Topic Introduction

Live Cell Imaging in *Drosophila melanogaster*

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INTRODUCTION

Although many of the techniques of live cell imaging in *Drosophila melanogaster* are also used by the greater community of cell biologists working on other model systems, studying living fly tissues presents unique difficulties with regard to keeping the cells alive, introducing fluorescent probes, and imaging through thick, hazy cytoplasm. This article outlines the major tissue types amenable to study by time-lapse cinematography and different methods for keeping the cells alive. It describes various imaging and associated techniques best suited to following changes in the distribution of fluorescently labeled molecules in real time in these tissues. Imaging, in general, is a rapidly developing discipline, and recent advances in imaging technology are able to greatly extend what can be achieved with live cell imaging of *Drosophila* tissues. As far as possible, this article includes the latest technical developments and discusses likely future developments in imaging methods that could have an impact on research using *Drosophila*.

RELATED INFORMATION

Specific protocols are available for the **Isolation of *Drosophila* Egg Chambers for Imaging** (Parton et al. 2010a), **Collection and Mounting of *Drosophila* Embryos for Imaging** (Parton et al. 2010b), ***Drosophila* Macrophage Preparation and Screening** (Parton et al. 2010c), and ***Drosophila* Larval Fillet Preparation and Imaging of Neurons** (Parton et al. 2010d). Additional information regarding the properties and uses of fluorescent probes is provided in **Fluorescent Protein Tracking and Detection: Fluorescent Protein Structure and Color Variants** (Rizzo et al. 2009a) and **Fluorescent Protein Tracking and Detection: Applications Using Fluorescent Proteins in Living Cells** (Rizzo et al. 2009b).

OVERVIEW

The fruit fly *Drosophila melanogaster* remains one of the most important model systems for basic research into the molecular mechanisms underlying cell function and development. It is also a major biomedical research tool: Approximately 75% of all human diseases that have been attributed to single genetic loci have homologous genes in *Drosophila* with similar functions (Botas 2007; Chintapalli et al. 2007). Indeed, many *Drosophila* organ systems and tissues perform similar basic functions to their equivalent human counterparts, and their developmental regulation involves highly related molecular mechanisms and pathways. This makes flies excellent models for numerous aspects of human physiology and disease states ranging from drug addiction to cancer metastasis. *Drosophila* is, arguably, the best understood complex multicellular organism, and this is particularly evident in the study of nervous system development and adult function (Clyne and Miesenböck 2008).

The success of *Drosophila* as an experimental system stems from numerous factors (summarized in Table 1). However, perhaps the most spectacular advantage of *Drosophila* is the ability to apply live cell imaging to an extensive variety of living tissues and organs that can be dissected and imaged in vivo, ex vivo, or in vitro (Table 2). The ease of dissection and the flexibility of the genetic tools mean that, in many cases, it has been possible to combine powerful genetic screens with live cell imaging assays to identify new components in developmental pathways (Martin et al. 2003; Ryder et al. 2009).

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Table 1. *Drosophila* as a model organism

Background	Large community of highly cooperative investigators and communal genome efforts easily accessible via FlyBase website (Drysdale and FlyBase Consortium 2008) Twelve sequenced drosophilid genomes Ready availability of mutant lines History of the application of imaging techniques to fixed and live material Online protocols available (see "Web Resources")
Size and diversity of tissue types	Small; easy to culture in useful quantities in the laboratory Prolific breeders with short life cycle (~10 d at 25°C) Diversity of most complex tissue types found in mammals, but more accessible to manipulation Good for imaging: small enough for whole organisms to be examined under microscope; large enough to isolate individual tissues (e.g., embryos, ~150 × 150 × 400 µm) Giant salivary glands with polytene chromosomes (easily identified bands); simple karyotype (four chromosomes)
Genetically tractable	Many simple genetic screens developed over many years have allowed identification of new mutations, and they continue to do so. Fluorescent trap screens Many genetic tricks, such as *P*-element transformation, germline clones, somatic clones; many tissue-specific expression lines; very easy to perform RNA interference (RNAi) on tissue-culture cells (Venken and Bellen 2007) A variety of existing fluorescent-protein-expressing lines available on request from individual research groups or stock centers Commercial production of transformed fly lines
Disadvantages	Cannot store lines frozen very easily Transgenic line development takes a few months. Homologous recombination still difficult Do not "self" as with hermaphrodite nematodes

PREPARATION OF MATERIAL FOR LIVE CELL IMAGING

A variety of different tissue types are routinely used to investigate different kinds of questions in axis specification, cell differentiation, and organogenesis through the use of live cell imaging techniques. The different tissues are derived from different stages of the life cycle of the flies: from fertilized eggs, through larvae, to adults (Table 2). A crucial first step before imaging is preparation of the experimental material. The main aims are to ensure physiological relevance and to achieve the best conditions for image quality. However, in the case of live cell imaging, it is often desirable to balance image quality against optimizing tissue viability.

Drosophila Tissues Amenable to Time-Lapse Imaging

The *Drosophila* tissues commonly used for experimental purposes are relatively simple to maintain in a physiological state on the microscope stage. Although there are many common considerations for the different kinds of tissues, such as temperature (generally 18°C-37°C), sensitivity to hypoxia, and dehydration, the optimal conditions for viability vary under different experimental circumstances (see Table 3). An exhaustive list of tissue preparation protocols is beyond the scope of this article, but some key examples are presented (see "Related Information").

Methods for Keeping Various Fly Tissues Alive on the Microscope Stage

To maintain tissue viability on the microscope stage, it is important to avoid dehydration, hypoxia, overheating, or medium deterioration. Early- to mid-stage egg chambers cannot be mounted in an aqueous-based medium because this causes a change in microtubule organization and follicle cell morphology. Such egg chambers survive better in Halocarbon oil, which allows free diffusion of oxygen, has low viscosity, and thus prevents dehydration and hypoxia. With a refractive index (RI) similar to glycerol, Halocarbon oil also has good optical properties for imaging. The properties of Halocarbon oil

Table 2. Key examples of live cell imaging of different *Drosophila* tissues

Tissue type	Subject	Approach[a]	References
Various tissues	Chromosomes/division/nuclei	nls-GFP, histone-GFP, H2Av-mRFP1, EGFP-Cid (kinetochores)	Davis et al. 1995; Yucel et al. 2000; Pauli et al. 2008
	For numerous cell components	Numerous stains and dyes	Molecular Probes (see "Web Resources")
Egg chambers	Border cell migration	GFP fluorescence	Tekotte et al. 2007; McDonald et al. 2008
	Golgi units	GalT, a GFP-tagged line	Morin et al. 2001; Nicolas et al. 2009
	Microtubules	τ-GFP, tubulin-GFP, Jupiter-GFP	Endow and Komma 1997; Micklem et al. 1997; Grieder et al. 2000; Karpova et al. 2006
	mRNA transport	MS2/MCP-RFP and GFP, Alexa Fluor 488/546-RN	MacDougall et al. 2003; Clark et al. 2007b; Weil et al. 2008; Zimyanin et al. 2008
Testes	Spermatogenesis, cell division	GFP, FRAP	Noguchi and Miller 2003
Gonads	Gonadal cell migration	Six4-nls-eGFP	Clark et al. 2007a
Embryos	Lipid droplet motility	DIC	Welte et al. 1998
	Movement of Wg signal	Wg-GFP	Pfeiffer et al. 2002
	Following the movement of individual chromosome sites	LacI-LacO-GFP	Marshall et al. 1997
	General labeling of embryos	Feeding adults rhodamine B	I Davis (unpubl.)
	Asymmetric cell division	τ-GFP	Kaltschmidt et al. 2000
		FRAP Llg-GFP	Wirtz-Peitz et al. 2008
	Macrophage migration, wound healing	Cytoplasmic GFP	Stramer et al. 2005
	Apoptosis, neuronal cell ablation	Caspase sensor (Apoliner), GFP	Hidalgo and Brand 1997; Bardet et al. 2008
	Marking clones	Caged-FITC-dextran	Vincent and O'Farrell 1992
	mRNA transport	Alexa Fluor 546-RNA	Bullock and Ish-Horowicz 2001; Wilkie and Davis 2001; Vendra et al. 2007
Larvae	Salivary gland chromosomes	DIC, GFP	Vazquez et al. 2001, 2002
	Imaginal discs	Argos-GFP, Dpp-GFP	Entchev et al. 2000; Greco et al. 2001
	Mitochondria transport	Mito-GFP	Pilling et al. 2006
	Brain neuroblast cell cycle	GFP trap line, centrosomin-GFP	Rusan and Peifer 2007; Siller and Doe 2008
	Synapse addition	GFP/RFP/FM dyes, FRAP-GFP	Rasse et al. 2005; Schmid et al. 2008
	Dendrite morphogenesis	Rab5-GFP, 2xFYVE-GFP	Satoh et al. 2008
	PNS	EB1-GFP, mCD8-GFP	Rolls et al. 2007
	NMJs	Calcium sensor	Reiff et al. 2005
	NMJs, vesicle trafficking	FM1-43	Verstreken et al. 2008
Pupae	Glial migration in wing disc	UV laser ablation	Gho et al. 1999; Aigouy et al. 2008
	Asymmetric cell division	GFP	
Adults	Eye in whole adult	Rhodopsin	Mollereau et al. 2000; Pichaud and Desplan 2001
	Brain	Calcium and camgaroo, Cameleon calcium sensor	Yu et al. 2003; Riemensperger et al. 2005; Hendel et al. 2008

