Chapter 20

Drosophila S2 Schneider Cells: A Useful Tool for Rebuilding and Redesigning Approaches in Synthetic Biology

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Abstract

Synthetic biology is an engineering approach to biology. A synthetic biologist wants to describe biological molecules and their subdomains as well-defined parts of a molecular machine. To achieve this goal, synthetic biologists rebuild minimal functional biological systems from well-defined parts or they design new molecules that do not exist in nature but have new and useful functions. In short, these engineering approaches can be summarized as "rebuild, alter, and understand." The *Drosophila* S2 Schneider cell is a useful tool for both rebuilding and redesigning approaches. S2 cells are phagocytic cells that easily take up large amounts of DNA from the cell culture. They, thus, have a high cotransfection rate, allowing the coexpression of up to 12 different proteins. We have developed a transient transfection protocol allowing the rapid and parallel analysis of wild-type and altered forms of a biological system. This chapter describes our methods to rebuild and better understand mammalian signaling systems in the evolutionary distant environment of Drosophila S2 cells.

Key words: S2 cells, Rebuilding, Protein complex

1. Introduction

Drosophila Schneider cells were isolated from late-stage *Drosophila melanogaster* embryos over 30 years ago (1). They are easily maintained at room temperature and do not require an incubator with a CO_2 supply. Among drosophilists, S2 continue to provide a good system to study many functional aspects of Drosophila cell biology. For example, the fact that these cells can be grown in large amounts in culture and that they also have an efficient silencing mechanism (RNAi) has permitted knockdown experiments for functional analysis of *Drosophila* proteins (2, 3). In the meantime, vectors for constitutive and inducible expression of proteins have been developed in the S2 cell system (4–8). Especially, the inducible vectors

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have turned out to be very useful since they allow regulated gene expression. These vectors are now used for the production of large amounts of proteins required for protein crystallographic studies (9, 10). In these applications, both transiently transfected S2 cells and selected stable lines are commonly used (11, 12). The same standard transient transfection protocol using large amounts of plasmids (10–20 μg per 10^6 cells) resulting in a high transfection efficiency (>30%) can also be used to establish stable lines. When our lab became interested in conducting rebuilding approaches, we were particularly intrigued by the high transient transfection efficiency of S2 cells. We asked whether this would also hold true for the transfection of several different vectors at the same time. Therefore, we modified the protocol not using microgram amounts of one plasmid, but rather nanogram amounts of multiple plasmids for the transient transfection. We noticed that, indeed, S2 cells not only take up large amounts of a single vector but they could also take up many different vectors without compromising the transfection efficiency. Most notably, expression of the proteins from different cotransfected vectors appears to be closely correlated (13, 14). Thus, in a single transient transfection experiment, one can rebuild a complex system composing up to 12 different components in S2 cells with a reasonably high efficiency. This unique feature made the S2 cells an ideal system for a detailed study of protein function in signaling subsystems. Using this approach, we have discovered the oligomeric nature of the B cell antigen receptor (BCR) on resting cells and have better characterized the interaction of the protein tyrosine kinase Syk with the signaling subunits of this receptor (13, 15). Herein, we describe our method for using S2 cell in synthetic rebuilding approaches.

2. Materials

1. Schneider's S2 cells: The cells can be obtained from the American Type Culture Collection (http://www.atcc.org/) or purchased from Invitrogen as *Drosophila* S2 Cells (Invitrogen, cat. no. R690-07).
2. Schneider's *Drosophila* Powder Medium, revised (SERVA, cat. no. 47521). Store powder dry at +2°C to +8°C.
3. Liquid S2 cell medium: All the steps should be done while stirring slowly. To prepare 2 L of medium, add ca. 1,600 mL millipore water (pure water from Millipore lab water purification system) in a 4-L flask and dissolve 48.8 g powder medium. Add 0.8 g sodium bicarbonate ($NaHCO_3$), the pH of the medium should be around 4.7. Adjust the pH to 9.2 ± 0.2 with NaOH pellets. Then, adjust the pH to 6.7 ± 0.2 with 37%

HCL. Dissolve 1.59 g calcium chloride dihydrate ($CaCl_2 \bullet 2H_2O$) in 100 mL millipore water. Add the calcium chloride solution dropwise to the medium slowly to avoid precipitation. Add millipore water to 2 L. Filter the medium through a 0.22-μm filter, aliquot in glass bottles (400 mL per bottle).

