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Thawing, culturing and cryopreserving Drosophila cell lines

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Dear Dr. Justis,

Thank you for the detailed editor and reviewers' comments.

We are submitting our revised manuscript titled Thawing, culturing, and cryopreserving *Drosophila* cell lines. With the revised submission, we hope it is suitable for publication at JoVE, and look forward to the video production stage of the manuscript. Thank you.

Yours sincerely,

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TITLE:

Thawing, Culturing and Cryopreserving *Drosophila* Cell Lines

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KEYWORDS:

Drosophila, cell culture, cryopreservation, culture media, cell line, best practices

SUMMARY:

Drosophila cell lines are important reagents for both fundamental and biomedical research. This article provides protocols for thawing, subculturing, and the cryopreservation of commonly used *Drosophila* cell lines to assist researchers in incorporating the use of these reagents in their research.

ABSTRACT:

There are currently over 160 distinct *Drosophila* cell lines distributed by the Drosophila Genomics Resource Center (DGRC). With genome engineering, the number of novel cell lines is expected to increase. The DGRC aims to familiarize researchers with using *Drosophila* cell lines as an experimental tool to complement and drive their research agenda. Procedures for working with a variety of *Drosophila* cell lines with distinct characteristics are provided, including protocols for thawing, culturing, and cryopreserving cell lines. Importantly, this publication demonstrates the best practices required to work with *Drosophila* cell lines to minimize the risk of contaminations from adventitious microorganisms or from other cell lines. Researchers who become familiar with these procedures will be able to delve into the many applications that use *Drosophila* cultured cells including biochemistry, cell biology and functional genomics.

INTRODUCTION:

The use of *Drosophila* cultured cells complements in vivo fly genetic analysis and serves as a primary inquiry tool for addressing many basic biological questions[1–3]. *Drosophila* cell lines offer unique homogenous populations of cells derived from different tissue sources with distinct genetic backgrounds. Cell lines are suitable for many applications including transgenic gene expression, genomics, transcriptomics, proteomics, metabolomics, high throughput RNA

interference (RNAi) screens, cell biology and microscopy. Importantly, the use of *Drosophila* cell culture facilitates the characterization of the immediate temporal responses to known stimuli. Furthermore, *Drosophila* cell culture is amenable to CRISPR-Cas9 genome editing, making it relatively easy to create new cell lines with specific genome modifications[4–7].

The Drosophila Genomics Resource Center (DGRC) serves as a repository and distribution center for *Drosophila* cell lines. One of the goals of the DGRC is to assist members of the research community in using *Drosophila* cell culture resources. This article presents basic protocols for the handling of *Drosophila* cell lines. It complements existing resources to help researchers become comfortable with handling *Drosophila* cell cultures and achieve a level of independence in their experiments[1,2,8–10].

The most commonly used *Drosophila* cell lines are: Schneider lines[11], Kc167[12], Mitsubishi/Miyake imaginal disc and central nervous system (CNS) lines[13,14], Milner laboratory imaginal disc lines[15], the adult ovarian cell lines[16,17], and the Ras lines[18] (**Table 1**). The Schneider and Kc167 lines are general all-purpose cell lines for use in biochemistry, recombinant transgenic gene expression, and reverse genetic screens. The Mitsubishi/Miyake laboratory (ML) lines were derived from either the larval imaginal discs or central nervous system (CNS) and they have been useful for studies related to neurosecretion, transcription regulation, and RNA processing. The Milner (CME) disc lines have been important for studying signal transduction. The fGS/OSS cell lines derived from mutant adult ovaries remain important reagents to study the impact of non-coding small RNA biology in germ cell maintenance and differentiation[17,19]. Lastly, the Ras lines are unique because these are cell lines derived from embryos ectopically expressing the Ras oncogene. They have the transcriptional signature of muscle precursor cells and expresses active piRNA machinery[20]. Recent review articles and book chapters cover the applications of these popular cell lines with more details[2,3,9].

All these cell lines can be subcultured and frozen. There are slight but important different requirements for how each cell line is maintained and prepared for cryopreservation. For example, distinct cell lines require different media and supplements (**Table 1**). The lines also vary in surface adherence properties, morphologies (**Figure 1 and Figure 2**), genotype and doubling time (**Table 2**). We present basic protocols and highlight the unique differences for handling the various widely used *Drosophila* cell lines.

PROTOCOL:

1. Thawing and reviving frozen *Drosophila* cell lines

1.1. Sterilize the hood by wiping the working surface with 70% ethanol. Dispense 5 mL of the appropriate medium (**Table 1**) into a 25 cm^2 T-flask (T-25).

1.2. Remove the cryovial/ampule from liquid N_2 or dry ice. Wipe the cryovial with 70% ethanol, carefully loosen and unseal the ampule.

1.3. Using a Pasteur pipette, withdraw 1 mL of room temperature (RT) media from the T-25 flask. Slowly add the media into the cryovial and gently mix to thaw the frozen cells, ensuring that the cell suspension does not overflow.

1.4. Transfer the entire volume of the thawed cell suspension from the ampule into the T-25 flask. Repeat to ensure the cell suspension has been completely transferred.

1.5. Place the flask in a 25 °C incubator, allowing the cells to settle and adhere for at least 2 h. Examine the cells under a microscope to ensure that most cells have settled on the growing surface. Gently remove old media and replace with 5 mL of fresh media. Return the flask into the incubator.

1.6. On the following day, gently remove the old media and replace with 5 mL of fresh media. Return the culture to the incubator.

2. Thawing and reviving frozen *Drosophila* cell lines (Alternative)

2.1. In a sterile hood, thaw the cells by resuspending the frozen pellet with 1 mL of RT media. Transfer all the thawed cell suspension into a 15 mL conical tube.

2.2. Pellet the cells by centrifugation at 1000 x *g* for 5 min. Discard the supernatant and resuspend the cell pellet in 5 mL of fresh media.

2.3. Transfer the entire volume of the cell suspension into a T-25 flask and incubate the culture at 25 °C.

2.4. One to two hours later, examine the cells under the microscope to ensure that most cells have settled on the growing surface. On the following day, replace the old media with 5 mL of fresh media and return the culture to the incubator.

3. Subculturing semi-adherent cells grown in 100 mm culture plates

3.1. Sterilize the hood by wiping with 70% ethanol. Bring the sterile materials for subculturing into the hood, including media bottles, pipettes, pipette aid, and culture plates.

3.2. Examine the morphology and confluence of the culture under a microscope. Look for clear signs of microorganismal contaminations in the culture. Determine whether the cells are ready to be passaged, based on the characteristics of the culture: cell density and doubling time, including the last time they were subcultured.

3.3. If the culture appears highly confluent (**Figure 1**), determine the cell density. In the sterile hood, dislodge the cells from the growing surface by pipetting up to 10 mL of the medium from the plate and dispensing it over the cells. Repeat a few times, ensuring not to create foam, until the growing surface becomes clear. Determine the cell density using a hemocytometer or an

automatic particle counter (section 5, **Figure 3**). Subculture the cells if the cell density is between 5 x 10^6 and 1 x 10^7 cells/mL.

NOTE: Do not subculture *Drosophila* cell lines to a cell density below 1 x 10^6 cells/mL.

3.4. Dilute the cell suspension accordingly using an appropriate medium to a final seeding concentration of at least 1 x 10^6 cells/mL.

3.4.1. For routine passaging and maintenance, add an appropriate volume of cell suspension to a pre-determined volume of medium in a new culture plate to achieve the desired seeding cell density.

3.4.2. For scaling up a culture, transfer all the cell suspension into a large flask. Dilute the cell suspension to the desired cell density with an appropriate volume of the medium. Distribute equal volumes of the diluted cell suspension to new plates. This method minimizes the variations in cell density between plates.

3.5. Cover and label the plates with the operator initials, date, split ratio, seeding cell density, cell line identifier, media, passage number and any media additions such as antibiotics.

3.6. Place the plates into a plastic container and return the box to the incubator.

NOTE: **Table 3** lists the culture vessels commonly used for culturing *Drosophila* cell lines and the associated working volumes.

4. Dislodging adherent cells grown in 100 mm culture plates

4.1. Transfer all the medium from the plate to a new sterile flask. Save the medium.

4.2. Rinse cells by slowly adding 1 mL (0.1 mL/cm^2) of 0.05% trypsin-EDTA to the plate. Swirl gently to ensure the trypsin solution covers the entire growth surface. Discard the trypsin solution.

