Time-Lapse Live Imaging of Stem Cells in *Drosophila* Testis

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ABSTRACT

This unit describes a protocol for time-lapse live-imaging of stem cells in *Drosophila* testis. Testis tips are dissected from *Drosophila*, sliced, and transferred to glass-bottom chambers where the stem cells residing in their native microenvironment can be monitored in real time. This protocol, facilitated with various fluorescence-labeled markers, allows dynamic cellular processes in stem cells to be characterized throughout the cell cycle. *Curr. Protoc. Stem Cell Biol.* 11:2E.2.1-2E.2.8. © 2009 by John Wiley & Sons, Inc.

Keywords: *Drosophila* gonad • stem cell • tissue culture • time-lapse live imaging • epifluorescence microscopy

INTRODUCTION

The *Drosophila melanogaster* male gonad has been one of the best model systems to study stem cell biology due to its easily identified anatomy and well-studied signaling pathways. Using this system, many important questions in stem cell biology have been addressed by studying fixed samples. However, the information that can be deduced from fixed samples is limited and potentially ambiguous when reconstructing a dynamic process from static images. This unit describes a protocol of performing time-lapse live imaging of *Drosophila* testes, which can be used to study the migration patterns of cells or cellular organelles throughout the cell cycle. We focus on the detailed procedures for performing time-lapse live-cell imaging with an inverted epifluorescence microscope, and then describe the method of dissecting and tissue-culturing *Drosophila* testes in glass-bottom chambers in the Support Protocol.

NOTE: This unit assumes that readers have the basic knowledge of laboratory culture of *Drosophila*; for a more detailed account on *Drosophila* culture, see Roberts (1998) and Greenspan (2004).

TIME-LAPSE LIVE IMAGING OF *DROSOPHILA* TESTES

BASIC PROTOCOL

This protocol describes the general procedure of performing time-lapse live imaging of *Drosophila* testes in the glass-bottom culture chamber.

Materials

Culture of *Drosophila* testes tips in petri dishes (Support Protocol)
A high-quality inverted microscope with epifluorescence capability (e.g., Zeiss Axiovert 200)
3-axis computer-controlled microscope stage (e.g., Madcity Labs)
Automated shutter in the epifluorescence light path (e.g., Uniblitz)
Plan-NEOFLUAR 40× objective with NA = 0.75 or AchroPlan 63× objective with NA = 0.8
A highly sensitive CCD camera [e.g., Hamamatsu Electron multiplier (EM) CCD camera]
Computer with software for controlling shutter, specimen stage, and image acquisition (e.g., Metamorph or ImageJ)

