

## Live imaging with *Drosophila* tissue culture cells (PROT25)



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### Introduction

Live imaging provides an important complementation to the "snapshot" view obtained in fixed tissue by immunofluorescence. It allows following dynamic cellular processes as they unfold "in vivo", and often reveals a degree of complexity impossible to study in still images. Thanks to the increasing interest in this technique, significant technical improvements have been made in the recent years in microscopy leading to more sensitive and faster cameras, more efficient filter sets and a variety of different microscope systems to choose from. The temporal dimension of cellular processes in microscopy is still often neglected due to the difficulties in technical setup and the necessity to fabricate your own tools. Now the majority of the required equipment is commercially available from microscope manufacturers and related companies including environmental chambers, CO<sub>2</sub>-control and temperature control.

Often used in conjunction with time-lapse microscopy are fluorescent proteins, such as the Green Fluorescent Protein (GFP) or the Red Fluorescent Protein (RFP; Shaner *et al.*, 2004; Shaner *et al.*, 2005; Wiedenmann *et al.*, 2004) and live dyes. Likewise this is a rapidly developing field of research, which has provided a variety of different "flavors" of fluorescent proteins for the scientific community, with properties specifically suited for different live-imaging applications (for a guide to choose the right one, see Shaner *et al.*, 2005).

### Procedure

#### Cells and Fluorescent Proteins

Growth conditions, transformation and selection of stable cell lines are as described (Cherbas *et al.*, 1994) using 100µg/ml hygromycin as a selection reagent. The following protocols can be used with most *Drosophila* tissue culture cells as long as they are semi-adherent.

I tested a variety of different *Drosophila*

Schneider S2 cells for their growth behavior and found that most of them had some difficulties undergoing and completing cytokinesis, producing membrane bubbles and remaining attached for a long time. These phenotypes were least pronounced in a Schneider S2 cell line called L2-4, which I have used for most applications (obtained from the Botchan Lab, 171 Koshland Hall, UCB, Berkeley, CA, 94720, USA).

- Fluorescent proteins (FP's) like eGFP (CLONTECH) represent a good choice for the green and mCherry for the red channel (Shaner *et al.*, 2004). Depending on the properties of the protein of interest, the FP's can be used as an N-terminal or C-terminal fusions and in general are non-toxic to the cells.
- FP's fused to histones (e.g. Histone H2B) are a good alternative for a chromosome counter stain. Hoechst 33342 and Draq5 are DNA specific live dyes, that don't stain significantly other nucleic acids. Unfortunately they are toxic to *Drosophila* tissue culture cells after prolonged exposure (> 2 hours) at concentrations that give a reasonable signal to noise ratio. Moreover the UV light to excite Hoechst 33342 dye is also damaging to the DNA in contrast to the lower wavelengths used for the FP's or Draq5.
- Although live imaging can be performed on transiently transfected cells, a significant proportion of cells displayed tri-polar spindles in mitosis (~30% vs. 5% in untransformed or stable cells), thus it is advisable to use stably selected cell lines for studies in mitosis.

### Growth and cell preparation

Cells are plated at  $1 \times 10^6$ /ml in Schneider's Medium (GIBCO/Invitrogen), 10% FCS, Antibiotics (Sigma A5955; used at 33x instead of 100x) at 25°C and used two days later, when the cell culture should be exponentially growing with a density of  $\sim 3 \times 10^6$ /ml (generation time is  $\sim 23$  hours).