4.3. Gently add 1 mL (0.1 mL/cm^2) of 0.05% trypsin-EDTA to the plate. Incubate the plate at 25 °C between 3−10 min while monitoring for visible signs of the cell layer detaching and sliding off the growing surface.

4.4. Add 9 mL of the saved medium to the plate to stop trypsin activity. Mix the cell suspension to dissociate cell clumps. Once all cells have been dislodged, the growing surface will be clear.

NOTE: The use of digestive enzymes such as trypsin aids in passaging strongly adherent cell lines. Trypsin is a mixture of proteases often derived from porcine pancreas and is commercially available in different grades of purity.

5. Manual cell counting using the Neubauer cell counting slide

5.1. Prepare the hemocytometer slide and coverslip by wiping the surface with 70% alcohol.

5.2. Mix the cell suspension and dispense 15 µL of the cell suspension into the grooved edge of the hemocytometer (**Figure 3A**) to fill the first chamber of the hemocytometer. Fill the second chamber of the hemocytometer. The cell suspension will be drawn into the counting chamber by capillary action.

5.3. Using a 10x microscope objective, count the cells within the 1 mm^2 area in the middle of the grid bound by the parallel lines (**Figure 3C,D**). To avoid duplicate counting, count the cells that overlay the top and left boundaries, but not cells that cross the right and bottom boundaries of the 200 μm^2 squares. Count between 100−200 cells. Repeat the count with the second chamber.

5.4. Calculate the average of the two counts and determine the cell density according to the following formula: Cell density (cells/mL) = Average cell count ($n_1 + n_2/2$) x 10^4.

NOTE: Cell viability is expressed as the percentage of viable cells over total cells. To determine cell viability, mix the cell suspension with an equal volume of trypan blue (0.4%) solution prior to manual or automatic cell counting. Live cells will not take up the dye, while dead cells will be stained blue.

6. Cryopreservation of *Drosophila* cell lines

6.1. Check the culture for healthy morphology, growth, and the lack of contamination. Harvest the cultures from the mid to late-log growth phase (step 3.3, or section 4). For many *Drosophila* cell lines, it is approximately between 4 x 10^6 cells/mL to 8 x 10^6 cells/mL.

6.2. Transfer the entire cell suspension into a 15 mL or 50 mL conical tube. Collect the cells by centrifugation at 1000 x *g* for 5 min and discard the supernatant.

6.3. Resuspend the cell pellet in a volume of freezing medium (**Table 4**) that will result in a final cell density of at least 4 x 10^7 cells/mL.

6.4. Add dropwise the appropriate amount of the cryoprotectant dimethyl sulfoxide (DMSO) into the cell suspension such that the final DMSO concentration is 10%. Gently mix the cell suspension.

6.5. Carefully dispense 0.5 mL of the cell suspension into aliquots of the pre-labeled cryovials (~ 2 x 10^7 cells/vial). Place the ampules into a freezing container filled with isopropanol (**Figure 4A**). Transfer the freezing container into a -80 °C freezer overnight to allow the temperature of the cryovials to drop slowly (-1 °C/min) to the freezer temperature.

6.6. Take out the frozen cryovials and rapidly attach them to canes (**Figure 4B**). Insert the canes containing cryovials into a canister (**Figure 4C**). Alternatively, place frozen cryovials inside a pre-cooled freezing box (**Figure 4D**). Store frozen cryovials in the liquid phase of N_2 freezers (**Figure 4E,F**).

NOTE: When using freezing media containing DMSO, a delay of up to 30 min at RT is not detrimental to the cells.

REPRESENTATIVE RESULTS:

It is important to thaw frozen *Drosophila* cells rapidly and culture them at a cell density that brings the culture back into the growth phase. If the procedures for cryopreservation and thawing are adhered to, the cell density in the T-25 flask will at least equal 4 x 10^6 cells/mL. One to two hours after thawing, most *Drosophila* cell lines will begin to attach to the growing surface. Under the circumstance in which most of the cells have not attached on the growing surface within two hours after thawing, it is recommended to incubate the cells overnight before changing the media.

The goal of subculturing is to maintain cells in the healthy exponential log-phase of the growth curve. The criteria for subculturing depend on the visible lack of microorganismal contamination, cell density, and the need to establish a regular maintenance schedule. It is important to first assess the health of the cells and determine the absence of adventitious contaminants prior to freezing. Most bacterial and fungal contaminants are easy to detect simply by visual inspections. Contaminated cultures can be identified by an increase in media turbidity. Under the microscope, contaminants may appear as bacterial rods, cocci, budding yeast cells or string-like fungal hyphae. Other sources of contamination such as the non-cytopathic mycoplasma cannot be visually detected and can be routinely tested by PCR-based assays[21].

The confluence of a cell line can be determined visually (**Figure 1**). Fast growing cell lines reach confluence early and need to be passaged regularly. Such lines are subcultured up to twice a week. In contrast, slow growing cells are passaged at least once every two weeks or longer. However, the cells need to be fed fresh media every week. This is to prevent media exhaustion and to dilute metabolic waste products from the cells. Cell lines derived from varying tissue sources differ in their morphology (**Figure 2**), adherence properties, media requirements (**Table 1**) and doubling time (**Table 2**). **Table 5**, **Table 6**, **Table 7**, and **Table 8** list the recipes for the various *Drosophila* cell culture media.

Cell counting ensures an accurate seeding density and a predictable routine for subculturing. For quantitative experiments, cell counting is essential. Cells are counted either by using a hemocytometer (**Figure 3A**) or an automated particle counter (**Figure 3B**). If using an automated counter, follow the manufacturer's instructions. Counting cells manually using a hemocytometer is economical and easy. The number of cells enclosed in the middle Neubauer grids are counted and the cell density is calculated; For example, n = 214 cells, resulting in a cell density of 2.14 x 10^6 cells/mL (**Figure 3D**).

Cell suspension from two 100 mm plates, each containing 10 mL of cell suspension at 4 x 10^6 cells/mL are collected and resuspended in 2 mL of freezing media to achieve a density of 4 x 10^7 cells/mL. Each frozen cryovial with 0.5 mL of cell suspension contains 2 x 10^7 cells. This will result in a culture with 4 x 10^6 cells/mL when thawed according to the protocol section 1.

FIGURE AND TABLE LEGENDS:

Figure 1: Representative images of three distinct *Drosophila* cell lines at different confluence and cell densities. (**A**) S2-DGRC culture at 1 x 10^6 cells/mL. (**A'**) S2-DGRC culture at 4.5 x 10^6 cells/mL. (**B**) ML-BG2-c2 culture at 2 x 10^6 cells/mL. (**B'**) ML-BG2-c2 culture at 8 x 10^6 cells/mL in which cells are piling and aggregating as foci. (**C**) OSS culture at 1 x 10^6 cells/mL. (**C'**) OSS culture at 4 x 10^6 cells/mL. Cells in suspension are not captured on the same focal plane. Scale bar = 100 µm.

Figure 2: Representative images of the eight distinct *Drosophila* cell lines. (**A**) Round embryo-derived S2-DGRC. (**B**) Round embryo-derived Kc167. (**C**) Round larval CNS-derived ML-BG2-c2. (**D**) Round larval spindle-shaped ML-BG3-c2. (**E**) CME L1, a cell line derived from the larval leg imaginal discs, is smaller and has round/fusiform morphology. (**F**) OSS, a cell line derived from adult ovaries, displays spindle-shaped morphology. (**G**) Spindle-shaped Ras^{V12} cell line expressing activated Ras. (**H**) $Ras^{V12}; wts^{RNAi}$ (WRR1), a cell line expressing activated Ras and double-stranded RNA targeting the tumor suppressor *warts* (*wts*), displays epithelial characteristics. Scale bar = 50 µm.

Figure 3: Cell density can be counted manually using a hemocytometer or automatically using an automated particle counter. (**A**) A hemocytometer with two chambers. (**B**) An automated cell counter displaying the output of a cell count. (**C**) The improved Neubauer cell counting grid viewed under a 10x objective. Count cells bound by the 0.1 mm^3 central grid (red dashed-line square). (**D**) The central grid on the hemocytometer filled with cells for counting. Scale bar in panel **C** = 1 mm. Scale bar in panel **D** = 0.2 mm.