[a]Abbreviations: DIC: differential interference contrast; eGFP: enhanced green fluorescent protein; FITC: fluorescein isothiocyanate; FRAP: fluorescence recovery after photobleaching; GFP: green fluorescent protein; NMJs: neuromuscular junctions; PNS: peripheral nervous system; RFP: red fluorescent protein; UV: ultraviolet.

Table 3. Optimal conditions for culturing *Drosophila* tissues during imaging

Tissue	Optimized culture conditions	Notes[a]
Early- to mid-stage egg chambers dissected from well-fed females	Halocarbon oil (series 95)	Avoids dehydration/hypoxia
		Early stages: problem of activation and loss of MT organization in aqueous media Oil has higher RI than water Good environment for injection
Late-stage egg chambers dissected from well-fed females	Grace's medium (Sigma)	Provides ionic and osmotic balance and nutrients
		Late stages not susceptible to problems of early stages
Embryos, dechorionated and dehydrated	Halocarbon oil (series 700)	RI similar to glycerol
		Halocarbon oil prevents excess dehydration while avoiding hypoxia (which causes changes to the cell cycle) Good environment for injection
	Halocarbon oil with breathable Teflon membrane	Better dehydration prevention for long-term development studies Better bright-field imaging Can help squash specimen for greater optical clarity (reduced spherical aberration)
	Aqueous medium	Can use WI objectives, which have longer working distance with high NA
Spread macrophages from third instar larvae	Culture-slide-mounted coverslips treated with ConA in a humidified overchamber	Inverted microscope, 100X 1.4-NA oil objective
	Six-well tissue-culture plate Schneider's insect medium with 5% FCS[b]	Upright microscope, 60X 0.9-NA dipping objective
Neuronal cell cultures from larval brains, overnight culture	Dissociation by enzyme cocktail (Kraft et al. 1998)	Inverted microscope, 100X 1.4-NA oil objective
	Schneider's insect medium with 5% FCS Coverslips treated with ConA (16 µg/mL) and laminin (5 µg/mL)	
Whole larval fillet	Schneider's insect medium with 5% FCS Sylgard mounting chamber	Upright microscope, 60X 0.9-NA dipping objective

[a]Abbreviations: MT: microtubule; RI: refractive index; WI: water immersion; FCS: fetal calf serum; NA: numerical aperture.
[b]From Sigma-Aldrich.

make it particularly useful for long-term imaging of embryos. The disadvantage of Halocarbon oil is that, unlike with aqueous solutions, changes in the medium are not possible. In contrast, late-stage egg chambers develop better in aqueous culture media (such as Grace's medium) than in Halocarbon oil, although the basis of this difference is not clear. When culturing cells, one also has to consider different substrates to assist cellular adherence and spreading (see ***Drosophila* Macrophage Preparation and Screening** [Parton et al. 2010c]).

In addition to choosing the appropriate medium, it is also useful to consider culture chambers and methods of securing the specimens to the stage. The choice of culture chamber is dependent on

whether an inverted or upright microscope is used, although most laboratories favor the former. A common method of imaging living embryos is literally to glue them to a coverslip (see **Collection and Mounting of *Drosophila* Embryos for Imaging** [Parton et al. 2010b]), cover them with Halocarbon oil, and secure the coverslip to the stage of an inverted microscope with slide clips (Davis 2000). Egg chambers can be similarly mounted, except glue is not required; they stick naturally to glass under Halocarbon oil. When using oil immersion objectives on such coverslips, an aluminum slide with a small hole (see Fig. 1) can be used as a support, thus avoiding unwanted focal changes caused by bending of the coverslip.

Maintaining the specimen and the stage at constant temperature is not only very important physiologically (e.g., when imaging temperature-sensitive mutations at the restrictive temperature) (Wilkie and Davis 2001), but can also dramatically reduce or eliminate focal shift problems. For controlling the temperature of the specimen, the use of either a stage-mounted system (such as the Bioptechs Delta T Open Dish System) or a temperature-controlled environmental chamber that encloses the microscope body is recommended. A variety of devices are commercially available in both cases.

FLUORESCENT REAGENTS FOR LIVE CELL IMAGING AND THEIR INTRODUCTION INTO CELLS

Most biological specimens, including *Drosophila* tissues, are relatively transparent, so that details of internal and intracellular morphology are difficult to image in untreated living specimens using simple bright-field techniques. Although there are methods of improving contrast in bright-field imaging (see below), fluorescence microscopy offers the greatest advantages and possibilities for increasing contrast and determining the specific localization of molecules in cells. Fluorescence microscopy has proven to be particularly well-suited to the study of live material now that an ever-increasing array of different vital probes is available for tracking cellular components and activities (see http://www.invitrogen.com/site/us/en/home/References/Molecular-Probes-The-Handbook.html), including many that have been applied to *Drosophila* (Table 2). Bright, stable dyes with narrow excitation and emission spectra that can be conjugated to a variety of probes are now available (e.g., the Alexa Fluor dye series [Invitrogen] or the latest generation of fluorescent proteins) (Shaner et al. 2005). By using different dyes, multiprobe experiments can be performed in living samples to investigate interactions at both the organelle and molecular level. Here we outline the three methods most commonly used to introduce an appropriate label into *Drosophila* tissue without unduly perturbing the biological processes under study.