The prepared liquid S2 cell medium can be stored at +2°C to +8°C in the dark for ca. 8 weeks.

4. Complete S2 cell medium: Add FCS (heat inactivated) to the liquid S2 cell medium to a final concentration of 10% (v/v) and L-Glutamine to a final concentration of 2 mM.
5. Conditioned medium: Collected from well-growing, nice-looking (viability higher than 90%) S2 cells.

   Prior to preparation, the cells should be tested for their transfection efficiency using, for example, a GFP expression vector. Ideally, S2 cells should have a transfection efficiency above 30%. Grow the cells to around 70% confluency. Collect the medium and centrifuge for 20 min at 500 × *g* to remove the cells. Filter the supernatant with a 0.22-μm filter, make aliquots, and freeze the prepared conditioned medium at –20°C for no longer than 1 year. The conditioned medium should be thawed at room temperature before use. If precipitates are observed, centrifuge again to remove the precipitates.
6. Schneider's *Drosophila* Medium, liquid (Invitrogen, cat. no. 11720067).
7. Serum-free medium (for transfection): Add L-Glutamine to Invitrogen's Schneider's *Drosophila* Medium to a final concentration of 2 mM.
8. Culture dishes and plates (Greiner Bio-One) (see Note 1).
9. Laminar flow hood for tissue culture.
10. Pipets, hemocytometer, and other standard equipment for tissue culture.
11. Materials for molecular cloning and plasmid preparation.
12. FuGENE HD transfection reagent (Roche cat. no. 04709705001).
13. Cell culture-grade dimethyl sulfoxide (DMSO).
14. 100 mM $CuSO_4$, prepared using millipore water and filtered through a 0.22-μm filter. Store at +2°C to +8°C for ca. 1 year.
15. 27°C incubator.
16. 1× PBS buffer.
17. 2% paraformaldehyde (PFA), for 100 mL, add 98 mL 1 × PBS to a 100 mL bottle, add 50 μL of 1N NaOH and 2g paraformaldehyde into the PBS. Fill a 2000 mL beaker with about 400 mL H_2O and put a thermometer into the beaker. Heat the

water on a hot stir plate to 64°C. Put the PBS bottle into the beaker with the warm water and stir the paraformaldehyde until it is clear. Remove the 2% PFA from the beaker on the hot plate and cool it down to room temperature. Immediately make 1 mL aliquots and freeze at –20°C.

18. Fluromount-G or Dapi-fluromount-G (SouthernBiotech, cat. no. 0100-01 or 0100-20).

3. Methods

3.1. S2 Cell Culture

We started our work with S2 cells by obtaining a cell line as a generous gift from Dr. K. Karjalainen (then at the Basel Institute of Immunology, Switzerland). S2 cells can be cultured with Schneider's *Drosophila* medium supplemented with 10% FCS at 27°C in a water-saturated atmosphere with atmospheric CO_2 levels in the dark (see Note 2). To maintain S2 cells in culture, cells should not be split below 5×10^5 cells/mL, as low-density cultures grow very slowly or arrest completely. For routine culture, we split the cells every 2 days to give approximately $0.5–1 \times 10^6$ cells/mL. If the cells need to be kept in culture for 3 days without splitting, we suggest making several different dilutions starting from about 0.5×10^6 cells/mL. We normally grow S2 cells in 15-cm petri dishes. Since S2 cells do not attach very well to the dish, they can be resuspended by gently pipetting up and down. Trypsinization is not required. Alternatively, using a cell lifter at the beginning might save some work. As with any cell line, it is highly recommended to keep track of the number of passages that have taken place since the cells were thawed (see Note 3).

3.1.1. Freezing S2 Cells

It is important to maintain adequate frozen culture stocks. Keeping S2 cells too long in culture can reduce their transfection efficiency (see Notes 3 and 4).