Figure 4: Equipment for cryopreservation. (**A**) A freezing container stores the ampules in an upright position for slow freezing. (**B**) A metal cane for holding frozen ampules. (**C**) A canister for holding canes. (**D**) A plastic freezing box (cryobox). (**E**) A canister holding multiple canes inserted into a liquid N_2 storage tank. (**F**) A liquid N_2 storage tank.

Table 1: The properties and media requirements of various *Drosophila* cell lines. Different isolates of semi-adherent Schneider lines including S2R+, S2-Drosophila RNAi Screening Center (DRSC), S2-Drosophila Genomics Resource Center (DGRC), and Sg4 are commonly used cell lines that proliferate robustly when cultured in M3 media + Bactopetone yeast extract (BPYE) supplemented with 10% fetal calf serum (FCS). Alternatively, Schneider's media (pH 6.7–6.8) is often used in place of M3+BPYE. The Kc lines proliferate in either M3 + BPYE (5% FCS) or serum-free CCM3 media. The ML imaginal disc and central nervous system (CNS) lines require insulin supplementation for proliferation. The Milner imaginal disc lines require both insulin and fly extract supplementation. Adherent fGS/OSS cell lines require insulin, a higher concentration of

fly extract as well as glutathione for growth. Adherent Ras^{V12} lines grow well in M3 + BPYE (10% FCS). Trypsin is used to dislodge adherent cell lines from the growth surface.

Table 2: Genotype, doubling time, and tissue sources of widely used *Drosophila* cell lines. The tissue genotype, source and population doubling time of commonly used cell lines are presented. Doubling time is based on growth in the recommended media at 25 °C.

Table 3: Culture vessels and the recommended media volumes. Culture vessels of various sizes are available for culturing *Drosophila* cells. The appropriate media volumes (mL) are recommended for each vessel. Seal multi-well plates containing less than 0.5 mL of cell suspension with paraffin film to reduce media loss due to evaporation.

Table 4: Recipe for preparing 100 mL of freezing medium (M3 + BPYE, 20% FCS, 10% DMSO). Prepare freezing media as needed and avoid storing freezing media containing DMSO for prolonged period.

Table 5: Recipe for preparing 1 L of M3 + BPYE tissue culture medium. Adjust pH to 6.6. Sterilize by passing the medium through a 0.22 µm filter.

Table 6: Recipe for 1 L of fGS/OSS M3 base medium. Adjust pH to 6.8. Sterilize by passing the medium through a 0.22 µm filter.

Table 7: Recipe for 1 L of Hyclone-CCM3 tissue culture medium. Adjust pH to 6.2. Sterilize by passing the medium through a 0.22 µm filter.

Table 8: Recipe for preparing 100 mL of various common *Drosophila* cell culture media. Incubate FCS at 56 °C for one hour and shake every five minutes to heat-inactivate complement proteins.

DISCUSSION:

Drosophila cell cultures are primary reagents for high throughput cell-based screens. Their use also complements in vivo genetic research by providing a homogenous population of cells suitable for biochemistry, rapid testing of transgenic constructs prior to injecting into flies, cell biology, microscopy and more recently somatic cell genetic manipulations by genome editing[1–3,8–10].

The viability and recovery of frozen *Drosophila* cells is sensitive to drastic fluctuations even at low temperatures. The DGRC stores frozen cell lines in the liquid phase of N_2 (-196 °C) and transports them in dry ice (-78.5 °C). Frozen ampules that have been transported in dry ice should not be transferred back into liquid N_2 or a -80 °C freezer for storage. Instead, the frozen cells should be thawed, reseeded at a high cell density as soon as possible upon arrival (protocol section 1) and cultured for their intended purposes (protocol section 3). If the cell lines are not immediately utilized for experiments, the cell lines should be cryopreserved (protocol section 6) until they are ready for use.

Some cell lines, such as the ML-BG2-c2 and Ras lines need several days to recover from the effects of being revived from the cryopreserved state. A significant amount of cellular debris accompanies these cell lines the first few days after thawing. Left undisturbed, the cells will recover and proliferate. Many *Drosophila* cell lines at the DGRC have been adapted to grow in M3 based media[22]. For cell lines that are slow to recover from the effects of thawing, the use of conditioned media may be useful. Conditioned media likely contain growth factors secreted by the cells into the media which may encourage the recovery and proliferation of the cells after thawing.

Cell lines generally follow a stereotypical growth curve consisting of a lag phase, exponential phase, plateau phase and a deterioration phase. Many *Drosophila* cell lines proliferate in the log-phase of growth when they are cultured at a density between 1 x 10^6 and 1 x 10^7 cells/mL at 25 °C. It is essential that cell lines are passaged such that they are always in the exponential growth phase.

The confluence of a culture, expressed as a percentage, describes the growth surface area that is covered by cells. Cell confluence for a cell line depends on its cell shape and size. Distinct cell lines have different morphologies and adherence properties. As a result, different cell lines at approximately similar confluence may have vastly distinct cell density (**Figure 1**). Culture confluence may not be an ideal indicator for passaging *Drosophila* cell cultures because *Drosophila* cell lines continue to proliferate either by piling on top of one another as foci or in suspension even after the growth surface has been covered (**Figure 1**). However, users experienced with specific cell lines may often use confluence as a rapid visual guide for when to subculture.

While it is possible to grow *Drosophila* lines at ambient RT between 19−25 °C, it is not recommended because ambient temperature fluctuations may affect the proliferation rate. The use of a dedicated 25 °C incubator is recommended. The incubator for *Drosophila* cell cultures does not need to facilitate CO_2 gas exchange because *Drosophila* cell culture media do not use CO_2 for buffering. The humidity inside the incubator for culturing cell lines is an important factor not to be overlooked when culturing cells in plates. Depending on the type on incubator and the working environment, it may be necessary to place a beaker of sterile water inside the incubator. To minimize media evaporation, use closed T-flask or store culture plates in a tightly sealed plastic container while inside the incubator.

It is important to develop a schedule for subculturing *Drosophila* cell lines. To estimate the growth rate and monitor consistency, it is convenient to subculture at an even geometric ratio (split ratio 1:2, 1:4, 1:8). For example, a 10 mL confluent plate of Kc167 cells at 8 x 10^6 cells/mL can be split at 1:8 ratio to achieve a seeding density of 1 x 10^6 cells/mL (1.25 mL of cell suspension diluted into 8.75 mL of fresh media). In 72 hours, Kc167 cultures are expected to proliferate to a density of 8 x 10^6 cells/mL, given its doubling time of 24 hours. The split ratio therefore is determined to facilitate a convenient subculture routine of up to twice a week, ensuring that the cells are always cultured in their exponential log phase of growth. This allows for a regular

schedule for subculturing the cells so that the time to confluence is neither too short nor too long. If the time to confluence is too short, the cells are subcultured at a lower cell density (higher split ratio). Similarly, if the time to reach confluence is too long, the cells are subcultured at a higher cell density (lower split ratio). It is important to note that most *Drosophila* cell lines are very sensitive to low cell densities (< 1 x 10^5 cells/mL), in which cells hardly proliferate and may eventually die.

Drosophila cell lines vary in growth characteristics and morphology. As a result, cell lines with distinct properties may have to be handled differently. Most *Drosophila* cell lines are semi-adherent. At lower cell density, they adhere stronger to the growth surface and as the culture becomes confluent, the cells become less adherent and easily detach. This gradual change in cell adherence facilitates easy subculturing of most widely used *Drosophila* cell lines (Schneider, Kc lines, imaginal disc and CNS lines) as it allows the operator to simply dispense media over the cell monolayer to dislodge them from the growth surface when the culture is dense. For lines that are surface adherent such as the female germ-line stem/ovarian somatic sheath (fGS/OSS) and Ras lines, it is essential to incubate the cells in trypsin for a short duration to aid in detaching the cells from the growth surface.

Media additions for most *Drosophila* cell lines include fetal calf serum (FCS). Insulin and adult fly extract (FEX) are required for some specific lines. FEX contains undefined components essential to the growth of specific larval imaginal disc lines and the adult ovarian cell lines. The DGRC prepares, and makes available adult FEX derived from one-week old Oregon-R-modENCODE flies (RRID: BDSC_25211) in 2.5 mL and 10 mL aliquots. The DGRC also provides instructions for small scale FEX preparation on its website <https://dgrc.bio.indiana.edu/include/file/additions_to_medium.pdf>. FEX preparation, however, is time-consuming and requires a large quantity of adult flies.