External Application of Dyes

In principle, the simplest method of introducing membrane-permeable reagents into *Drosophila* embryos or other tissues is to soak the tissue in the reagent. However, in practice, this is only easily achieved in imaginal discs, stage 10-12 egg chambers, and other tissues that can be cultured or dissected into aqueous-based media. The waxy vitelline membrane surrounding late-stage egg chambers and embryos prevents membrane-permeable reagents from accessing the plasma membrane, and early- to mid-stage egg chambers must be dissected directly into Halocarbon oil, making it difficult to add aqueous reagents after dissection. There are solutions to these problems, but they come at a cost. Embryonic vitelline membranes can be permeabilized with heptane or octane before adding the

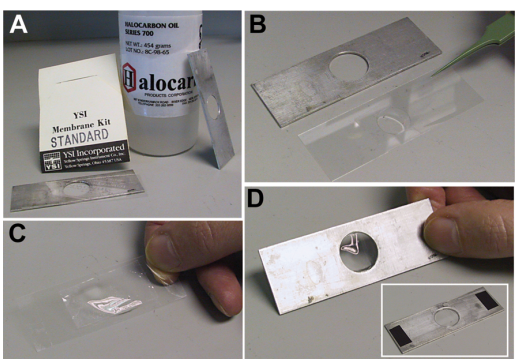


FIGURE 1. Mounting material in Halocarbon oil. (*A*) Halocarbon oil, Teflon membranes, and aluminum coverslip mounts. (*B*) Laying a piece of membrane over the mounting medium. (*C*) Embryos on a coverslip, held by heptane glue, mounted in Halocarbon oil (series 700), and covered with a Teflon membrane. (*D*) Mounting the coverslip on an aluminum coverslip mount, forming an aluminum "slide," which can fit certain stage plates or be used on an upright microscope. (*Inset*) View of the reverse side.

membrane-permeable solutions, but this treatment yields embryos with very fragile membranes that exhibit nonspecific lethality and sickness. Thus, results using this procedure must be interpreted with care. Membrane-permeable reagents can be introduced into early- to mid-stage egg chambers either by feeding adult female flies on a sugar and dye mixture on filter paper or by injecting the dyes directly into the abdomen of the mothers using, for example, the Drummond Nanoject II (Drummond Scientific).

Microinjection

The most direct method of introducing any fluorescent reagent into certain living *Drosophila* tissues is microinjection (Movie 1). A major experimental advantage of microinjection over other approaches is that it allows the delivery of a high concentration of material at a very precisely controlled time and location. For embryos and egg chambers, with practice and suitable equipment, injection is the fastest, most reliable, and quantitatively reproducible technique. Micromanipulation and injection apparatuses are now available from a number of suppliers; joystick-operated manipulators with very positive movement control, which allow a fast stabbing motion to be executed to aid penetration, are particularly useful. An additional along-the-needle-axis movement is also beneficial.

Injection systems fall into two basic types: gas-pulse systems and direct displacement syringe barrel types. The latter offer more control of injection volume and can handle finer needles and more viscous materials, but needle mounting can be tricky. For best results with injection, it is worth taking several precautions:

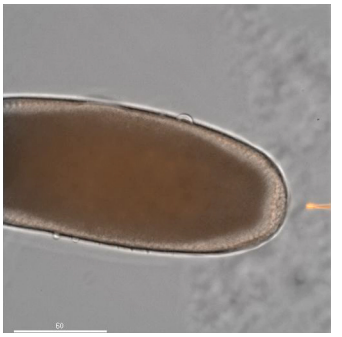
- Briefly centrifuge the material to be injected to avoid aggregates that can block the injection needles. Needles can often be unblocked by breaking the needle tip on a broken edge of a piece of coverslip under Halocarbon oil, thereby allowing the needle to be used for multiple injections.
- Use only newly pulled or commercially supplied prepulled needles (e.g., Eppendorf Femtotips).
- If possible, ensure that the needle has a slight positive "back pressure" from the injection system to reduce the chances of it blocking with cytoplasmic debris.
- Anchor or immobilize the cells in some way during microinjection. For example, a section of coverslip can be glued down on the main coverslip and can be used to trap tissues against it.
- Embryos should be neither too dehydrated nor too turgid. The right extent of dehydration can be judged with experience.

There are also disadvantages to microinjection:

- It is not appropriate for every tissue.
- There is a limit to the number of specimens that can be processed.
- There is a limit to the final concentration of reagent once it is injected into the cell (injected volume is usually no more than 5% of the total volume of the cell).
- There is a danger of physically damaging the cells, especially when they are small in size.

Fluorescent Proteins

Arguably, the most important and versatile method of tagging fluorescent molecules in living *Drosophila* cells is the use of endogenously expressed recombinant fluorescent proteins (Table 2; see Spector and Goldman 2010). There is a wide array of different colors, combinations, and permutations



MOVIE 1. Injection into a stage 4 syncytial blastoderm *Drosophila* embryo (approximately cycle 14-15). Imaging by DIC and wide-field fluorescence microscopy, 20X NA-0.75 objective.

of fluorescent proteins to choose from (see **Fluorescent Protein Tracking and Detection: Fluorescent Protein Structure and Color Variants** [Rizzo et al. 2009a]; see also Shaner et al. 2005), all of which have the advantage of interfering less with cell function than is the case with microinjection or permeabilization. Fluorescently tagged proteins can be expressed under ubiquitous or high-level promoters, with tissue or temporal specificity (e.g., using the UAS/Gal4 driver combinations) (Brand et al. 1994) or under the endogenous promoter. Often it is possible to incorporate the tagged protein of interest into a null fly line for that protein and demonstrate normal function. Combinations such as enhanced cyan fluorescent protein (ECFP)/enhanced yellow fluorescent protein (EYFP), enhanced green fluorescent protein (EGFP)/mCherry, and ECFP/EYFP/mCherry allow multiple labeling experiments.

The diversity of available fluorescently tagged proteins is ever increasing, as are fluorescent protein-based reporter molecules that can be used as indirect probes for non-protein cellular components (Zhang et al. 2002), such as the calcium-ion-sensing camgaroo (Yu et al. 2003). It is also possible to tag DNA and RNA motifs indirectly with GFP-tagged nucleic-acid-binding proteins. For example, using the MS2 viral-coat-protein-binding region on a nucleic acid of interest, in combination with a fluorescent-protein-tagged coat protein (i.e., mRNA-MS2/MCP-fluorescent protein), transport of mRNAs has been tracked successfully in *Drosophila* egg chambers (Movie 2; Weil et al. 2006; Zimyanin et al. 2008). Photoactivatable or photoswitchable fluorescent proteins (Diaspro et al. 2006; Subach et al. 2009) have also opened up a range of experimental techniques to follow the distributions and fates of specific subsets of molecules (see below).

A major disadvantage of fluorescent proteins is the lead time for making the constructs and transgenic flies, if they are not already available (see Roberts and Standen 1998; Davis 2000; Spector and Goldman 2010). However, it has recently become possible to have transgenic fly lines generated commercially from a construct of interest (Genetic Services, Inc., http://www.geneticservices.com/), and with the advent of new cloning techniques, such as the Gateway cloning system (http://www.ciwemb.edu/labs/murphy/Gateway%20vectors.html), it is easier than ever to develop new tagged lines. There is also an accumulation of ready-made fluorescently tagged lines in the fly community through trapping projects (Ryder et al. 2009; http://flyview.uni-muenster.de/html/searchpage.html.

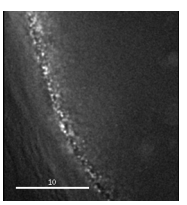
Although it is possible to transiently express constructs instead of making transgenic flies, this method has not been used extensively in *Drosophila* (Bossing et al. 2002). It is possible to inject mRNA constructs, including those coding fluorescent-protein-tagged proteins, into embryos or oocytes and obtain expression within ~2 h (R Oliveira and J Soetaert, pers. comm.).

SELECTION OF APPROPRIATE IMAGING EQUIPMENT AND METHODOLOGY

The past two decades have seen many revolutions in light microscopy techniques made possible by improvements in optics, detector technology, and computers, and there is no indication that the rate of development is slowing. A description of the full range of imaging possibilities is beyond the scope of this article; an overview of available options and important considerations applicable to imaging *Drosophila* cells and tissues is presented in Table 4. It is assumed that the reader is familiar with the basics of bright-field and fluorescence microscopy. Numerous excellent comprehensive references and web resources are available on the subject (Murphy 2001; Stephens and Allan 2003; Pawley 2006) (see also "Web Resources").

Evaluating a Microscope System

When deciding which kind of system to use or purchase, the first consideration is to determine what the critical parameters are: speed, sensitivity, resolution, multi-wavelength discrimination, or cell viability. Many of these parameters are conflicting, so compromises must usually be made. The second



MOVIE 2. *Bicoid* mRNA-transport dynamics followed by *bcd*-MS2/MCP-GFP labeling of *bcd* mRNA particles at the anterior of a stage 11 *Drosophila* egg chamber. Images were collected on a DeltaVision Core wide-field deconvolution microscope at 3 frames per second; movie is 15 frames per second.