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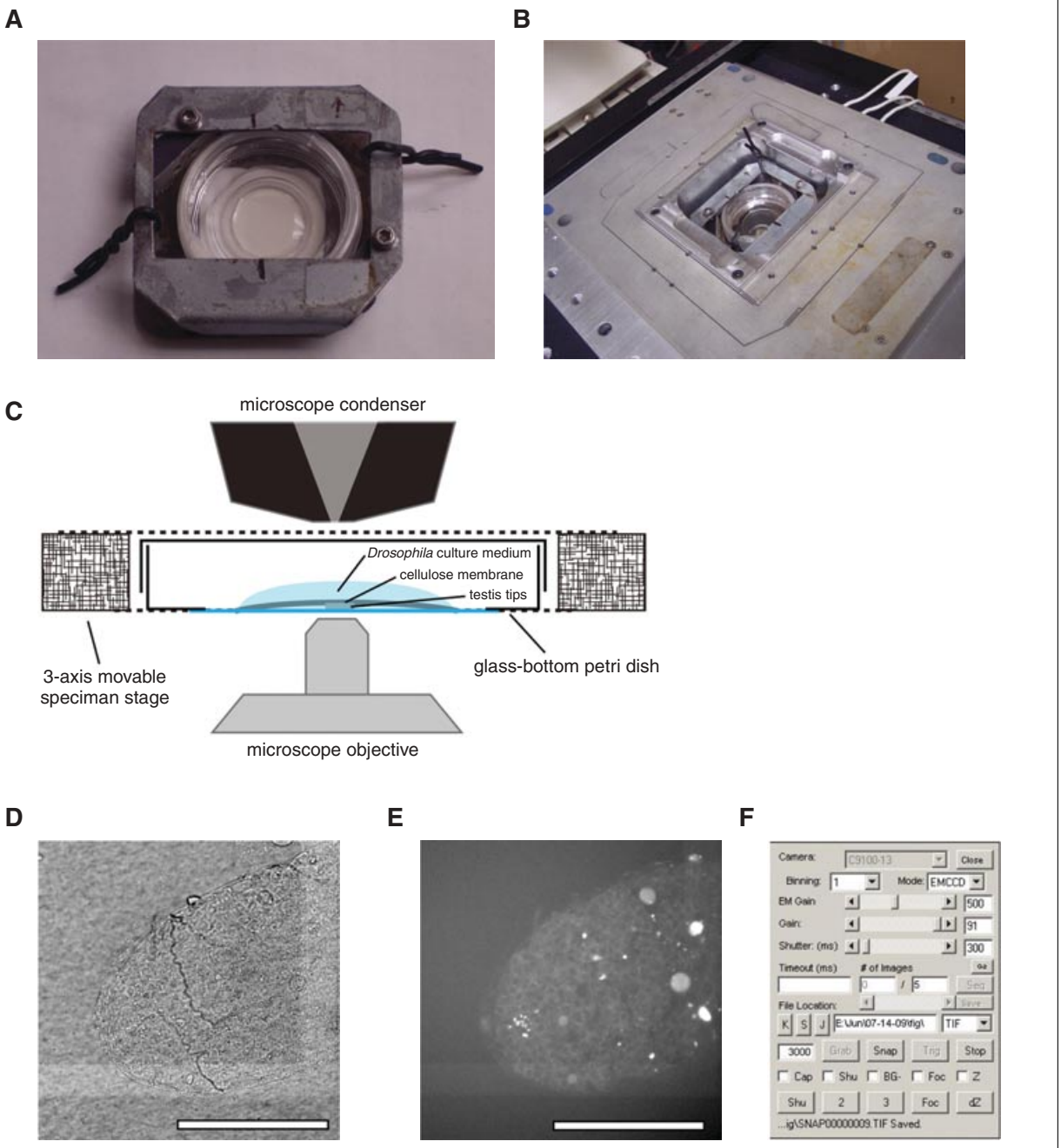

Figure 2E.2.1 Time-lapse live imaging setup. (**A**) The petri dish is placed into the chamber holder and assembled. (**B**) The chamber holder is transferred into the microscope specimen stage. (**C**) Schematic of the microscope setup, illustrating the relative positions of the sample chamber, the 3-axis specimen stage, the microscope objective, and the microscope condenser. The brightfield image (**D**) and the epifluorescence image (**E**) of the *Drosophila* testis tip of mCherry-Sas6 flies. Scale bar = 100 μm. (**F**) Snap shot of EM-CCD setting parameters for mCherry-Sas6 flies.

NOTE: 40× NA = 0.75 and 63× NA = 0.8 objectives provide the necessary depth of field while maintaining sufficient resolution and epifluorescence intensity.

NOTE: To prevent any photo-bleaching and potential photodamage to the testis tissue, exposure time is minimized. To achieve this goal, a highly sensitive CCD camera and a fast-response shutter in the epifluorescence light pathway are necessary. Moreover, software control of the shutter and CCD camera is required to synchronize the shutter open/close with the timing of the image acquisition.

1. Place the petri dish with testes (Support Protocol) into the chamber holder (Fig. 2E.2.1A).
2. Place the chamber onto the 3-axis specimen stage (Fig. 2E.2.1B,C).
3. Select the target testis with bright-field microscopy (Fig. 2E.2.1D).
4. Adjust the focus by imaging the target testis with epifluorescence microscopy (Fig. 2E.2.1E).

   Open and close the shutter promptly to minimize the exposure time when adjusting the focus.

5. Set the proper exposure time and gain for the EM-CCD camera (Fig. 2E.2.1F).

   To minimize photo bleaching and maintain constant brightness and contrast for fluorescent images over an extended time, the exposure time should be set as short as possible. Although increased camera gain can partially compensate for decreased exposure time, the gain should not be set unnecessarily high, as it increases background noise and EM-CCD dark current. For example, the EM gain is set at 500 and exposure time is set at 300 msec for mCherry-Sas6 fly testis (Rusan and Peifer, 2007).