The cryopreservation of *Drosophila* cell lines saves time and reagents for the maintenance of cell lines not in immediate use. Cryopreservation is achieved by slowly freezing the cells (-1 °C/min) to -80 °C in a medium containing DMSO, a cryoprotective agent. The slow cooling step is critical for successful cryopreservation. In a -80 °C freezer, the ampule of cells is cooled at a rate of -1 °C/min when placed in a freezing container filled with isopropanol. Starting at 25 °C ambient temperature, it will take up to two hours for the temperature in the ampules to reach -80 °C. It is recommended to leave the ampules to freeze overnight.

Frozen ampules must then be rapidly transferred into the liquid phase of nitrogen for prolonged storage. At ambient temperature, the cryovial will reheat rapidly at approximately 10 °C/min and the viability will be compromised at above -50 °C[23]. To keep the transfer rapid, handle ampules in small batches to minimize the exposure to ambient temperature. Alternatively, place the frozen cryovials on dry ice while preparing for their transfer into liquid N_2. If liquid nitrogen is not available, the cells may be stored in a -80 °C freezer, although with a risk of significant deterioration over time.

Cell density is critical for successful cryopreservation and the subsequent revival of cell lines. In

general, new cell lines should be frozen to create the initial freeze (1−3 ampules) as soon as an excess of cells becomes available. Once the cell line has been further cultured stably, a frozen stock of 10−20 ampules should be created. This stock is then thawed to check for its post-freeze cell recovery and viability, after which it is propagated for experimentations or to replace the stock when the number of frozen stock ampules falls below five. Finally, it is important to validate that the thawed cells retain the characteristics of its parental stock as cell lines are known to evolve[3,24].

In conclusion, this article presents a primer for working with *Drosophila* cell cultures by providing the fundamental information on the various lines, best practices, and audiovisual protocols for the basic handling of *Drosophila* cell lines. This accessible resource is meant to smoothly ease the introduction to working with cultured *Drosophila* cells and to complement existing training guides at any research laboratory.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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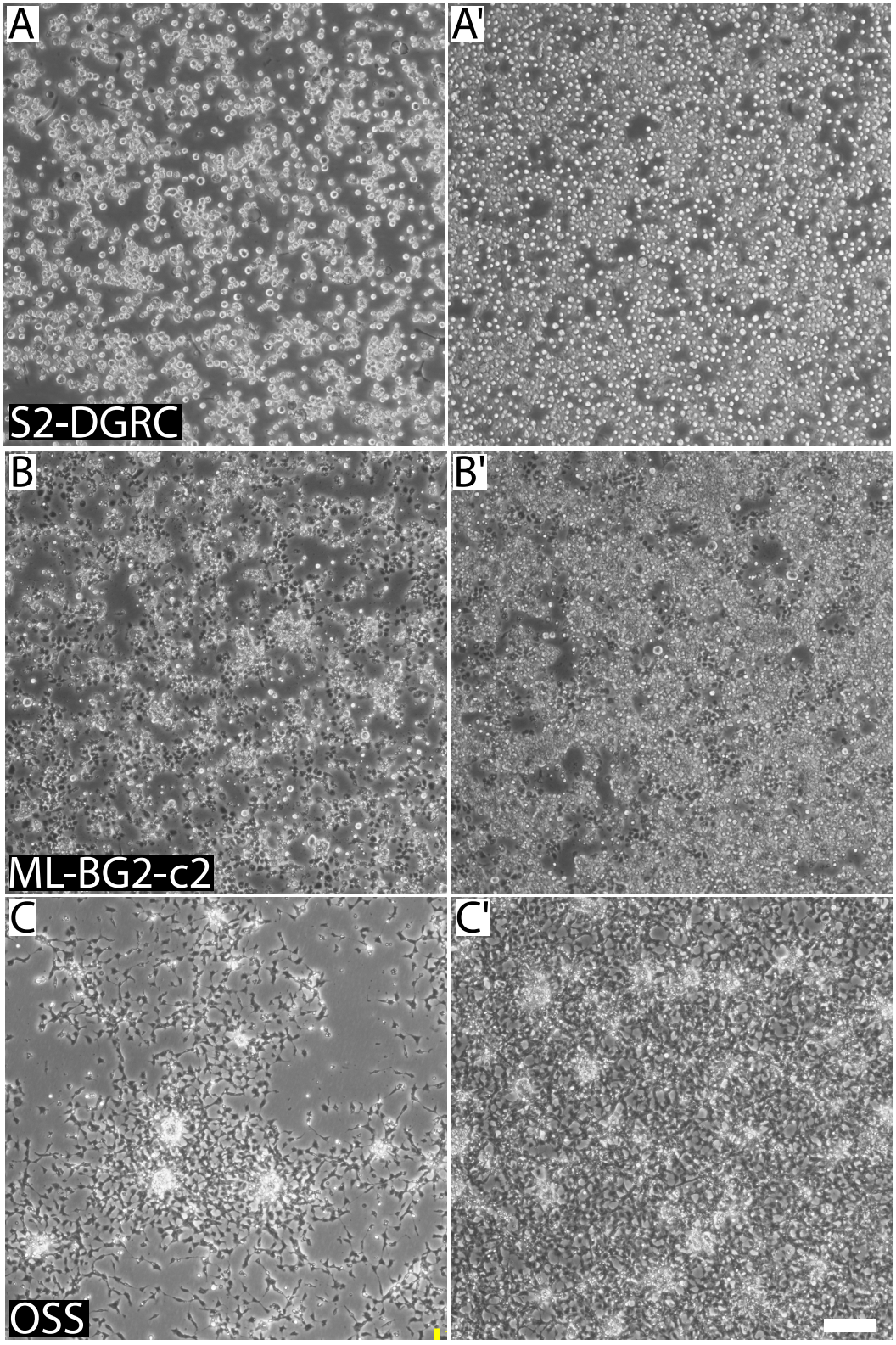