- A. This is a simple method for live imaging of Schneider S2 cells for short periods of time (< 6 hours), and can be used on both upright and inverted microscopes (see [note 1](#), [note 2](#) and [comment 1](#)):
  1. 22x22mm coverslips and special glass slides with a circular depression are cleaned with 70% Ethanol. Vacuum grease is applied to the rim of the circular depression of the slide. Due to the short time of imaging, sterility is less of an issue here;
  2. 10 $\mu$ l of cell suspension are added to the center of the coverslips;
  3. The depression slide is put on top to "sandwich" the drop as described for the "hanging drop" method (see [figure 1](#); [comment 2](#); Heun *et al.*, 2006; Shields and Sang, 1970);
  4. Cells are allowed to settle for  $\sim 10$  minutes before imaging.
- B. For time-lapse imaging of cells over multiple days the following protocol can be used (only for inverted microscopes) (see [note 3](#)):
  1. Culture dishes or slides with coverslip bottom are used. Some providers produce round culture dishes (e.g. Willco Wells B.V.®) or 8-well or single well culture slides (e.g. Nunc™ or ibidi®, see [figure 2](#));
  2. To follow cells for multiple days it is advisable to pre-treat the culture dishes or slides with Poly-L-Lysine to minimize cellular movement. In short the slides or dishes are incubated 30 minutes in 0.01% Poly-L-Lysine, drained and allowed to dry for 1 hour at 60°C or at RT overnight;
  3. Particularly helpful for overnight experiments is the autofocus option. It is part of many microscope-controlling software (Metamorph®, Velocity®, softWoRx® etc.) and can be setup to refocus after longer time-intervals (e.g. once every hour). Also the cell tracking option can be useful if cells move a lot over time, although high cell density can confuse the program.

## Image acquisition and analysis

The protocol presented here is optimized for a Deltavision Spectris® and Deltavision RT® system using an inverted Olympus™ IX71 microscope. This microscope system is based on wide field microscopy. It uses an algorithm to calculate and remove the out-of-focus light in a process called deconvolution. The advantage of this and similar systems compared to Laser Confocal Microscopy is a reduced risk of photo damage to the cells and the possibility to follow a larger field of cells. In contrast these systems are limited for imaging objects with depths > 30 $\mu$ m, because of high amounts of scattered out-of-focus light. A comprehensive summary for different options of image acquisition and choice of microscope is given in protocol 15 on <http://www.epigenome-noe.net/researchtools/protocols.php> (Neumann *et al.*).

- Time-lapse microscopy is done by taking z-stacks with step sizes from 0.2-1 $\mu$ m for each channel using an Olympus™ Planapo 60x oil-immersion objective for  $\sim 3$  hours. Exposure times, number of z-sections and time-intervals between the image acquisitions depend on the signal to noise ratio of the fluorescence and the time resolution required to follow the cellular process of interest. These conditions have to be determined empirically for different applications. To follow a cell through mitosis I found it sufficient to take 7 z-stacks of 1 $\mu$ m step size for each channel once a minute. Under these conditions exposure time should not exceed more than 100 ms in the green (eGFP) and 50 ms in the red channel (mRFP) to reduce bleaching and photo damage (see [note 4](#) and [note 5](#)) (an example of results that can be obtained upon observation times of about 3 hours is shown in [figure 3](#)).
- *Drosophila*  
Schneider S2 cells are fairly sensitive to light. To observe cells for > 3 hours the number z-stacks or the exposure time have to be reduced or time-intervals between images should be increased empirically to minimize photo-toxicity. For longer periods of time-lapse analysis, I increased the time intervals to one z-stack every 5 minutes.
- Images are deconvolved on a softWoRx® analysis workstation. If 3D information is not part of the analysis, deconvolved time-lapse movies are quick projected to facilitate the analysis in 2D and for display purposes. Bleaching of the FP's can be significant over time and often requires auto scaling to display the images with approximately similar brightness from beginning to end. Metamorph® allows this with very acceptable results, while other software's tested (softWoRx®) perform less convincingly.
- For analysis in 3D, many software packages (Metamorph®, Bitplane Imaris®, Velocity®) allow the taking of a variety of different measurements such as the distance between two points of interest, volumes, distance traveled over time, etc. Whenever possible image analysis should be done as automated as possible to reduce the possibility of human error and bias towards expected results. A high image quality (= high signal-to-noise ratio) is a prerequisite for automated tracking, counting of objects and position information based on thresholding algorithms. However in reality lower image quality or high image complexity continues to present difficulties for commercially available image analysis software and needs to be done manually on a frame-to-frame basis.