6. Set the interval between exposures.

   The exposure interval should not be set shorter than necessary; otherwise, photo bleaching may be a problem. The proper exposure interval depends on several factors, at least including the timescale of cellular processes, the stability and brightness of the imaged fluorescent proteins, and the exposure time set in the previous step. For example, the exposure interval is set at 2 min for mCherry-Sas6 fly testis.

7. Start acquiring the image sequence. Multiple image planes can be followed (XYZT sequence) by taking images at different *z*-focal planes.

   The movement of the 3-D specimen stage can be controlled using custom-designed software, or integrated with standard imaging packages (Metamorph, ImageJ), and is synchronized with the shutter open/close and image acquisition. Next, the acquired images can be processed and analyzed depending on the experiment. For example, 4-D image sequences (x, y, z, and t) are acquired for mCherry-Sas6 fly testis, and then the centrosome locations (labeled by mCherry-Sas6) are tracked by semi-automatic tracking software (Cheng et al., 2008).

DISSECTING AND TISSUE-CULTURING *DROSOPHILA* TESTES

SUPPORT PROTOCOL

This protocol describes how to dissect *Drosophila* testes and how to tissue-culture the testis tips in the glass-bottom petri dish.

Materials

70% ethanol
Drosophila culture medium (DCM; see recipe)
Drosophila male flies

Regenerated cellulose membrane (Spectrum Lab)
Carbon dioxide flowbed (Genesee Scientific)
Stereomicroscope (Leica)
Standard dissecting equipment including:
- Forceps (Dumont #5)
- Scalpel (Feather #15)
- Scissors