To freeze cells:

1. Grow cells to a density of $3–5 \times 10^6$ cells/mL (log phase) in 20–30 mL of medium in a 15-cm petri dish. Collect the cells and centrifuge them for 5 min at $300 \times g$.
2. Prepare freezing medium: 90% FCS and 10% DMSO. Filter the freezing medium through a 0.22-μm filter.
3. Resuspend the cell pellet in freezing medium at a final cell concentration of $0.5–1 \times 10^7$ cells/mL.
4. Aliquot the cells into vials (0.5 mL per vial) for freezing.
5. Place the vials into a container with dry ice, and allow freezing for 24–48 h at –80°C before transferring to liquid nitrogen.

3.1.2. Thawing S2 Cells

1. Thaw a vial of frozen S2 cells rapidly by exposing it to running hot water.
2. Immediately after the cells are thawed, move the vial to tissue culture hood and sanitize the vial with 70% ethanol.
3. Pipet the cells into a fresh 15-mL falcon tube containing 5 mL complete S2 cell medium (prewarmed to room temperature).
4. Pellet the cells by centrifuging at 300 × *g* for 5 min.
5. Resuspend the cells in 6 mL of fresh complete S2 cell medium and distribute the cells to three wells in a 6-well plate as follows:

	Well no.		
	1	2	3
Resuspended cells	1 mL	2 mL	3 mL
Complete S2 cell medium	2 mL	1 mL	–
Conditioned medium	1 mL	1 mL	1 mL (final concentration: 25%, see Note 5)

6. Place the cells in an incubator and replace the medium every 2 days.

Normally, after 4–6 days, the cells start to expand. Reduce the amount of conditioned medium after several passages.

3.2. Using S2 Cell for Synthetic Rebuilding Approaches

To rebuild a part of the cell machinery or a signaling pathway in S2 cells, one must first clone the related genes of interest into S2 cell expression vectors and introduce the expression plasmids into the S2 cell by transfection.

3.2.1. Choice of Expression Vector

Expression vectors with a variety of different promoters and polyadenylation signals have been tested in *Drosophila* S2 cells. While both the *Drosophila* hsp70 promoter and the *Drosophila* metallothionein (Mtn) promoters are strongly inducible, the hsp70 promoter has considerable basal activity in S2 cells (6, 16). Although the mRNA stability is somewhat affected by various polyadenylation signals, the polyadenylation signals derived from late SV40, *Drosophila* Mtn, early SV40, or *Drosophila* alcohol dehydrogenase (Adh) all result in significant levels of target gene protein expression (4, 5). We are currently using the expression vector pRmHa3, which is based on pUC18 and contains a *Drosophila* Mtn promoter and *Drosophila* Adh polyadenylation signal (5).

3.2.2. Construct the Expression Plasmids

1. Kozak sequence: *Drosophila* uses a different Kozak sequence than vertebrates (17). However, for synthetic rebuilding approach, overexpression of the target protein normally should

be avoided. For this reason, an optimization of the Kozak sequence is not always necessary. We, therefore, often insert the cDNA or genes of interest directly into the multicloning site of pRmHa3 without modifying the sequence flanking the natural ATG. In many cases, carrying over a short (<100 base) native 5′ UTR with the cDNA still allows reasonable expression of the target protein (see Note 6).

2. Signal peptide: We suggest using the original signal peptide from the genes of interest unless a problem with protein expression is encountered (see Note 7).
3. Tags: Most of commonly used tags, such as flag-tag, HA-tag, work fine in S2 cells (see Note 8). The position of the tag has to be tested with the special genes of interest.
   All the constructs should be verified by sequencing prior to use.

3.2.3. Preparing the Expression Plasmids for Transfection

It is critical to prepare the plasmids using a commercial kit to obtain high-quality plasmids necessary for achieving a high transfection efficiency and cotransfection rate. The plasmid DNA concentration should be determined using the 260-nm absorption (estimates of DNA content based on the intensity of gel bands are not sufficiently accurate). Determine the DNA purity using a 260/280-nm ratio; the optimal ratio is 1.8.

Prepare the plasmid DNA solution in sterile Tris/EDTA (TE) buffer or sterile water, and store at −20°C for no longer than 2 years. A working solution of the plasmid DNA should be prepared at a concentration of 0.1 μg/μL and also stored at −20°C (see Note 9).

3.2.4. Transfect the S2 Cells with Fugene6 HD

One day prior to the transfection, plate out S2 cells in 3 ml of complete S2 cell medium at 1×10^6 cells/well in 6-well plates. Transfect the S2 cells 18–24 h later.