Table 4. Summary of major imaging techniques

Imaging technique	Features	Notes[a]	General references
Wide-field microscopy	Best sensitivity, but limited image quality resulting from the contribution of blurring from above and below the focal plane.	Most useful with thin tissues or culture cells or with low-NA, low-magnification dry objectives.	Davis 2000; Wallace et al. 2001
Deconvolution Wide-field microscopy (3D imaging)	Post-image acquisition processing of 3D *x-y-z* data. Constrained iterative deconvolution algorithms act by reassigning out-of-focus light to its point of origin to deliver increased signal-to-noise, resolution, and contrast. Requires multiple *z* sections.	The most commonly applied imaging technique for high-resolution analysis of structure and dynamic processes.	Parton and Davis 2005
Deconvolution Wide-field microscopy (2D imaging)	Limited deblurring or deconvolution approaches applied to 2D *x-y-t* data to sharpen image detail.	Useful increase in signal-to-noise and contrast when speed requirements/ tissue sensitivity preclude *z*-stack collection.	MacDougall et al. 2003; Parton and Davis 2005
OMX fast live wide-field deconvolution	Wide-field imaging system with increased sensitivity, temporal resolution, and extremely low background. Has the ability to image up to four fluorescence channels simultaneously.	Useful for fast live imaging of multiple components interacting in sensitive biological processes.	Dobbie et al. 2010
Point-scanning confocal microscopy (or LSCM)	An optical arrangement that eliminates the contribution of out-of-focus light to the image by a physical pinhole arrangement to produce sharp optical sections. Images one spot at once, and slowly builds an image from these spots.	Useful in brightly labeled thick, hazy, or scattering material. Very good for high-resolution structural studies but suffers from speed limitations and is significantly less sensitive than wide-field techniques.	Pawley 2006
Slit-scanning confocal microscopy	Similar to LSCM but increases speed by imaging one line rather than one spot at a time. Some compromise in optical sectioning.	An alternative to spinning disks when simultaneous multichannel imaging is essential.	
Spinning-disk or multifocal confocal microscopy	Similar to LSCM and slit-scan confocal but increases speed and sensitivity by imaging multiple points simultaneously and detecting with a CCD camera. Some compromise in optical sectioning and flexibility.	A compromise between wide-field and confocal microscopies for fast imaging in sensitive tissues. Much faster than confocal microscopy but less sensitive than wide-field microscopy.	Basto et al. 2006
Multiphoton imaging	Achieves optical sectioning by the principle of multiple low-energy photon absorption (at 740-1100 nm) that occurs at very high illumination intensity, limiting excitation to a <1-µm-thick focal plane.	Is able to image deeper in thick, hazy, or scattering material with improved cell viability. Limited application so far in *Drosophila*.	Amos 2000; Diaspro et al. 2006

(continued)

Table 4. *Continued*

SPIM (or Theta imaging, DSLM)	Alternative to confocal optical sectioning with improvements in 3D imaging and tissue viability.	Useful for detailed 3D mapping of dynamic processes in living specimens. Significantly reduces photodamage compared with either confocal or wide-field microscopy.	Huisken et al. 2004; Keller et al. 2008; Keller and Stelzer 2010
STED (and STED 4∏)	True optical super-resolution imaging in *x*, *y* (and *z* with 4∏)	Useful to resolve fine structures and interactions of components at beyond conventional optical resolution.	Willig et al. 2006

[a]Abbreviations: 2D: two-dimensional; 3D: three-dimensional; CCD: charge-coupled device; DSLM: digital scanned laser light sheet microscopy; LSCM: laser-scanning confocal microscopy; NA: numerical aperture; OMX: optical microscope experimental; SPIM: single-plane illumination microscope; STED: stimulated emission depletion.

consideration is whether the system should be tailored to a specialized application or should be a general-purpose instrument. In general, systems with more flexibility in the choice of hardware can be better customized for specialized tasks but are likely to be more technically demanding to set up and run. Choosing off-the-shelf solutions with tried-and-tested software can be more time-efficient. Whatever type of system is being considered, the need to carry out tests using your own particular experimental material must be emphasized. Company representatives demonstrating equipment should be advised of the choice of fluorochromes and magnifications, so that they can bring the appropriate filter sets, excitation sources, and objective lenses. In addition to the biological material of interest, it is worth using sets of slides of fluorescent beads such as InSpeck and TetraSpeck (from Invitrogen) to provide quantitative data for comparison of different systems (Davis 2000; Swedlow et al. 2002).

The Microscope

The first decision is whether to use an upright (view from above) (Fig. 2) or an inverted (view from below) (Fig. 3) microscope. In general, for live cell work, an inverted microscope is advantageous when high-resolution three-dimensional (3D) imaging is required. On an inverted microscope, the specimen is generally more accessible, simplifying the microinjection procedure and the use of growth chambers and environmental control chambers. Inverted microscopes also offer better mechanical stability for mounting charge-coupled device (CCD) cameras.

Contrast-Enhancing Bright-Field Methods

Bright-field imaging is desirable in addition to fluorescence because it provides a reference for the location of the fluorescent signal. Contrast-enhancing bright-field methods such as differential

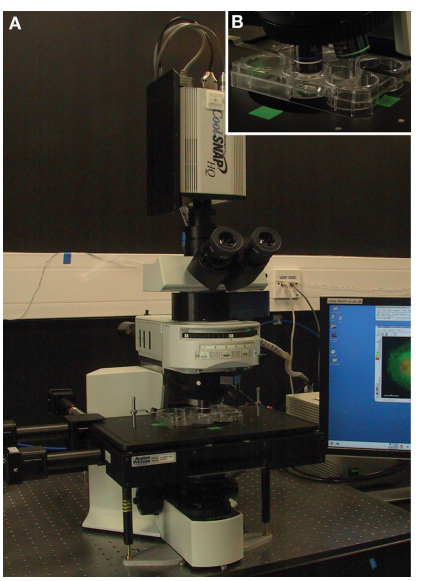


FIGURE 2. An upright wide-field deconvolution fluorescence-imaging system. (*A*) A DeltaVision Core (Applied Precision) was custom-adapted to an Olympus BX51 stand (Imsol). (*B*) Detail of the motorized DeltaVision stage, objectives, and a six-well plate during macrophage screening.

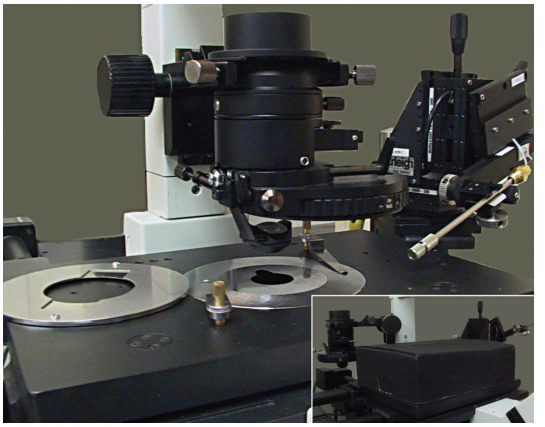


FIGURE 3. An Olympus IX70 inverted microscope with a DeltaVision motorized stage (Applied Precision). Note the condenser and the micromanipulator, which can be rotated out of the way to accommodate a stage cover (*inset*).

interference contrast (DIC; also known as Nomarski) and phase contrast are very useful in specific cases. For example, DIC can be used to observe lipid droplet motility (Welte et al. 1998) or patterning in living embryos, and phase contrast works well for imaging mitosis in the developing testis (Yamashita et al. 2003). However, it should be noted that combining either of these techniques with fluorescence imaging requires forethought. Phase-contrast objectives reduce fluorescence intensity significantly (up to 30%), and the combination of DIC and fluorescence imaging requires either that the DIC analyzer be mounted on a filter wheel (and observations alternate between fluorescence and DIC) or that a custom DIC/fluorescence filter cube be used so that the polarizing filter does not diminish the signal in the fluorescence path.