35-mm glass-bottom Petri dish with 20-mm microwell (MatTek)
Parafilm

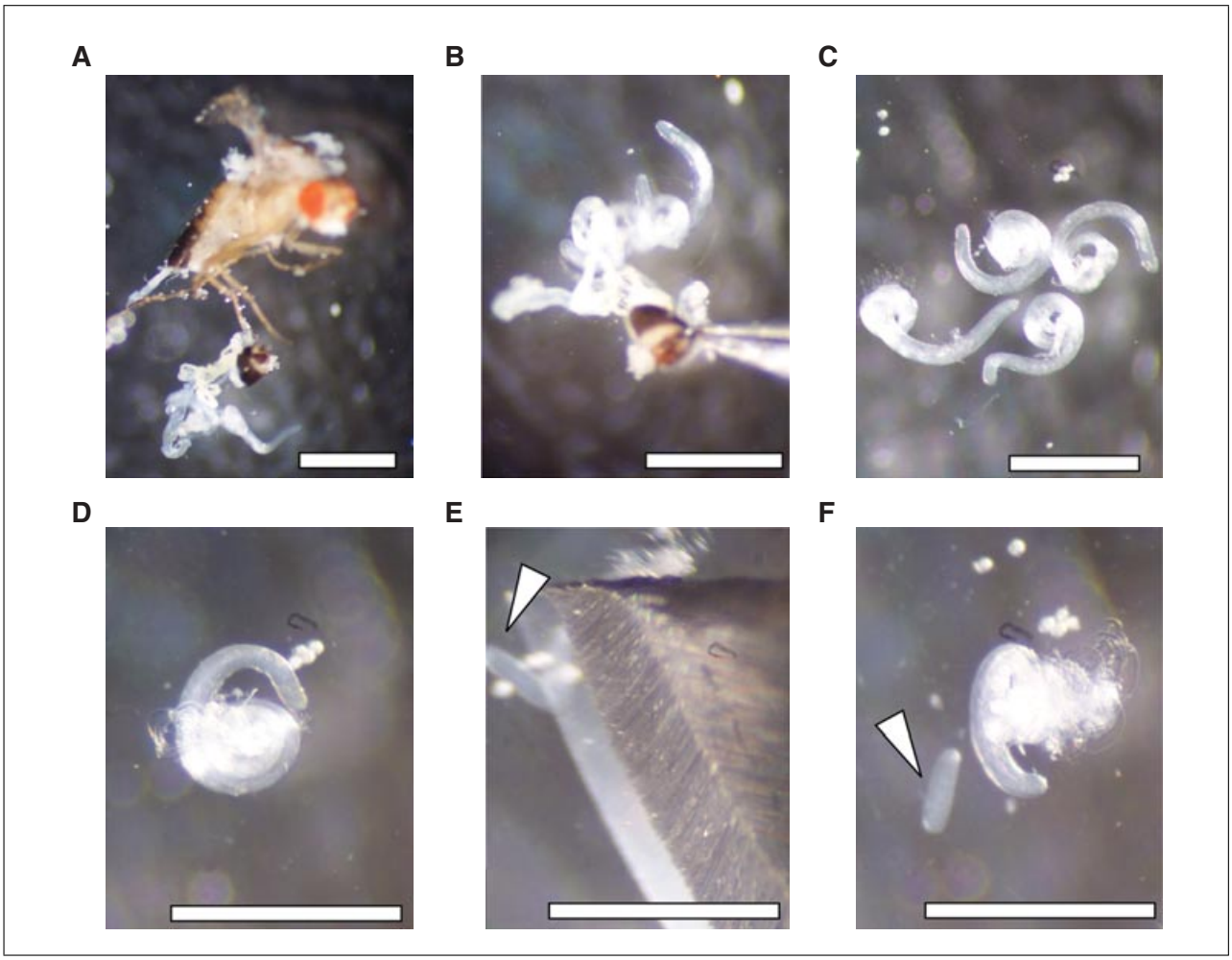

Figure 2E.2.2 *Drosophila* testis tips are prepared for tissue culturing. (**A,B**) Male *Drosophila* is dissected and testes are removed in DCM. (**C,D**) Dissected testes are transferred to new DCM. (**E,F**) Testis tip (arrowhead) is cut by scalpel. Scale bars = 1 mm.

Prepare for dissection

1. Sterilize all dissecting equipment with 70% ethanol.

   Sterile dissecting equipment is critical for maintaining a healthy tissue culture condition.

2. Cut regenerated cellulose membrane into 16-mm diameter circles and soak them in DCM at 4°C for 24 hr prior to use.

   The circular regenerated cellulose membranes can be stored in DCM up to 7 days at 4°C.

3. Warm DCM to room temperature before use.

   Prewarming DCM minimizes the temperature shock to Drosophila testes.

Dissect and cut Drosophila testes

4. Anesthetize the fly on a carbon dioxide flowbed.

5. Dissect testis out of the fly abdomen under a stereomicroscope in prewarmed DCM (Fig. 2E.2.2A,B)

   DCM instead of PBS is used here to minimize the physiological stress on testis tissue during dissection.