1. For each transfection, add 100 μL serum-free medium (see Note 10) in an Eppendorf tube.
2. Add corresponding plasmids (300 ng each) to each Eppendorf tube and mix well by pipetting (see Notes 11 and 12).
3. Add 4 μL of Fugene HD to each tube and vortex briefly. Incubate for 25 min at room temperature (see Notes 13 and 14).
4. During the incubation time, remove the culture medium of the S2 cells and add 2 mL fresh serum-free medium to wash the cells.
5. After the incubation, remove the washing medium from the cells, add 900 μl serum-free medium to the DNA–Fugene HD mixture, and transfer to the cells (see Note 15).

6. Incubate the cells for 18 h and replace the Fugene HD-containing medium with 3 mL of complete S2 cell medium.
7. 8–24 h later, start to induce the expression of the target proteins.

3.2.5. Induce the Expression

One of the greatest features of the S2 cell system is the inducible expression of the target proteins. Since we are using the pRmHa3 expression vector, which contains the *Drosophila* Mtn promoter, the expression of the genes of interest can be regulated by adding or removing $CuSO_4$ to the culture medium. To achieve the desired expression level for the target proteins, a pilot experiment should be done with various amounts of $CuSO_4$ (final concentration ranges from 0.1 to 2 mM) and different induction times (from 6 to 24 h) (see Note 16). In certain case, one might even want to stop the expression after a defined time by removing the $CuSO_4$ from the culture and track the cells for additional times. A general consideration is to express the target gene close to its physiological expression level.

3.2.6. Analyze the Resulted S2 Cells

Depending to the biological question being addressed, different methods or method combinations could be used to analyze the S2 cells after the rebuilding. A western blot assay can be used to study the steady-state expression level of the target protein(s). FACScan analysis of extra- or intracellularly stained S2 cells can give information about the distribution of the target protein(s). An imaging experiment can provide information about the subcellular localization and the relative abundance of the target protein(s) in different organelles. As an example, we describe below a detailed protocol for examining S2 cells using a microscope.

Prepare the S2 Cells for Imaging Experiment

The subcellular localization of target proteins can be studied by expressing fluorescence protein (FP)-tagged genes in S2 cells and examining the resulting cells by confocal or epifluorescence microscopy. Both living or fixed S2 cells can be used in this approach.

1. After inducing expression of the target genes, recover the S2 cells from a 6-well plate using a cell lifter.
2. Transfer the cells to a FACS tube. Centrifuge at 300 × g for 5 min to collect the cells.
3. Wash the cells twice with 1× PBS, and resuspend the cells in 500 μL PBS.
4. Transfer 100 μL of the cells to another FACS tube. Perform FACSscan analysis to verify the transfection efficiency (see Note 17).
5. To image living S2 cells, count the cells and dilute to 1×10^6 cells/mL in 400 μL of PBS. Mix well, transfer to 35-mm

μ-Dish, and visualize the cells directly using a microscope equipped with an inverted stage.

6. To fix the cells prior to imaging, count the cells, transfer 5×10^5 cells to an Eppendorf tube, and centrifuge at $3{,}000 \times g$ for 5 min.
7. Remove the supernatant, and resuspend the cells in 500 μl of 2% PFA (see Note 18). Keep the cells in the dark at room temperature for 8–10 min.
8. Centrifuge at $3{,}000 \times g$ for 10 min to collect the cells.
9. Resuspend the cells in 40 μL of Fluromount-G or Dapi-fluromount-G. Mix well and transfer to a microscope slide. Place a coverslip over the cells and slightly press the center of the coverslip with the blunt end of a pencil to allow it to settle down evenly (see Note 19).
10. Keep the slides at a clean and dark place overnight to dry the slides (see Note 20).
11. Visualize the slides using either an inverted or conventional microscope.