Figure 2

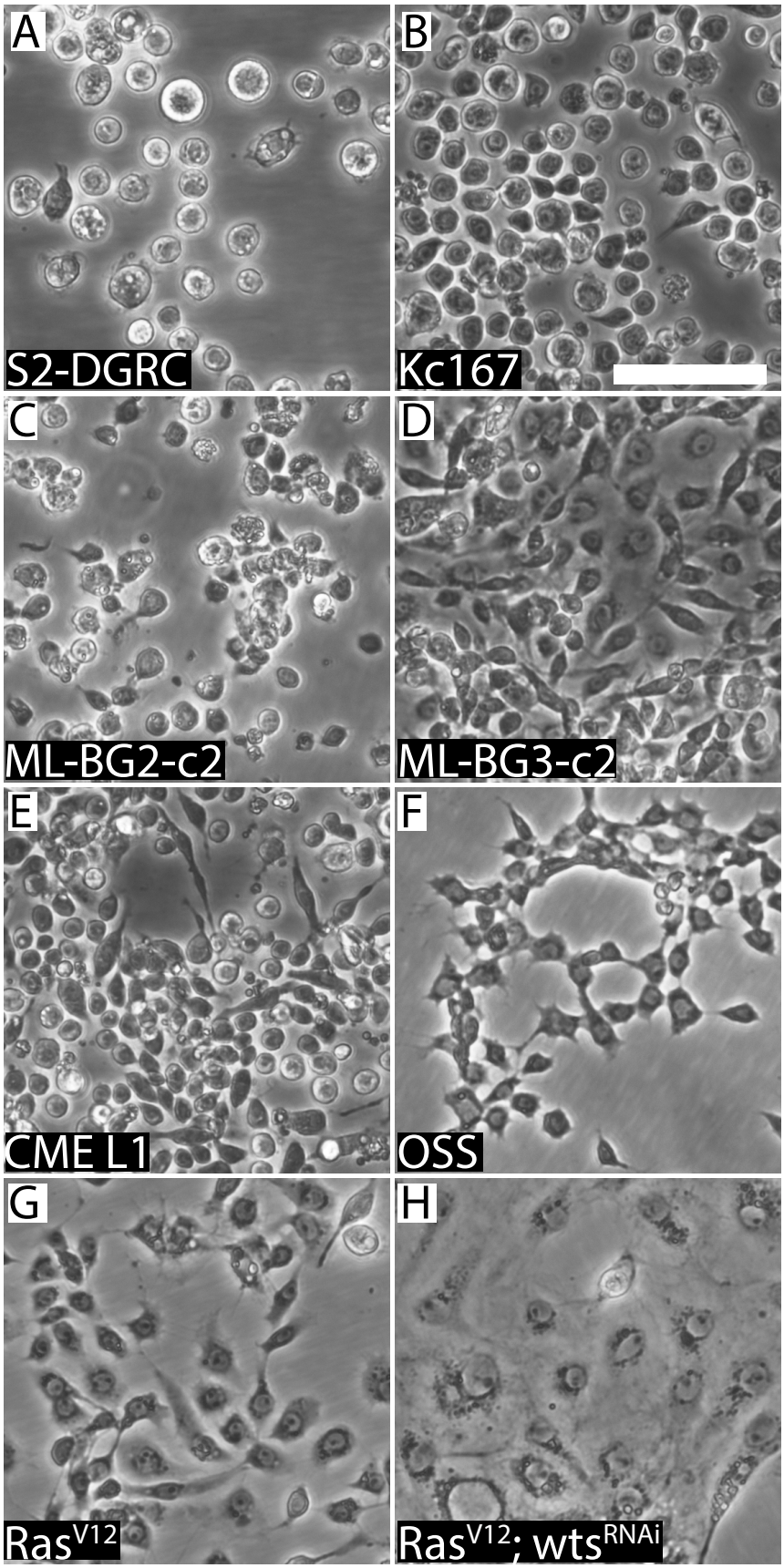

Figure 3

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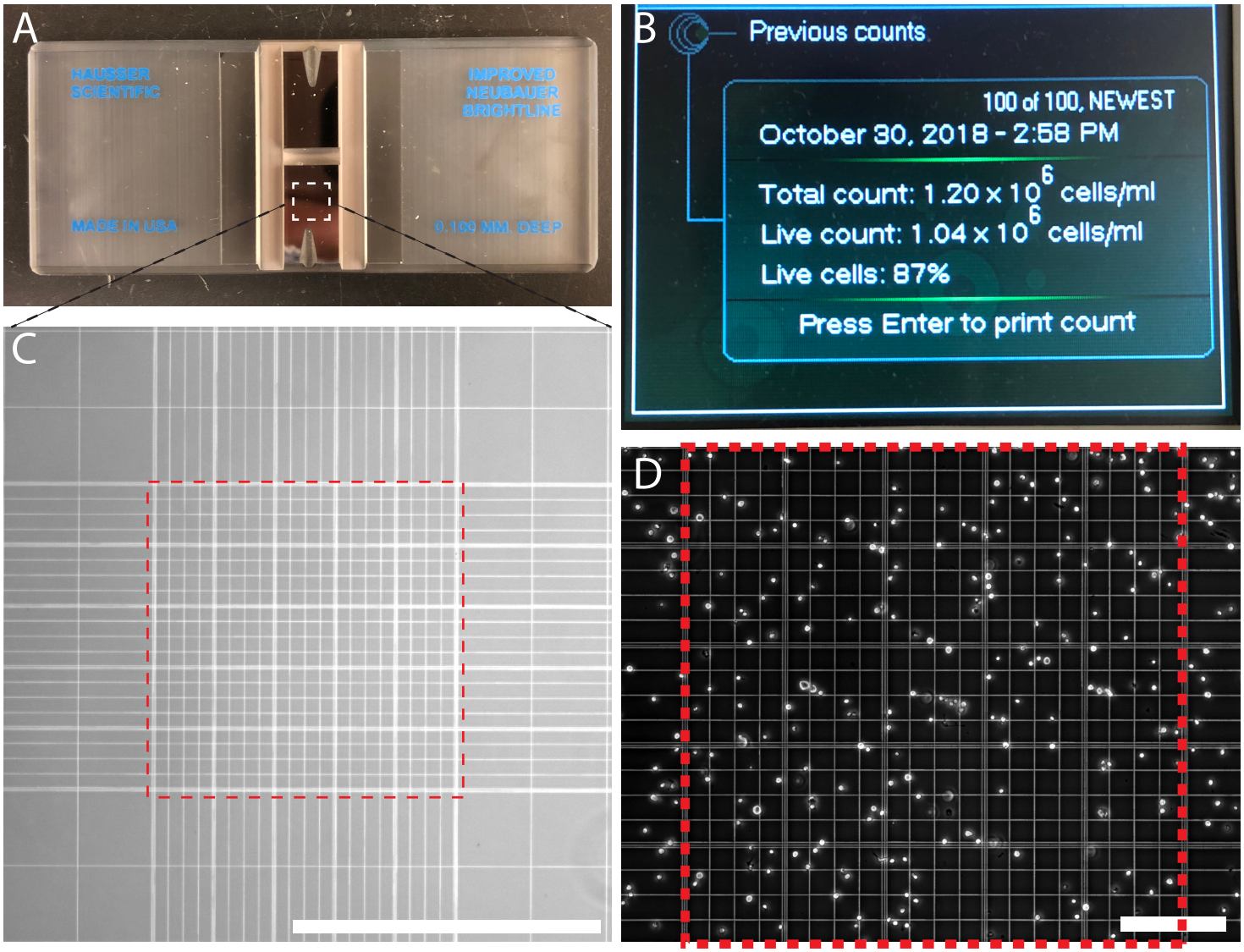

Figure 4

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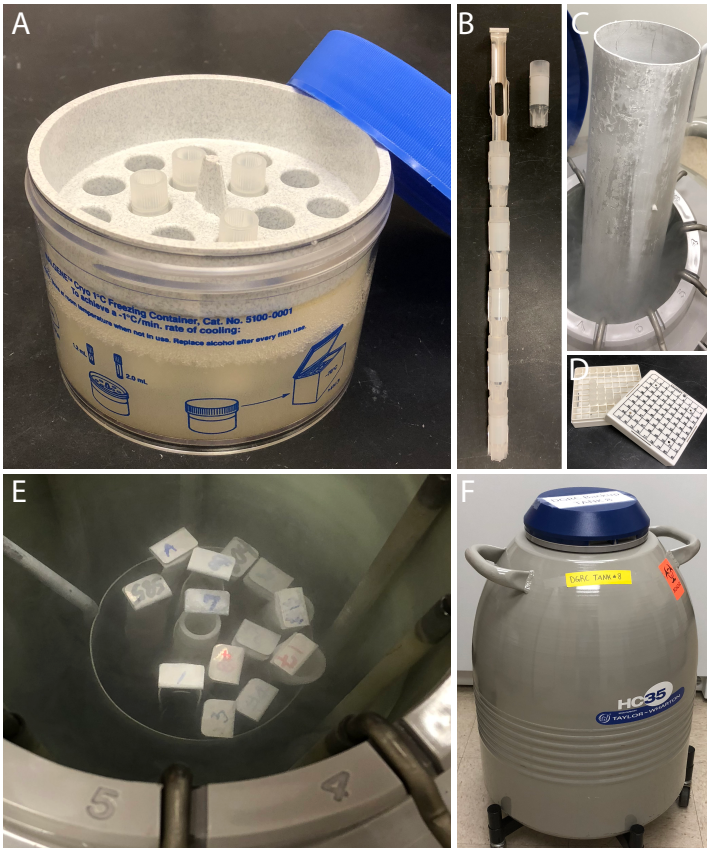

Table 1. The properties and media requirements of various *Drosophila* cell lines

Cell Strain
Schneider Lines (S2R+, S2-DRSC, S2-DGRC, Sg4)[11]
Kc lines (Kc167, Kc7E10)[21,22]
Imaginal disc and CNS lines (ML-lines)[13,14]
Milner imaginal disc lines (CME-lines)[15]
fGS/OSS[16]
Ras^{V12} lines[18]