6. Transfer the testes into fresh DCM (Fig. 2E.2.2C,D).

7. Cut off the testis tip with a scalpel (Fig. 2E.2.2E,F) and use the testis tip for experiments.

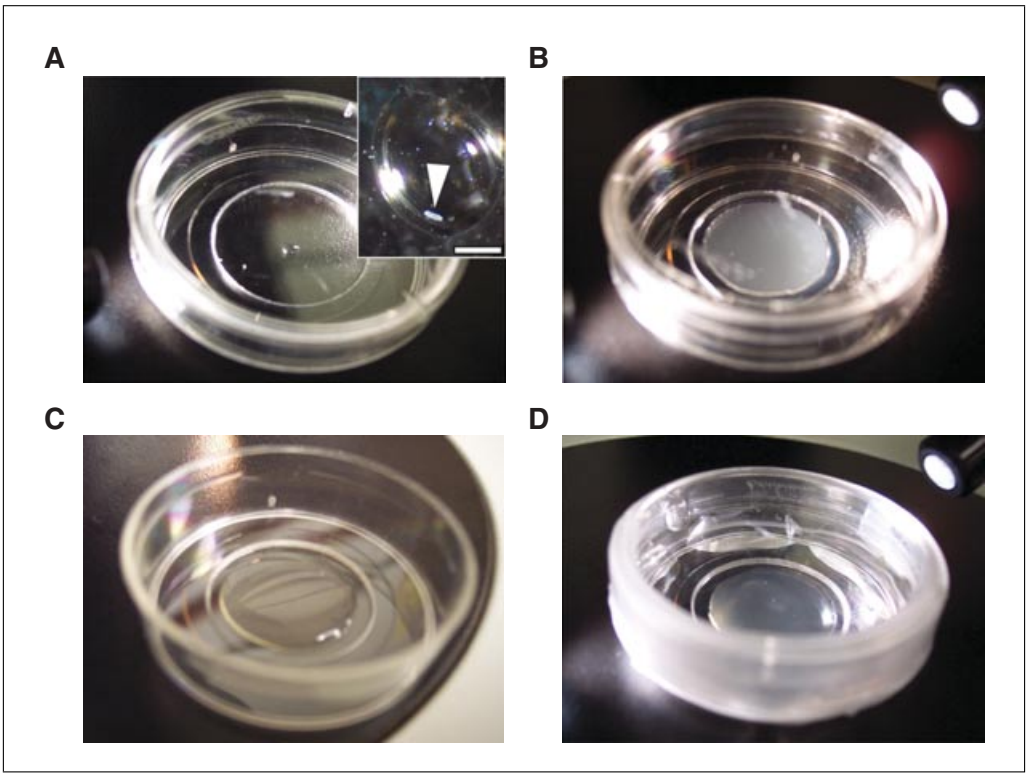

Figure 2E.2.3 Tissue culturing of *Drosophila* testis tip in the glass-bottom petri dish. (**A**) *Drosophila* testis tip is transferred to glass-bottom petri dish in DCM. Inset: a zoom-in view of the testis tip in a drop of DCM. Scale bar = 1 mm. (**B**) The testis tip is covered by the DCM-presoaked circular regenerated cellulose membrane. (**C**) DCM is added on the top of the membrane. (**D**) Additional DCM is added in the petri dish, and the dish is sealed with Parafilm.

Using only the testis tips for experiments eliminates the peristaltic motion associated with the testis tube.

Culture Drosophila testis tip in the glass-bottom chamber

8. Transfer the testis tip into the glass-bottom petri dish (Fig. 2E.2.3A).

   It may be beneficial that several testis tips are prepared and transferred into one chamber, and then the one with best imaging quality is selected for the experiment.

9. Cover the testis tips with DCM-presoaked circular regenerated cellulose membrane (Fig. 2E.2.3B).

   The membrane prevents testis tip from floating in the DCM while allowing exchange of dissolved gases and nutrients to sustain the testis tissue.

10. Carefully add 200 to 300 µl DCM to cover the regenerated cellulose membrane (Fig. 2E.2.3C).

    DCM is added to provide nutrients. By adding DCM on the top of the regenerated cellulose membrane, the membrane is prevented from floating, thus pressing down and immobilizing the testis tissue.

11. Slowly add another 700 to 800 µl DCM inside the periphery of the petri dish, and seal the dish with Parafilm (Fig. 2E.2.3D).

    Additional DCM inside the petri dish and the seal by Parafilm prevent changes in the concentration of DCM due to evaporation during long-term observation.

    Testis tips can be kept in the chamber for up to 20 hr in the dark at room temperature before commencing the time-lapse live-imaging experiment.

REAGENTS AND SOLUTIONS

For culture recipes and steps, use sterile tissue culture–grade water. For other purposes, use deionized, distilled water or equivalent in recipes and protocol steps. For suppliers, see ***SUPPLIERS APPENDIX.***

Drosophila culture medium (DCM)

Schneider's *Drosophila* medium (Invitrogen, cat no. 11720-034)
10% (v/v) fetal bovine serum (FBS; Lonza, cat no. 14-501E)
50 U/ml penicillin
50 μg/ml streptomycin
Store up to 6 months at 4°C

COMMENTARY

Background Information

With remarkable prolonged self-renewal ability and the potency to differentiate into specialized cells, stem cells have been the subject of intensive study for their potential medical applications (Morrison et al., 1997; Watt and Hogan, 2000; Morrison and Kimble, 2006). This self-renewal capability also confers on stem cells an intrinsic risk of cancer formation (Groden et al., 1991; Pece et al., 2004; Singh et al., 2004; Clevers, 2005; Clarke and Fuller, 2006). Indeed, a subset of cancer cells has been shown to have stem cell characteristics (Al-Hajj et al., 2003; Singh et al., 2004). On the other hand, excessive differentiation of stem cells is believed to contribute to tissue degeneration and ageing (Van Zant and Liang, 2003; Kirkwood, 2005; Rando, 2006; Brunet and Rando, 2007). Therefore, a better understanding of the regulatory mechanisms balancing self-renewal and differentiation offers the possibility to provide new perspectives on treating cancer and ageing-related diseases. Local microenvironments known as niches govern the fates of the stem cells in many systems (e.g., gonads, hematopoietic system, skin and gut epithelium; Watt and Hogan, 2000; Spradling et al., 2001; Fuchs et al., 2004), and in these tissue architectures, regulatory signals secreted by the niche cells regulate the adjacent stem cells' self-renewal ability and suppress their differentiation.