4. Notes

1. We use culture dishes and plates from Greiner Bio-One. S2 cells partially attach to culture dishes and attach reasonably well to the 6-well plates. This makes it easy to change the medium during the transfection and is not too difficult to recover the cells, especially with the help of a cell lifter. We do not recommend using culture dishes and plates from Corning since the cells attach tightly to the surface. Extensive use of the cell scraper or trypsinization can resuspend the cells in this situation, but subsequent culture and transfection are suboptimal.
2. In the past, we successfully used Schneider's *Drosophila* Medium from Invitrogen to grow our S2 cells. However, after their recent changes in the composition of the medium, our S2 cells grow more slowly in this medium and have a low transfection efficiency. This is probably because we do not grow our S2 cells in suspension to high densities but rather grow them partially attached at a relatively low concentration. Currently, we use complete S2 cell medium prepared from the SERVA powder medium to grow our S2 cells.
3. During the passage of the S2 cells, we noticed changes in the endogenous protein phosphorylation level by western blot probably due to changes in the expression level of endogenous S2 cell kinases. Newly thawed cells normally have less

background. Thus, depending on the question being asked, it may be important to avoid comparing data from newly thawed cells with cells that have been in culture for long periods. Generally, cells should be discarded 8–10 weeks after thawing or whenever an unexpected high-background phosphorylation level is encountered.

4. Antibiotics, such as penicillin, are often used in S2 cell culture for protein production on a preparative scale. However, we have found that it slows down the growth of S2 cell in our culture conditions and reduces the transfection efficiency. However, without the protection of the antibiotics, the cells are more easily contaminated. Thus, all solutions and equipment coming into contact with living cells must be sterile and verified frequently. Aseptic techniques should be rigorously applied.
5. Since S2 cells may release insect growth factors into the medium, the use of conditioned medium when thawing cells could help the cells to recover.
6. When comparing the protein expression level from wild-type and mutant constructs, identical upstream and downstream sequences should be used when constructing the expression vectors.
7. The influenza virus hemagglutinin signal sequence (18) and the N terminus of human tissue plasminogen activator (tPA) (19) have been shown to be efficient signal sequences in S2 cell system.
8. We noticed that the anti-flag M2 antibody recognizes an endogenous S2 protein. It runs at around 47 kD on a reducing SDS-PAGE.
9. It is suggested to thaw the working solution of the plasmids on ice before the experiment and keep it on ice during the transfection. To control the quality of the plasmids, we normally run an agarose gel after thawing the plasmids. If a plasmid shows faint additional bands or strong degradation, a new working solution has to be prepared.
10. Serum-free medium is made from the Invitrogen's liquid Schneider's *Drosophila* Medium. Using medium prepared from SERVA power medium in this step very often leads to the death of most cells.
11. Depending on the goals of the experiment, it is normally useful to monitor the transfection efficiency. We do this by cotransfecting an EGFP expression plasmid.
12. The total amount of the DNA used for each transfection should be equalized with the empty vector pRmHa-3.
13. To achieve high transfection efficiency, do not allow undiluted FuGENE HD to come into contact with any plastic surfaces

other than pipette tips. Directly add the FuGENE HD reagent into the serum-free medium. Do not use siliconized pipette tips or tubes. A minimum 25-min incubation time is required to achieve a good transfection efficiency. We observed a slight increase in the transfection efficiency when the incubation time is extended to 45 min.

14. We previously used Cellfectin from Invitrogen for our transfection experiments. However, Invitrogen discontinued Cellfectin and introduced the new Cellfectin 2. We experienced low transfection efficiencies with the new Cellfectin 2. This is also probably related to the way we culture our cells.
15. S2 cells are quite sensitive to tonicity changes. Thus, it is recommended not to remove the medium from all wells of all plates if one has many samples in one experiment. It is best to process one plate (six samples) at a time.
16. Beginning around 0.5 mM, $CuSO_4$ shows considerable toxicity in S2 cells. Thus, lower $CuSO_4$ concentrations and longer induction time is a better choice compared with higher $CuSO_4$ concentrations and shorter induction times.
17. An FACScan analysis provides not only information regarding the transfection efficiency, but also the distribution of the expression level in the cells. Thus, one could anticipate the signal intensity for the microscopy experiment.
18. Use PFA that has never been previously thawed.
19. Fluromount-G is very viscous, so cut the head of the pipette tips before pipetting. To avoid the formation of air bubbles on the slide, resuspend the cells in 5 μl PBS before adding the Fluromount-G. Precooling the slide might also help to reduce the amount of air bubbles.
20. The prepared slides can be stored in the dark for several weeks. Keeping the slides at a lower temperature may elongate their lifetime.

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