Media	Adherence	Trypsin
M3 + BPYE + 10% fetal calf serum (FCS), pH 6.6	Semi-adherent	No
Schneider's media^{+} + 10% FCS M3 + BPYE + 5% FCS, pH 6.6	Semi-adherent	No
Hyclone-CCM3, pH 6.2		
M3 + BPYE + 10% FCS, pH 6.6 10 µg/mL insulin	Semi-adherent	No
M3 + 2% FCS 5 µg/mL insulin 2.5% fly extract	Semi-adherent	No
M3 + 10% FCS, pH 6.8 10 µg/mL insulin 1 mg/mL $C_5H_8KNO_4$ 0.5 mg/mL $KHCO_3$ 0.6 mg/mL glutathione 10% fly extract	Adherent	*
M3 + BPYE + 10% FCS, pH 6.6	Adherent	Yes

Table 2. Genotype, doubling time and tissue sources of various widely used *Drosophila* cell lines

Cell line (Stock #)	Genotype	Doubling time (h)*
S2R+ (150)	*OreR*	39
S2-DGRC (6)	*OreR*	23
S2-DRSC (181)	*OreR*	46
Kc167 (1)	*e/se*	22
ML-BG2-c2 (53)	*y v f mal*	48
ML-BG3-c2 (68)	*y v f mal*	104
ML-DmD8 (92)	*y v f mal*	66
CME W1 Cl.8+ (151)	*OreR*	46
CME L1 (156)	*OreR*	47
OSS (190)	*bamD* [86]	45
Ras^{V12} lines	*UAS-GFP; P(UAS-Ras85D.V12)/ P(Act5C-GAL4)17bFO1*	41–65

Tissue Source
Late embryos
Late embryos
Late embryos
6−12 h embryos
3rd instar larval CNS
3rd instar larval CNS
3rd instar larval wing disc
3rd instar larval wing disc
3rd instar larval leg disc
Adult *bam* mutant ovaries
Embryo

Table 3. Culture vessels and the recommended media volumes

Culture vessel
12.5 cm^2 T-flask
25 cm^2 T-flask
75 cm^2 T-flask
35 mm plate
60 mm plate
100 mm plate
384-well plate*
96-well plate*
48-well plate*
24-well plate*
12-well plate
6-well plate

Volume of media (mL)
2.5
5
15
1
4
10
0.04/well
0.1/well
0.3/well
0.5/well
1.0/well
2.0/well

Table 4. Recipe for preparing 100 mL of freezing medium (M3 + BPYE, 20% FCS, 10% DMSO)

	Volume
M3 + BPYE, pH 6.6	70 mL
Heat inactivated FCS	20 mL
Sterile filtered DMSO*	10 mL

Table 5. Recipe for 1 L of M3 + BPYE tissue culture medium

M3 + BPYE medium	Amount
Shields and Sang's M3[23]	1 bottle
$KHCO_3$	0.5 g
Select yeast extract	1.0 g
Bactopeptone	2.5 g
Sterile purified water	1000 mL

Table 6. Recipe for 1 L of M3 base medium for culturing fGS/OSS (fGS/OSS M3)

Base M3 medium for fGS/OSS cell line	Amount
Shields and Sang M3	1 bottle
$KHCO_3$	0.5 g
$C_5H_8KNO_4$	1.0 g
Sterile purified water	1000 mL

x

Table 7. Recipe for 1 L of Hyclone-CCM3 tissue culture medium

Hyclone-CCM3	Amount
CCM3 powder	28.6 g
$NaHCO_3$	0.35 g
10 N NaOH	2.5 mL
$CaCl_2$	0.5 g
Sterile purified water	1000 mL

Table 8. Recipe for preparing 100 mL of various common *Drosophila* cell culture media

	M3 + BPYE + 10% FCS	**Miyake disc and CNS lines medium**
M3 + BPYE, pH 6.6	90 mL	90 mL
Heat inactivated FCS*	10 mL	10 mL
Insulin (10 mg/mL)	-	100 µL
Fly extract	-	-
Glutathione (60 mg/mL)	-	-
M3, pH 6.6	-	-
fGS/OSS M3, pH 6.8	-	-

Milner disc lines medium	fGS/OSS complete medium
-	-
2 mL	10 mL
50 µL	100 µL
2.5 mL	10 mL
	1 mL
97.5 mL	-
	79 mL

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number
100 mm tissue culture plates	Corning	430167
25 cm2 T-flask	Corning	430168
35HC Liquid Nitrogen Storage Tank	Taylor-Wharton	35HCB-11M
Automated Cell counter	BIO-RAD	TC20
Bactopeptone	BD BioSciences	211677
Counting Slides	BIO-RAD	145-0011
Cryovial 1 mL	Greiner	123263
DMSO	Sigma Aldrich	D5879
Freezing Box	Nalgene	5029-0909
Freezing Container	Fisher Scientific	15-350-50
Hematocytometer	Fisher Scientific	#0267110
Human Insulin	Millipore Sigma	I9278
Hyclone CCM3 media	GE Healthcare Life Sciences	SH30061.03
Hyclone Fetal Bovine Serum	GE Healthcare Life Sciences	SH30070.03
L-Glutamic acid potassium salt monohydrate	Millipore Sigma	G1149
L-Glutathione reduced	Millipore Sigma	G6013
Potassium Bicarbonate	Millipore Sigma	237205
Select Yeast Extract	Millipore Sigma	Y1000
Shields and Sang's M3 Insect medium	Millipore Sigma	S8398
Trpsin-EDTA (0.05 %), phenol red	ThermoFisher Scientific	25300054
Trypan Blue (0.4%)	BIO-RAD	145-0013

Comments/Description

Subculturing
Subculturing
Cryopreservation
Counting
Medium additions
Counting
Cryopreservation
Cryopreservation
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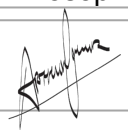
Signature:	
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We thank the editor and reviewers for comments and suggestions that improved the clarity of this manuscript. Our point-by-point responses are in blue.

Editorial comments:
Changes to be made by the author(s) regarding the manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have proofread the manuscript for spelling and grammar issues.

2. Please remove the embedded table(s) from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.

We have removed the tables and created separate .xls file for each Table.
Each table has a title, both in the xls.file and in the FIGURE and TABLE LEGENDS section.

3. Please number the tables in the sequence in which you refer to them in the manuscript text.

We have changed the numbering of the Table according to the sequence in which they are referred to in the text.

4. Tables 4-6: Please replace commercial language (Milli-Q) with a generic term.

We have replaced Milli-Q with the generic term “sterile purified water”.

5. Table of Equipment and Materials: Please sort the items in alphabetical order according to the name of material/equipment.

We have sorted the items in alphabetical order.

6. Figure 3B: Please crop the image to so that the brand name (BIO-RAD) is not shown.

We have amended Figure 3B as suggested.

7. Please revise the title to avoid the use of colon.

We have revised the title to avoid the use of colon.

8. Keywords: Please provide at least 6 keywords or phrases.

We have provided 6 keywords.

9. Please define all abbreviations before use.

We have checked the manuscript and defined all abbreviations before use.

10. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We have revised the entire manuscript to eliminate the use of personal pronouns.

11. Lines 92-96, 132-152, 214-219, etc.: Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

We have revised the identified sections, included several notes, and moved most of the content to the Discussion.

12. 1.5: Please specify the volume transferred each time.

We have specified to transfer the entire volume of the cell suspension.

13. 2.1.4: Please provide more specific guidance here.

We have expanded this in Protocol 2, steps 2 and 3.

2. Examine the morphology and confluence of the culture under a microscope. Look for clear signs of microorganismal contaminations in the culture.
Determine whether the cells are ready to be passaged, based on the characteristics of the culture: cell density and ~~coun and~~ doubling time, including the last time they were subcultured.
~~1.~~
3. If the culture appears highly ~~is~~ confluent (Figure 1), ~~prepare to~~ determine the cell density. In the sterile hood, dislodge the cells from the growing surface by pipetting up to 10 mL of the medium from the plate and dispensing it over the cells. Repeat a few times, ensuring not to create foam, until the growing surface becomes clear.
Determine the cell density using a hemocytometer or an automatic particle counter (Figure 3) (Protocol 3).
Subculture the cells if the cell density is between 5 X 10^6 – 1 X 10^7 cells/ mL.

Note: Do not subculture *Drosophila* cell lines to a cell density below 1 X10^6 cells/ mL.

Formatted: Normal, Indent: Left: 0.25", No bullets or numbering, Tab stops: Not at 1"

14. 2.1.8: Please specify the concentration of trypsin used.

We have specified the concentration of Trypsin-EDTA (0.05%)

15. 2.1.10: Please specify the incubation temperature.