Choosing a Fluorescence Imaging System to Use with *Drosophila* Tissues

The two predominant types of epifluorescence imaging used in biological research are wide-field and confocal microscopies (summarized in Table 4). Both have been used to image *Drosophila* tissues, and many excellent descriptions of these different technologies can be found (e.g., Stephens and Allan 2003; Pawley 2006). In general, optical sectioning techniques (including multiphoton and spinning-disk confocals) should be used to image bright signals with a lot of blur in thick specimens, such as embryos (see, e.g., Fig. 4; Movie 3) and tissues dissected from larvae and adults. Although confocals are more convenient because the results are ready without lengthy processing and a need to image many *z* sections, they are significantly less sensitive than wide-field imaging. For rapid image capture of sensitive processes (e.g., cytoskeleton dynamics) or following fast RNA particle movements in *Drosophila* embryos and egg chambers, spot-scanning instruments are too slow and not sensitive enough. In these cases, only spinning-disk confocals or wide-field imaging systems (Fig. 4) are suitable. Wide-field deconvolution tends to outperform confocal microscopy on fainter signals and more sensitive

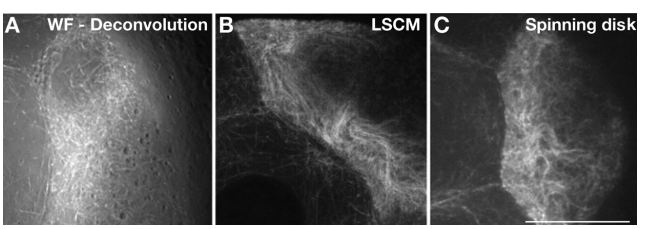
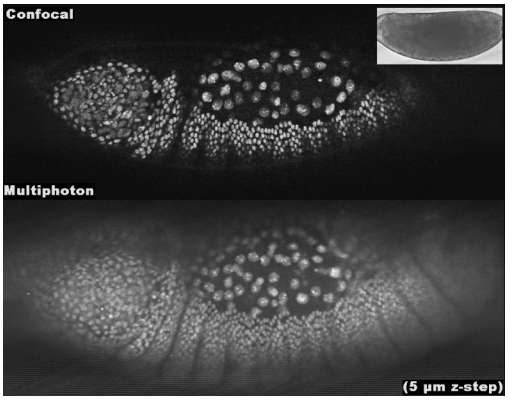


FIGURE 4. Comparison of wide-field deconvolution and confocal microscopy for live cell imaging. Stage 8 egg chambers expressing τ-green fluorescent protein (GFP) (associated with the microtubule cytoskeleton) were imaged by (*A*) wide-field (WF) deconvolution using a DeltaVision Core (Applied Precision), (*B*) laser-scanning confocal microscopy (LSCM) using a Leica SP5 resonant scanner, or (*C*) spinning-disk microscopy using a PerkinElmer Ultraview/CSU21 Yokagawa head. Scale bar, 20 μm. Images were collected at 1 *z*-stack/sec, 1 *z*-stack/1.5 sec, and 1 *z*-stack/sec, respectively. Pixel sizes are 0.066, 0.084, and 1.2 μm, respectively. Note the difference in contrast and detail between wide-field deconvolution and confocal microscopy that can be achieved at fast imaging rates. Confocal images provide better contrast but struggle to achieve detail without excessive imaging times and bleaching.



MOVIE 3. Confocal (*top*) and multiphoton (*bottom*) imaging deep into a late-stage (12-18 h after laying) *Drosophila* embryo labeled with histone-H2A GFP using a 20X, 0.75 multi-immersion objective. Confocal excitation of the GFP was at 475 nm, and multiphoton excitation was at 870 nm. Emissions were collected above 520 nm.

material (such as following mRNA dynamics) (see Movie 2; Wilkie and Davis 2001; MacDougall et al. 2003) where an image, however hazy, is still visible on the original unprocessed data. In practice, wide-field combined with deconvolution is more sensitive and quantitative and can achieve higher resolution than confocal imaging (Fig. 4; Swedlow et al. 2002). Numerous deconvolution algorithms are available (Parton and Davis 2005); the best are the constrained iterative restoration algorithms.

Optimizing Excitation and Emission for Imaging *Drosophila* Tissues

Optimizing the collection of emitted photons for a given dose of illumination light is largely a matter of selecting appropriate combinations of dye, excitation source, filter sets, detector, and objective. Failure to do this has an impact on image quality and cell viability. *Drosophila* cells and tissues are no exception, so the rules of good imaging practices apply; for an overview, see Pawley (2006). Correct system alignment is essential. On wide-field systems, ensure that the field iris in the fluorescence excitation path is correctly set to just cover the field of interest.

In general, longer excitation wavelengths cause less damage to biological specimens and induce less autofluorescence, but the exact characteristics depend on the *Drosophila* tissue being imaged. For example, yolk is particularly fluorescent in ultraviolet (UV) and blue light. It is better to replace UV excitation with excitation at 405-440 nm, use alternative dyes, or use multiphoton excitation. When imaging in the presence of yolk, the use of dyes with redshifted excitation spectra (e.g., rhodamine, Alexa Fluor 546, Alexa Fluor 568, mCherry) is recommended.

Imaging dynamic processes in living cells and tissues is limited by a combination of photodamage (direct damage by excitation light), phototoxicity (toxic effects associated with dye irradiation), and photobleaching (irreversible loss of dye fluorescence). It is now well documented that even under modest imaging regimes, cellular processes are adversely affected (Keller et al. 2007; Stelzer 2009; see also http://www.cef-mc.de/fileadmin/user_upload/Seminars/20081002_Abstract_Stelzer.pdf). To reduce these effects when imaging live cells, it is best to minimize exposure times and to attenuate the excitation power as much as possible, for example, by using neutral-density filters. The exact mechanisms of photobleaching and phototoxic effects are complex and involve nonlinear effects. It is possible that the short-irradiation-time excitation regimes used by spinning-disk and resonant-scanner confocals can produce improvements in cell viability and photostability. As noted above, it is important to maximize the efficiency of the imaging by matching the choice of fluorescent molecule with the excitation and emission filter sets and the bright peaks of the illumination source and the peak quantum efficiency of the detector. Protective infrared and UV filters can also be useful to reduce the damaging effects of excitation light. Filter sets, particularly excitation filters (UV-blue), should be treated as consumables and checked regularly for damage. It is important to note that the excitation and emission curves of fluorescent molecules reported in the manufacturer's product information can be generated under conditions very different from in vivo imaging conditions. This is particularly important with multiple dyes when trying to ensure that their spectra are sufficiently separated to allow them to be covisualized. The spectral detection options now available on several confocal systems (such as the Leica SP5 acousto-optical beam splitter [AOBS] and Zeiss LSM 710) offer increased light efficiency and much greater freedom to optimize excitation and emission. These systems also allow a larger number of simultaneous detection channels using linear unmixing.

Choice of Objective

The choice of objective lens and its mode of use are paramount for image quality. It is important to choose an appropriate microscope objective for imaging in the mounting medium used. Microscope objectives are available that are designed to work dry (generally at low magnification), with water immersion, or with glycerol (80%), silicone oil, or conventional immersion oils (RIs in the range of 1.50-1.534). It is essential to take care of objectives; they are the most important optical component of the microscope and should be cleaned and inspected regularly. The specifications of a range of objectives and their applications for imaging in specific *Drosophila* tissues are presented in Table 5.

