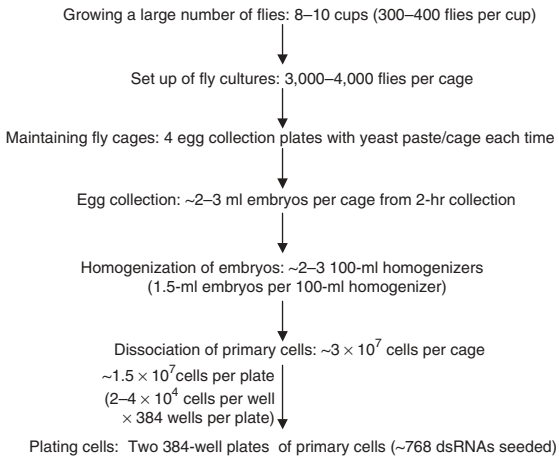

Figure 5 | Scale of mass culturing of primary cells for RNAi screening.

magnification. This is particularly useful for quantifying the muscle sarcomere size. For example, the gene *sarcomere length short (sals)* is required for thin filament lengthening[4], and *sals* RNAi primary muscles have a dramatic reduction in the sarcomere length compared with that of control RNAi primary muscles (**Fig. 4b**).

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