We have specified the incubation temperature (25 °C)

16. 2.1.11: What volume of medium is added?

We have specified the volume added. (9 mL)

17. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

We have combined the shorter steps of the protocol as recommended. Each protocol now contains on average 5 steps.

18. Please include single-line spaces between all paragraphs, headings, steps, etc.

We have included single-line spaces between paragraphs, headings, and protocol steps.

19. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

We have highlighted the essential steps portion of the Protocol (including headings and spacing). The highlighted sections now comprise 2.5 pages. We did not highlight actions that are repeated between protocols.

20. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Notes cannot usually be filmed and should be excluded from the highlighting.

We have highlighted only complete sentences and ensured that the highlighted step includes at least one action written in the imperative tense.

21. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

We have taken this into account when highlighting the protocol steps.

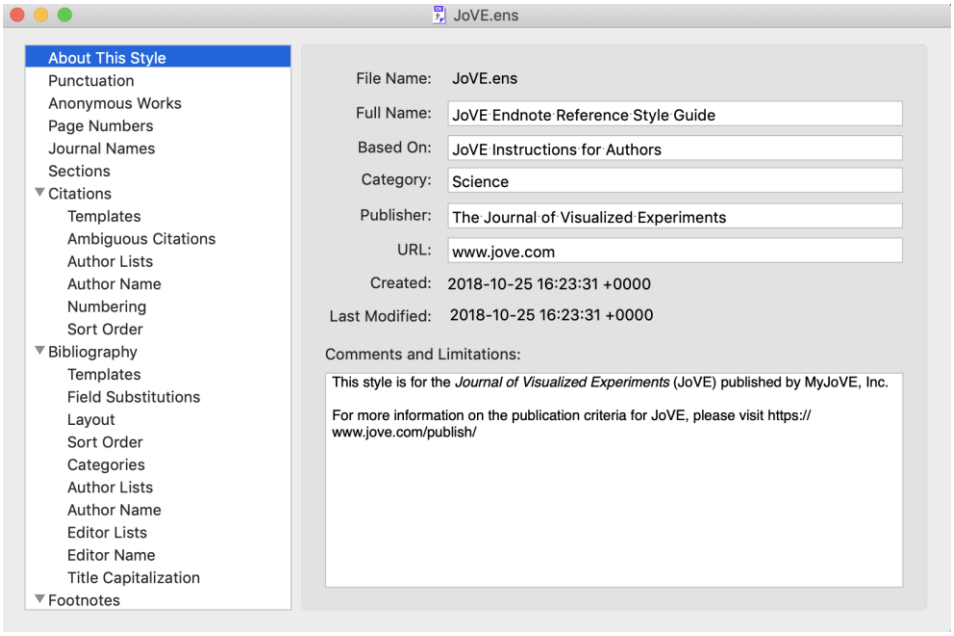
22. Lines 169, 204: Figure 2 shows images of cell lines, not the cell counter. Please reference the correct figure here (i.e., Figure 3B).

We have rectified the error.

23. References: Please do not abbreviate journal titles.

We are not completely sure what we needed to do for this point.

We have utilized the JoVE plugin for EndNote (JoVE.ens), which was downloaded from JoVE (Please see the screen shot below). We have not altered the way journals were referenced. In addition, we checked the plugin properties and it is set to display the Journal Titles using their full journal names, and not the abbreviated version.



Reviewers' comments:

Reviewer #1:
The authors outline basic methods of storing and culturing Drosophila cell lines. There are clear and well described methods for thawing, subculturing, counting and cryopreserving cell lines as well as detailed information on required media and doubling times. If I were a novice this would be an incredibly useful resource. I have a few small comments about the text.

We thank the reviewer for the positive comments.

67 -78
As this is aimed at novices, it may be useful to include information as to which cell types these are, e.g. epithelial, adherent etc.

We have directed the reader to Table 1 for important details regarding the different cell lines.

101-104
We find it useful to thaw vials using RT media dispensed into a 10ml tube. This avoids having to change tips, and dipping the pipette in and out of the stock bottle.

We thaw the cells in a similar fashion in Protocol 1, using media pre-dispensed into a T-25 flask, instead of a 10 mL tube to thaw the frozen cells. We have edited Protocol 1, Step 3. to reflect this similarity.

116
Do you mean to thaw cells as described above, or leave the vial to thaw by itself?

Protocol 1.1 uses centrifugation to collect thawed cells. The thawing step is similar to the step 3 in Protocol 1. We have amended the sentence to reflect thawing cells by resuspending cells in room temperature media. (Protocol 1.1, Step 1).

158, 187
Some visual descriptions of common contaminations might be useful to novices - eg cloudiness, a basic description of how bacteria and yeast look under the scope.

We have added the following sentence in the discussion line 257.

“Contaminated cultures can be identified by an increase in media turbidity. Under the microscope, contaminants may appear as bacterial rods, cocci, budding yeast cells or string-like fungal hyphae.”

232
'count cells that lie on the top and left sides' is a bit confusing, as it doesn't specify that these are cells that cross the boundary. Maybe 'count cells that partially cross the top and left boundary grooves' instead?

We have edited the sentence to read as follows:

“To avoid duplicate counting, count cells that overlay the top and left boundaries, but not cells that cross the right and bottom boundaries of the 200 μm^2 squares.”

271 - 272
'Freezing container' in 4.11 becomes 'cooler' in 4.12, which may be confusing

We have kept to using the term freezing container to maintain consistency.

279
You could mention that they can be briefly stored in dry ice whilst preparing for transfer to liquid nitrogen.

We have added the following statement in the Discussion, line 455:

Alternatively, place the frozen cyrovials on dry ice while preparing for their transfer into liquid N_2.

Reviewer #2:
Manuscript Summary:
This protocol provides best practices for thawing, culturing and freezing Drosophila cell lines from an established cell culture lab.

Major Concerns:
None

Minor Concerns:
Protocol step

1.7: Mention here that 25 degree incubator does not need CO2. Mention about whether flasks should be closed or with breathable filter and humidity levels of 25 deg incubator or provide note reference to later text description.

We have added the following sentences to address the above in the discussion section, line 401.

The incubator for Drosophila cell cultures does not need to facilitate CO_2 gas exchange because *Drosophila* cell culture media do not use CO_2 for buffering. The humidity inside the incubator for culturing cell lines is an important factor not to be overlooked when culturing cells in plates. Depending on the type on incubator and the working environment, it may necessary to place a beaker of sterile water inside the incubator. To minimize media evaporation, use closed T-flasks or store culture plates/vessels are stored in a tightly sealed plastic container while inside the incubator.

1.1.1 Thawing cells be done at what temperature?

We have amended the sentence to reflect thawing cells by resuspending the frozen cell pellet in room temperature media. (Protocol 1.1, step 1).

"In a sterile hood, thaw cells in the hood by resuspending the frozen pellet with 1 mL of room temperature media. Transfer all the thawed cell suspension into a 15 mL conical tube."

1.1.2 Specify range of g's (only one mentioned but the word between is used)

We have edited Step 2 of Protocol 1.1

"Pellet the cells by centrifugation at 1000 x g for at 5 minutes."

1.6.2 Define what is considered fully confluent and whether passaging should be 100% confluent or slightly less.

In the manuscript, we avoid recommending passaging based on confluence. We use confluence status as a visual guide to estimate cell density for different cell lines. We recommend cell counting to determine cell density accurately and passage cells such that the seeding density does not fall below 1 $X10^6$ cells/ mL.

We added this paragraph in the Discussion section.

The confluence of a culture, expressed as a percentage, describes the growth surface area that is covered by cells. Cell confluence for a ~~single cell~~cell line depends on its cell shape and size. Distinct cell lines have different morphologies and adherence properties. As a result, different cell lines at approximately similar confluence may have vastly distinct cell density (Figure 1). Culture confluence may not be an ideal indicator for passaging *Drosophila* cell cultures because *Drosophila* cell lines continue to proliferate either by piling on top of one another as foci or in suspension even after the growth surface has been covered (Figure 1). However, users experienced with specific cell lines may often use confluence as a rapid visual guide for when to subculture.

Table 2: Define abbreviation for FCS here rather than Table 7.
We have defined FCS in the Table legend as suggested.

Fig. 2: Discuss the morphologies and how they are distinct for those readers who might be new to cell culture.