Detectors

The detector is arguably the most fundamental aspect of hardware choice in determining the quality of the eventual image obtained. Wide-field and spinning-disk confocal systems rely on CCD cameras, where there is a wide freedom of choice. Detailed comparisons of different CCD cameras have been covered elsewhere (see http://www.andor.com/learning/digital_cameras/?docid=327; Amos 2000; Coates et al. 2003; Salmon and Waters 2010). Where a choice is available, do not skimp on the cost of the detector! For live cell work, where speed (to follow dynamic events) and sensitivity (to reduce the necessity for damaging excitation exposure) are paramount, a high-quality cooled CCD is the usual choice. Increased sensitivity and speed can be achieved with the latest generation of electron multiplying CCD (EMCCD) cameras (see below). Historically, intensified cameras (http://www.andor.com/learn/digital_cameras/?docid=326) have also had a significant place, but have been largely superseded by EMCCDs. Most often, EMCCD cameras are used in conventional mode, as a high-quantum-efficiency back-thinned detector. Only under very-low-signal conditions, or when rapid readout for fast imaging is desired, is the EMCCD used in gain mode. In gain mode, images have poorer signal relative to noise; this can be improved by post-acquisition "de-noising" techniques (see below). Modern point-scanning confocal systems generally come with optimized cooled photomultipliers, although some systems offer the option of avalanche photodiodes that can be used for very-low-light-level photon detection.

Table 5. Choice of objectives for *Drosophila* live cell imaging

Objective type	Examples[a,b]	Specifications	Applications
Dry	20X 0.75-NA, 40X 0.95-NA, 50X 0.5-NA LMPLFLN	#1.5-coverslip-corrected, long working distance	Deep imaging in thick tissues (e.g., embryos); micromanipulation and injection (upright microscope)
WI	60X 1.2-NA WI	Coverslip correction collar, sensitive to coverslip angle, good working distance	Deep imaging in thick tissues (e.g., embryos); multiphoton imaging
Water dipping	60X 0.9-NA, 100X 1.0-NA LUMPFL	Upright microscopes, no coverslip, long working distance	High-resolution imaging of live material in an upright configuration
Multi-immersion (glycerol)	20X 0.75-NA, 60X 1.3-NA	80% glycerol (better matched to RI of cytoplasm), coverslip correction collar, sensitive to coverslip angle	Deep imaging in thick tissues (e.g., embryos); multiphoton imaging
Silicone oil	60X 1.3-NA silicone	Requires silicone oil (better matched to RI of cytoplasm), coverslip correction collar, sensitive to coverslip angle	Improved deep imaging in thick tissues (e.g., embryos)
Conventional oil	40X 1.3-NA to 100X 1.4-NA	#1.5-coverslip-corrected, use a range of oils of varying RI to correct for spherical aberration	Tissue-culture cells; imaging within maximum 30 µm of the coverslip; high-resolution imaging; fixed material

[a]Examples refer to Olympus objectives; in most cases, similar objectives are available from other manufacturers.
[b]Abbreviations: NA: numerical aperture; RI: refractive index; WI: water immersion.

OVERCOMING THE SPECIAL PROBLEMS OF IMAGING THICK *DROSOPHILA* SPECIMENS

Imaging large multicellular structures presents challenges that are not encountered when imaging single-celled organisms. In this respect, *Drosophila* tissue tends to be easier to work with than many larger model systems, such as *Xenopus laevis* and mouse, but harder to image than *Caenorhabditis elegans*. Most objective lenses, with some notable exceptions, are designed by the microscope manufacturers for optimum performance at the inner surface of a #1.5 coverslip (0.16-0.19-mm thick), often used with an immersion medium with an RI matching that of the glass (usually 1.515-1.518). The optical quality of the image is compromised as soon as structures within the specimen (with its variable optical properties) are imaged: The deeper the imaging, the greater the aberration, the most important of which is spherical aberration (SA). SA is caused by light rays passing through different parts of the lens (i.e., through the center or outer edge) having different path lengths and consequently focusing at different points along the microscope axis (Davis 2000). This is generally a consequence of RI mismatch along the light path from the specimen to the objective. In addition to imaging into biological material, SA can also result from poorly corrected objectives, RI mismatch between the immersion and mounting medium, or incorrect setting of coverslip thickness-correction collars. The consequence of SA is a loss of resolution or blurring of the image at increasing imaging depth (Davis 2000).

With confocals, signal attenuation with imaging depth is aggravated by SA because some light from the plane of interest is excluded by the pinhole. This does not happen with multiphoton imaging using direct detectors and no pinhole. However, SA will decrease the power in the focal spot and lead to reduced excitation with depth. RI mismatch between immersion medium, mounting medium, and specimen also leads to reduction of useful working distance, often experienced with high-numerical-aperture (NA) oil-immersion objectives. Chromatic aberration also increases with depth, causing different wavelengths of light from the same focal depth in the specimen to be focused at different positions or imaging planes on the detector (a problem for colocalization studies) (Davis 2000).

Because RI inhomogeneities and the scattering properties of each kind of *Drosophila* tissue are different, SA should be reduced using a number of combined approaches, which must be determined empirically:

- Image as near as possible to the surface of the coverslip. Partially squash living specimens with a breathable Teflon membrane (see **Collection and Mounting of *Drosophila* Embryos for Imaging** [Parton et al. 2010b]). Squashing can improve the imaging quality considerably and reduce the depth required for imaging the structures of interest (Davis 2000).
- Choose the objective lens carefully (see Table 5). A dry 20X/0.75-NA lens is excellent for deep imaging, if high spatial resolution is not required. Higher-NA lenses suffer from the problems with deep imaging to a far greater extent. Try to use water immersion lenses (e.g., 60X/1.2 W) or oil-immersion lenses (e.g., 40X/1.35) that have a coverslip correction collar. The coverslip correction collar can be used empirically to correct SA. SA can be judged by eye on a wide-field microscope: When viewing a very small bright feature, the so-called Airy rings above and below the focal plane should look symmetric. If they are asymmetric, then SA is present.
- When using immersion lenses, the RI of the immersion media should be matched as far as possible to the mounting media and cell content (water, glycerol, or immersion oil).
- Immersion oils of higher RI can be used to correct for SA (Hiraoka et al. 1990). Standard oils have an RI that matches the glass in the coverslip (1.515-1.518). Cargille oils are sold in a range of RIs (1.512-1.534). Oils with even greater RI do exist (e.g., 1.65), but these are expensive, toxic, and inconvenient to use.
- Thinner coverslips (#1.0 or #0) can be used to compensate for SA.
- Adaptive optics (automated mechanical devices) are available for the correction of aberrations, including SA (Kam et al. 2007; Azucena et al. 2009).

Unfortunately, the problems of imaging at greater depth are not confined to SA. *Drosophila* tissues reflect, absorb, and disperse light as it passes through the specimen, mostly owing to variations in RI across the tissue in three dimensions. Embryos, late-stage egg chambers, and salivary glands are among the worst tissues to image from this point of view. A very laborious solution to this problem has been developed, involving mapping the RI of living tissues in three dimensions using DIC and applying these variations to deconvolution algorithms (Kam et al. 2001), but routine application of this strategy would probably require more than a 10-fold increase in computer processing speeds.

Imaging with longer wavelength visible dyes (Kam et al. 2001) has been used to improve deep imaging by reducing scatter. Multiphoton imaging using infrared irradiation can also reduce problems caused by SA and scattering and consequently improve depth penetration (Movie 3). Modern multiphoton systems are relatively easy to set up, and the lasers are now easier to tune, but they are still an expensive option (Diaspro et al. 2006).

Another major problem with imaging thick *Drosophila* tissues is autofluorescence. Embryos and late-stage egg chambers suffer from this problem greatly, owing to the presence of yolk granules. The chorion and vitelline membranes also contribute to autofluorescence and lack of transparency, although the chorion can be removed using bleach (see **Collection and Mounting of *Drosophila* Embryos for Imaging** [Parton et al. 2010b]). Autofluorescence can also be reduced by choosing appropriate filter sets and imaging at longer wavelengths. For example, the fluorescein isothiocyanate (FITC) HQ filter set from Chroma removes at least some yellow autofluorescence when imaging GFP. Imaging systems with spectral detectors make it easier to assess autofluorescence and optimize emission collection; they also allow linear unmixing to be used to separate signal from autofluorescence.