## Materials & Reagents

- *Drosophila* Schneider S2 cells
- Schneiders Medium (GIBCO/Invitrogen), 10% fetal calf serum, Antibiotics (Sigma A5955)
- Depression slides (Karl Hecht GmbH)

- Coverslips No. 1.5 (thickness on average 170  $\mu\text{m}$ ) are optimal for the Olympus™ microscopes and also recommended by other microscope manufacturers.
- Culture slides/dishes (ibidi®, Lab-Tek™ Chambered Coverglass/Nunc™, Wellco Wells®)
- Optional: 0.1% Poly-L-Lysine Stock (Sigma, P8920)
- Optional: Concanavalin-A (Sigma, C2010-25MG)
- Deltavision Spectris® and Deltavision RT® system using an inverted Olympus™ IX71 microscope.
- Filter sets from Chroma™ or Semrock™ (Sedat filter set).
- Temperature controlled environmental chamber from Solent Scientific™, PeCon™, Zeiss™ or Olympus™.
- Image analysis software used (softWoRx® Explorer, Applied Precision™, Bitplane Imaris®, Metamorph®, Microsoft Excel™).

## Authors Notes

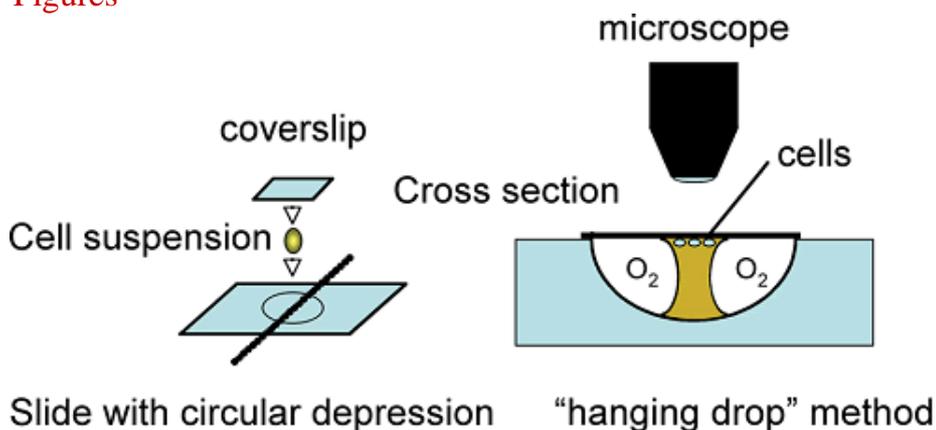
1. Cells keep dividing under these conditions for at least a day. For longer periods of time, cells should be grown in culture dishes with coverslip bottoms (see Protocol B for long periods of time).
2. Schneider S2 cells are semi-adherent, so specific treatment of the coverslips is not required. If cells should be immobilized further, acid-washed coverslips can be coated using 0.01% Poly-L-Lysine Solution (see Protocol B). If flattened cells are desired, acid-washed coverslips should be coated with Concanavalin-A. Acid washed 22x22mm coverslips that are treated with 100 $\mu\text{l}$  0.5mg/ml concanavalin A (Sigma) in water and allowed to air dry (Rogers *et al.*, 2002). However many cells fail to complete cytokinesis and become bi-nucleated under these conditions (see [comment 1](#)).
3. In all these experiments stable conditions around the microscope are mandatory as slight deviations in temperature can change the focus dramatically. This can be achieved separating the microscope from strong airflow (e.g. in climatized rooms through curtains - also useful to create a dark partition of the room) and the use of an environmental chamber that encloses most of the microscope. A constant temperature in a temperature-controlled chamber can only be guaranteed if the microscope room is at least 5°C lower than the chamber temperature. Alternatively, but less precise is the use of a heated stage with temperature control. Unless the environmental chamber can be both cooled and heated, the above-described setup almost always requires an air-cooling system in the room.
4. Culture wells mounted on slides have the advantage, that slides can be positioned in a reproducible manner on the microscope stage. This allows taking the slide on and off and identifying previously observed cells by coordinates.
5. The use of the Olympus™ Planapo 60x objective is preferable over the 100x objective, because it is more light sensitive yet provides a decent optical resolution to visualize substructures in the cell (chromosomes, nuclear bodies, microtubules).