With well-developed genetic manipulation techniques, *Drosophila melanogaster* has been one of the most commonly used model organisms in biological research for over a century. Moreover, the stem cell niche in the *Drosophila* testis is among the best characterized in signaling pathways and anatomy (Brinster, 2002; Lin, 2002; Fuller and Spradling, 2007), offering an ideal model system to study stem cell regulatory mechanisms in the niche microenvironment. Hub cells, a major component of the stem cell niche, are located at the apical tip of *Drosophila* testis and are surrounded by two different types of stem cell populations: germline stem cells (GSCs) and somatic stem cells [i.e., cyst stem cells (CySCs)]. Hub cells specify the stem cell identity of both GSCs and CySCs by secretion of the ligand Unpaired (Upd), which activates the JAK-STAT signaling pathway (Kiger et al., 2001; Tulina and Matunis, 2001; Leatherman and Dinardo, 2008). Each GSC is encapsulated by a pair of CySCs, and this germ-soma important interaction has been shown to play a role in guiding stem cell self-renewal and differentiation (Kiger et al., 2000; Tran et al., 2000; Schulz et al., 2002; Leatherman and Dinardo, 2008). Because of the importance of the interactions among different cell types in regulating functions and fates, the native stem cell niche in *Drosophila* testis instead of individual cell lines must be cultured and maintained to observe and study normal physiological behavior.

Due to the technical challenges of observing stem cells alive in their native niche environment in *Drosophila* testis, the biomechanical and morphological understanding of stem cell division and mitotic spindle formation had been mostly inferred from examinations of fixed samples. Although these approaches have revealed a great deal, they are limited and potentially misleading due to reliance on reconstructing the dynamics of biomechanical and morphologic events from loosely correlated static images. Following the protocol outlined in this unit, healthy stem cells within the intact niche from *Drosophila* testis can be maintained for extended time (at least 24 hr), providing a means to study new processes in stem cell biology by time-lapse live imaging. This described protocol was first applied to study the centrosome orientation dynamics in GSCs (Cheng et al., 2008).

Critical Parameters and Troubleshooting

The workstation and all dissecting equipment must be maintained under good sterile conditions to prevent potential contamination of the cultures. Prior to use, the DCM should be warmed up to room temperature to minimize any temperature shock to the tissue. Extra care must be taken when applying DCM onto the regenerated cellulose membrane. Either applying too much DCM or applying DCM too vigorously may float the membrane, and thus the membrane would not serve the function of immobilizing the testis tissue.

Exposure time, interval, and total observation time must be coordinated with the characteristics of the targeted fluorescent protein, and every effort must be taken to shorten exposure time and lengthen exposure interval to avoid the potential problems associated with photobleaching and photodamage. For extended periods of observation (e.g., overnight), microscope drift can be a serious challenge. We find drift is most easily suppressed by taking steps to maintain constant temperature in the microscope room.

Anticipated Results

This protocol is applicable to study various dynamic processes when relevant molecules are marked by fluorescence proteins. The maintenance of the healthy culture condition over extended time makes it possible to monitor a particular cellular process throughout the cell cycle. Furthermore, we anticipate that this protocol may be modified to obtain time-lapse live imaging of other tissue types from *Drosophila* or other organisms, providing a complimentary means of studying broader biological questions.

Time Considerations

To estimate the time necessary for this protocol from dissecting flies to taking time-lapse live imaging, the following four steps need to be taken into account: material preparation, *Drosophila* dissection and testis preparation, sample chamber preparation, and time-lapse live imaging. In the material preparation, the regenerated cellulose membrane should be cut and soaked in DCM 24 hr before experiment. With experience, it generally takes ~2 min per fly to perform *Drosophila* dissection and testis preparation. Five min may be needed to prepare the sample chamber. The duration of time-lapse live imaging is selected in accordance to the experiment.

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