LIVE CELL IMAGING AS AN EXPERIMENTAL APPROACH IN *DROSOPHILA*

At its simplest, imaging can be used for visual genetic screens such as analysis of morphological characteristics (Schüpbach and Wieschaus 1986; Merabet et al. 2002) or of the distribution of fluorescently tagged proteins in living embryos (Martin et al. 2003; Ryder et al. 2009). The ability to examine the distribution of proteins in living cells can save considerable labor compared with similar kinds of screens using antibody staining (Seeger et al. 1993). Such simple observations of GFP-fusion proteins can also provide very valuable complementary information for the use of antibody staining, which can suffer from fixation artifacts and penetration or accessibility problems (Micklem et al. 1997; Newmark et al. 1997). However, live cell imaging becomes essential when studying dynamic biological processes that cannot be analyzed in fixed material. This is most important in the study of very rapidly evolving processes. Examples (see also Table 2) include lipid droplet motility in embryos (Welte et al. 1998), the movement of chromosomal loci within the nucleus (Vazquez et al. 2001, 2002), diffusion of protein signals in imaginal discs (Greco et al. 2001), rapidly moving protein particles in the oocyte (Theurkauf and Hazelrigg 1998; Shimada et al. 2006), and RNA particles (Wilkie and Davis 2001; MacDougall et al. 2003; Weil et al. 2006, 2008).

Live cell imaging is particularly powerful in *Drosophila*, when used in combination with a number of well-established genetic tricks (St. Johnston 2002). Simply studying a process in mutant tissue can be extremely revealing, especially when studying dynamic processes in hypomorphic viable alleles, which appear normal in fixed material. For example, the speed of motility of RNA particles is lower in flies expressing mutant motor protein, despite the finding that their localization appears normal by in situ hybridization (Zimyanin et al. 2008). In such cases, strong alleles cannot be studied, as they are lethal. However, lethal alleles can be studied in the correct circumstances if germline clones (Chou and Perrimon 1992) can survive until the stage required for analysis (Brendza et al. 2000). The UAS/Gal4 activation system (Brand et al. 1994) can also be used to activate a dominant negative protein and study its effects at a particular time and location, despite its lethality (Duncan and Warrior 2002).

RNA interference (RNAi) is a technique that is routine in tissue-culture cells. Expression of an RNAi construct corresponding to the gene of interest allows selective reduction of expression at the RNA level (Carthew and Sontheimer 2009). It has also been applied to *Drosophila* primary neurons (Sepp et al. 2008) and preblastoderm embryos (Koizumi et al. 2007) to identify genes important in neural development.

Advanced fluorescence imaging techniques have allowed the design of very sophisticated experiments to answer difficult biological questions in dynamic living tissues:

- Fluorescence recovery after photobleaching (FRAP) (see Bancaud et al. 2010) can be used to study the dynamics of labeled structures. It can measure diffusion rates of freely moving fluorescent objects or turnover within larger protein complexes. In systems with a bright, freely diffusing background, photobleaching can also be used to visualize structures that would otherwise be lost in the bright background (Entchev et al. 2000; Berdnik and Knoblich 2002; Noguchi and Miller 2003; Delanoue et al. 2007). The technique is available with wide-field systems to follow rapid recovery kinetics as well as with point-scanning confocal systems that permit complex-patterned FRAP.

- Caged FITC has been used to mark cells in embryos and follow their fate in real time (Vincent and O'Farrell 1992). Careful quantification of the signal and analysis of its movement over time have led to conclusions regarding the mechanism of movement of chromosomes in the nucleus (Vazquez et al. 2001). Photoactivatable or photoswitchable proteins can be used in a similar way to follow redistribution and turnover of proteins within and between cells (see **Fluorescent Protein Tracking and Detection: Applications Using Fluorescent Proteins in Living Cells** [Rizzo et al. 2009b]). Multiphoton excitation can be applied in uncaging or photoconversion of precisely defined 3D volumes (Patterson and Lippincott-Schwartz 2002; Diaspro et al. 2006).
- Fluorescence resonance energy transfer (FRET) (see Grecco and Bastiaens 2010) is used to establish close molecular interactions of <10 nm by monitoring the effective quenching of fluorescence emissions from the donor fluorochrome and emission from the acceptor in the donor-acceptor FRET dye pair. The technique can be very powerful but is notoriously tricky in its application. Fluorescence lifetime imaging (FLIM) follows how long it takes for the fluorescence emission to last after excitation. This is affected strongly by the local environmental conditions of a fluorescent molecule and, in particular, can be used as a monitor of FRET that is less sensitive to intensity bias, cross talk, and bleed-through issues than spectral FRET measurement.

NEW AND EMERGING FLUORESCENCE MICROSCOPY TECHNIQUES AND THEIR APPLICATION TO *DROSOPHILA*

Microscopy is generally a fast-developing field, but in the last decade, there has been a revolution allowing cellular and molecular events to be studied in greater detail than ever before. The fundamental constraints on imaging, including the long-standing diffraction limit to resolution (in *x*, *y*, and *z*), speed of acquisition (*t*), imaging multiple probes, and maintaining cell viability, are being pushed to new levels. These advances are driven by a combination of basic hardware development and technical innovation.

There is a common thread in much of the current hardware development in making the best use of the photon budget and increasing contrast through reducing background and noise:

- Advances in detector technology have seen the introduction of a new generation of deep-cooled, back-thinned, EMCCD cameras with low read noise and high quantum efficiency (Coates et al. 2003). This has had a big impact on wide-field systems and spinning-disk confocals. Detectors used in laser-scanning confocal microscopes (LSCMs) and slit scanners have also been improved (e.g., http://www.olympusfluoview.com/java/channelpmt/index.html).
- The latest generation of optical filters (ET filter sets; Chroma Technology Corp.) has reduced light loss by reflection, drastically improving light throughput. There are now filter-free imaging systems (such as the Leica SP5 AOBS) that optimize the selection of excitation for rapid multichannel imaging and customize emission selection for optimal signal collection.
- Excitation sources have improved in stability and flexibility with the introduction of improved xenon lamps, solid-state lasers in a range of useful wavelengths and power outputs, tunable lasers, and white-light lasers. The wider availability of resonant-scanner LSCM systems and faster spinning-disk confocals has the added bonus of reduced photodamage and photobleaching when dye molecules are repeatedly excited with very small doses of irradiation during image capture.
- There are now improved fluorescent markers with better spectral properties and increased quantum efficiency (i.e., number of photons emitted/photons absorbed). These include the Alexa dye series (Invitrogen) and the various new forms of fluorescent proteins (Shaner et al. 2005; Resch-Genger et al. 2008).
- Improved speed and synchronization of hardware (shutters; filter wheels; camera triggers; and *x*, *y*, and *z* movement control) maximize useful excitation exposure by ensuring that camera exposure matches excitation and increases speed performance (e.g., the new DeltaVision Core, compared with the DeltaVision RT [Applied Precision]; see http://www.api.com/pdfs/lifescience/DVspecs.pdf).
- Motorized SA correction lenses hold promise for correcting SA but are only just beginning to be available (http://www.intelligent-imaging.com/spherical/Monks_SIM0304.pdf). Adaptive optics has applications in both aberration correction and rapid focusing and so has considerable potential applications to very fast multidimensional imaging (Kam et al. 2007; Azucena et al. 2009).

In addition to these important developments in hardware, there have recently been some truly innovative new imaging concepts that have emerged from research laboratories. Although many are currently only available to those able to put together their own custom imaging systems, they are rapidly being adopted for commercialization by the major vendors.