## Reviewer Comments

Reviewed by: [Helder Maiato](#), Institute for Molecular and Cell Biology, University of Porto, Portugal.

1. The use of the hanging drop method to leave oxygen available may just be important for long-term recordings. An alternative for short-term recordings (up to 6 hours) would be to use closed chambers (like Rose chambers) full with medium.
2. An alternative to Poly-L-Lysine, which just helps to stick S2 cells to the glass, and to Concanavalin-A, which flattens cells by using membrane receptors and may compromise cytokinesis, is to use S2R+ cells. This clone was originally isolated by Imogene Schneider (Schneider, 1972) and grows adherently on glass without any coating and as a loose monolayer, predominantly epithelial-like in appearance. As opposed to regular S2 cells, this clone expresses both Wingless receptors Dfrizzled-1 and Dfrizzled-2, hence the new nomenclature of "S2 receptor plus" (S2R+; Yanagawa *et al.*, 1998). Another alternative to image flat S2 cells for short periods (2-3 hours) is to use an agar overlay (Maiato *et al.*, 2004). For some purposes, this method results better than all the others, since it works based on the surface tension of the fluids and a pressure gradient that allows the visualization of cells that are flat enough for microscopy, without compromising cell viability.

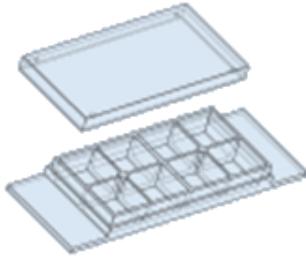
## Figures



**Figure 1.**

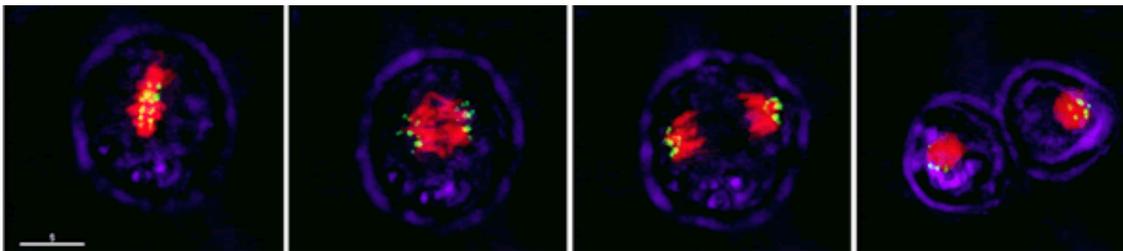
Mounting semi-adherent cells according as a "hanging drop" on a circular depression slide for live-imaging on both upright or

inverted microscopes.



**Figure 2.**

Example of an 8-well culture slide, where the bottom is made as thin as a cover slip. The plastic bottom is only 180 $\mu$ m thick and very similar to the cover slip thickness of No. 1.5 cover slips with  $\sim$ 170 $\mu$ m average). Such slides can be used for growing cells over long periods of time and for imaging on an inverted microscope at the same time.



**Figure 3.** Different frames from time-lapse microscopy are shown for CID-GFP (*Drosophila* CENP-A, centromere marker) coexpressed with H2B-mRFP (chromosome counter stain) at 1, 64, 83 and 138 minutes. Purple indicates cell outlines taken by transmission light.

## References

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