We have described the cell morphologies in Figure 2 legend. \

Figure 2. Representative images of the eight distinct *Drosophila* cell lines. **A** and **B.** Round embryo-derived S2-DGRC and Kc167, respectively. **C** and **D**. Round larval CNS-derived ML-BG2-c2 and spindle-shaped ML-BG3-c2, respectively. **E.** CME L1, a cell line derived from the larval leg imaginal discs, are smaller and have round/fusiform morphology. **F.** OSS, a cell line derived from adult ovaries, displays spindle-shaped morphology. **G.** Spindle-shaped Ras^{V12} cell line expressing activated Ras. **H**. Ras^{V12}; wts^{RNAi} (WRR1) cell line expressing activated Ras and double-stranded RNA targeting the tumor suppressor *warts* (*wts*) displays epithelial characteristics. Scale bar = 50 μm.

Discussion, line 363: list out the advantages.

We have reworded the sentence as follows:

Drosophila cell cultures are primary reagents for high throughput cell-based screens. Their use also ~~offers many advantages~~complements in vivo genetic research by providing: a homogenous population of cells suitable for

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biochemistry, ~~v The ability to~~ rapid testing of transgenic constructs prior to injecting into flies, cell biology, microscopy and more recently somatic cell genetic manipulations by genome editing [1-3,8-10].

Some typos were noticed, including mismatches in verb tenses and lack of prepositions. A careful read is needed.

We have proofread our manuscript for grammar and spelling.

Reviewer #3:
Manuscript Summary:

Cell lines are an important resource that make the Drosophila model ideal for investigating basic problems in cell and developmental biology. The present manuscript, from Luhur and colleagues at the Drosophila Genomics Resource Center, is an informative guide for both experienced and novice users of Drosophila cells that should further advance their use for standalone studies or as a complement to the more prevalent genetic studies.

It should be published and is very appropriate for JoVE, but below are a few questions/comments/suggestions for the authors to consider:

We thank the reviewer for the positive comments.

1. In Protocol Section (line 93), DGRC is mentioned without defining it. More importantly, a sentence in the Introduction to mention Drosophila Genomics Resource Center (DGRC) and its activities, especially as it relates to cell lines, would be useful to readers.

We added the following statements in the Introduction, line 59

The Drosophila Genomics Resource Center (DGRC) serves as a repository and distribution center for *Drosophila* cell cultures. One of the goals of the DGRC is to assist members of the research community in using *Drosophila* cell culture resources.

2. In step 1.8-1.9 of the thawing protocol, it is stated: "1.8 One to two hours later, examine the cells under the microscope to ensure that most cells have settled on the growing surface. 1.9. Remove all the media from the flask and replace with 5 mL of fresh media." In our experience, with only 2 hrs of plating, the majority of cells will not have attached, especially given the weakly adherent behavior of some Drosophila cell lines, therefore changing all media might risk the loss of many viable cells. The "1-2hrs" could be extended to 6-8hrs or even overnight to minimize cell loss, while mentioning that media change is important to remove residual DMSO. Maybe this can be addressed in a footnote or by some other means.

We have not had any problems with letting the cell sit for 1-2 hours after thawing. We agree with the reviewer's suggestion to be on the conservative side and we have added the following statement in the Representative Result section:

Under the circumstances in which most of the cells have not attached on the growing surface within two hours after thawing, it is recommended to incubate the cells overnight before changing the media.

3. In the Alternate Protocol for thawing, a mention of temperature or method of thawing the vial would be useful, eg. in waterbath, at room temp, with mixing etc.

We have edited the sentence to clearly describe thawing by resuspending the frozen pellet with room temperature media.

"In a sterile hood, t~~T~~haw the cells by resuspending the frozen pellet with 1 mL of room temperature media. Transfer all the thawed ~~t~~ the cell ~~thawed~~ suspension into a 15 mL conical tube."

4. In the Alternate Protocol for thawing cells (line 113-123), the numbering system is slightly confusing (1.1 vs. 1.1.1). Maybe "Alt1.1" or similar would be better. A similar numbering system is used in Section 2, so if changed, then a similar change should be made in Section 2.

We note this suggestion and have changed the way we number the protocol steps.
All protocol steps are numbered from Step 1 onwards.

5. When removing DMSO by centrifugation, in line 117, the authors recommend 1000xg for 10 minutes. We routinely use 5 minutes and cells are well-pelleted. Less time will minimize damage to the fragile thawed cells.

We have changed this accordingly.

6. Depending on the type of incubator and environmental conditions of the culture room, maintaining humidity can be an issue. When culturing cells in 60mm or 100mm plates, we often face problems with media evaporation. Into the closed plastic box, we usually include an open beaker or vessel with sterile distilled water to generate humidity, which should be changed periodically and handled carefully to avoid spillage. If authors think that humidity control is necessary and worth mentioning, perhaps a phrase should be included.

We agree with the reviewers that the humidity issue may depend on the type of incubator and environmental conditions. At the DGRC, we have not had the need to humidify. In addition, we have 96 plates (with parafilm) containing cells incubating for 3-4 weeks without much loss of media volume. We elaborate on this in the Discussion section, line 402.

"Note: The humidity inside the incubator for culturing cell line is an important factor not to be overlooked. Depending on the type on incubator and the working environment may be necessary to place a beaker of sterile water inside the incubator. Depending on the type on incubator and the working environment, it may necessary to place a beaker of sterile water inside the incubator. To minimize media evaporation, use closed T-flask or store culture plates in a tightly sealed plastic container while inside the incubator."

7. In line 182, there begins a subsection called "Subculturing adherent cells using trypsin in 100 mm culture plates." This is first mention of trypsin. If the article is for novices, it is worth briefly mentioning just before this section, 1) what trypsin is and why it is used and that 2) some cell lines are more adherent and require trypsin for dissociation and passage (see Table 2 for requirements). This theme is mentioned later in Discussion, but would be better mentioned ahead of the Protocol with Trypsin.

We have added a note regarding trypsin to Protocol 2.1

Note: The use of digestive enzymes such as trypsin aids in passaging strongly adherent cell lines. Trypsin is a mixture of proteases often derived from porcine pancreas and is commercially available in different grades of purity.

8. For some of the culture media formulations, "fly extract" is included, but there is no mention of what this is, how to prepare it, or where to obtain it. Some mention of this should be made, providing a suitable accessible reference or URL for novice users. This could be placed in Discussion, in paragraph which discusses media and serum (line 416). Also, regarding serum, the authors mention "reserving a batch of serum from a single lot". While this is a good idea, especially for a distributor of cell stocks, it may not be necessary or financially feasible for occasional users.

We have added a paragraph in the Discussion regarding fly extract. We have also removed the statement regarding serum batch reservation.

Media additions for most *Drosophila* cell lines include fetal calf serum (FCS). Insulin and adult fly extract (FEX) are required for some specific lines. FEX contains undefined components essential to the growth of specific larval imaginal disc lines and the adult ovarian cell lines. of The DGRC prepares, and makes available adult FEX derived from one-week old Oregon-R-modENCODE flies (~~RRID:BDSC~~RRID: BDSC_25211) in 2.5 mL and 10 mL aliquots. The DGRC also provides instructions for small scale FEX preparation on its website <https://dgrc.bio.indiana.edu/include/file/additions_to_medium.pdf>. FEX preparation, however, is time-consuming and requires a large quantity of adult flies.

9. There is no mention of the use of "conditioned media". We have encountered protocols that recommend production and use of conditioned media to facilitate recovery from freezing and encourage proliferation when culturing at lower density (e.g. when selecting stable populations or clones). Perhaps the authors can include some words about the conditioned media and whether or not it is helpful or recommended.

We have added the following sentences discussing the use of conditioned media (line 377).

For cell lines that are slow to recover from the effects of thawing, the use of conditioned media may be useful. Culture media conditioned by a cell line likely contains growth factors secreted by the cells into the media which may encourage the recovery and proliferation of the cells after thawing.

10. In line 422, the authors mention the preparation of a working stock of "50-100 ampules". Again, for a distribution center, this may be a reasonable number, but for novice and/or occasional users, 10-20 ampules may be sufficient. Maybe text can be revised to reflect that numbers/quantities can be adjusted according to needs.

We have revised the quantity to better reflect the need for the occasional user/novice.