The single-plane illumination microscope (SPIM), a technique pioneered by Ernst Stelzer at the European Molecular Biology Laboratory (Heidelberg, Germany), produces optical sectioning by excitation with a light sheet (Huisken et al. 2004) to produce stunning 3D reconstructions of whole *Drosophila* embryos (see Keller and Stelzer 2010). SPIM has undergone further developments, emerging as a digital scanned laser light sheet microscope (DSLM) (Keller et al. 2008; Stelzer 2009; see also http://www.cef-mc.de/fileadmin/user_upload/Seminars/20081002_Abstract_Stelzer.pdf). This technique offers full 3D imaging of large living specimens with reduced photodamage compared with other techniques. There are, however, limitations in the way specimens are prepared and mounted and the speed of acquisition of the necessarily large data sets, making this technique worthwhile but not trivial.

For many years, the diffraction limit of resolution was thought to be a fundamental barrier to light microscopy. This barrier has now been completely overturned with developments that significantly enhance resolution in both fixed and, most recently, live specimens (Gustafsson 1999; Hell 2003). These techniques are now finding application in imaging *Drosophila* tissues. Stimulated emission depletion (STED) is a true optical technique developed by Stefan Hell (and commercialized by Leica; see http://www.leica-microsystems.ru/website/products.nsf?open&language=english&path=/website/products.nsf/(allids)/d0af715211cfbe90c1257370002f68b0) (Willig et al. 2006; Nägerl et al. 2008). The technique works by engineering the effective point-spread function of the imaging system. An infrared laser beam with a torus-like profile coirradiates the specimen and de-excites, by stimulated emission, all but the very small central region of the illumination spot. The result is increased resolution in *x-y*, but only conventional *z* resolution. In practice, there are issues with maintaining alignment of the system, the dyes that can be used, and low signal. Even so, live imaging with unprecedented *x-y* resolution has been achieved in *Drosophila* tissues (such as neurons). A modification of the technique, STED 4∏, uses two opposing objectives to increase *z* resolution, but as a consequence, there are increased complications, particularly in alignment and restrictions on suitable specimens (Hell 2003).

Photoactivated localization microscopy (PALM), fluorescence PALM (F-PALM), and single-particle tracking PALM (SPT-PALM) (Subach et al. 2009) and the similar stochastic optical reconstruction microscopy (Bates et al. 2007) are clever techniques that break the resolution limits in *x-y* by using an innovative acquisition approach on conventional hardware. It effectively uses centroid analysis to identify the exact locations of individual dye molecules from the spatial distribution of photons detected for that molecule. The trick is to use selective activation of subpopulations of dye molecules to be able to effectively image single molecules at concentrations of much less than one molecule per pixel. This is done iteratively to build up an overall picture; for a full description of the technique, see Betzig et al. (2006). Initially only possible on fixed material in one channel, improvements in the technique have permitted multichannel PALM, live imaging, and particle-tracking PALM (Manley et al. 2009).

Structured illumination (SI), developed by Gustafsson and Sedat (Gustafsson et al. 2008; Schermelleh et al. 2008), allows detail beyond the diffraction limit to be determined by imaging with conventional optics but with a patterned (i.e., structured) excitation. Whereas PALM and STED put considerable restrictions on sample preparation and choice of probes, SI can be applied to standard samples and dyes, although it is more modest in its super-resolution (doubling resolution in *x-y* and *z*) (see Dobbie et al. 2010). In the Davis laboratory, SI imaging has been applied to look at microtubules in live macrophages, although with the current setup, the imaging rate is too slow to follow dynamic structures effectively. Because the technique is very sensitive to SA, in practice, it is limited to within 10-15 µm of the coverslip. The speed of SI imaging can be greatly improved by using total internal reflection fluorescence (TIRF) illumination at the coverslip interface to achieve *z* resolution; in this way, it has been possible to image live tissue culture cells.

Optical microscope experimental (OMX), developed in the Sedat laboratory at the University of California, San Francisco, is a reinvention of the basic microscope-stand design for improved stability and control (see Dobbie et al. 2010). OMX incorporates two imaging modalities: (1) high sensitivity, multichannel, fast live imaging and (2) super-resolution SI imaging. In the fast live mode, OMX offers simultaneous multichannel (up to four) *x-y-z-t* data collection with increased photon efficiency and contrast. In the SI-imaging mode, the OMX-stand design provides the thermal and mechanical stabilities to allow resolution beyond the diffraction limit in *x*, *y*, and *z* with conventional sample preparation

and labeling. Currently, the SI-imaging mode is too slow to image rapid dynamic processes at high resolution, but developments are under way that should make this possible. OMX is an ideal platform for further development, and work is under way at various sites to incorporate TIRF, PALM, and SPIM on the OMX platform. A post-acquisition processing approach to improving imaging is the use of so-called de-noising algorithms. The aim is to allow imaging with reduced excitation illumination (ultimately by orders of magnitude) to increase cell viability. The resulting poor signal-to-noise images are processed to reduce the effects of noise that dominate images at low photon flux. There are different strategies for de-noising, but the patch-based algorithm of Boulanger et al. (2009), which works across *x-y-z* and time, shows promise.

Drosophila is a very good experimental system for rapidly taking advantage of new technical developments in imaging. This is because of the diversity, ease of preparation, and flexibility of *Drosophila* as an experimental system, as well as the amenability of *Drosophila* tissues to microscopy, despite their level of biological complexity. Imaging in *Drosophila* has enabled, and will continue to enable, researchers to answer the most challenging biological problems leading to significant progress in cell and developmental biology.

WEB RESOURCES

Drosophila Resources

http://flystocks.bio.indiana.edu/Fly_Work/media-recipes/media-recipes.htm (Fly food recipe; Bloomington *Drosophila* Stock Center)
http://flyview.uni-muenster.de/html/searchpage.html (FlyView; *Drosophila* image database)
http://www.ceolas.org/fly/protocols.html (WWW virtual library; *Drosophila* Protocols)
http://www.ciwemb.edu/labs/murphy/Gateway%20vectors.html (The *Drosophila* Gateway Vector Collection)
http://www.geneticservices.com/ (Genetic Services, Inc.; custom genetic services for small model organism research)
https://dgrc.cgb.indiana.edu/vectors/store/vectors.html?product_category=3 (*Drosophila* Genomics Resource Center)

General Microscopy

http://rsb.info.nih.gov/nih-image/ (home page of NIH Image)
http://www.w3.org/1999/xlink" xlink:href="www.andor.com/learn/digital_cameras/?docid=326 (Andor Learning Center; intensified CCD cameras, ICCD structure, ICCD camera)
http://www.andor.com/learning/digital_cameras/?docid=327 (Andor Learning Center; CCD, EMCCD, and ICCD camera comparisons)
http://www.api.com/pdfs/lifescience/DVspecs.pdf (Applied Precision; DeltaVision Imaging Systems, technical specifications)
http://www.bioptechs.com/ (Bioptechs; temperature control for live cell microscopy)
http://www.cairn-research.co.uk/ (Cairn Research Ltd.)
http://www.chroma.com/ (Chroma Technology; optical filters)
http://www.intelligent-imaging.com/ (Intelligent Imaging Innovations, Inc.)
http://www.intelligent-imaging.com/spherical/Monks_SIM0304.pdf (Intelligent Imaging; optical spherical aberration correction)
http://www.invitrogen.com/site/us/en/home/References/Molecular-Probes-The-Handbook.html (Invitrogen; *Molecular probes: The handbook*)
http://www.leica-microsystems.com/ (Global home page of Leica Microsystems)
http://www.microscopy.fsu.edu/ (Molecular Expressions; images from the microscope)
http://www.microscopyu.com/ (Nikon; MicroscopyU)
http://www.olympusmicro.com/primer/index.html (Olympus Microscopy Resource Center; introduction to optical microscopy, digital imaging, and photomicrography)
http://www.olympusfluoview.com/java/channelpmt/index.html (Olympus FluoView Resource Center; channel photomultipliers, interactive Java tutorial)
http://www.openmicroscopy.org/ (OME, the Open Microscopy Environment)
http://www.prior.com/uk/products/product.html (Prior Scientific; microscope automation)
http://www.roperscientific.com/ (Roper Scientific)

http://www.sutter.com/ (Sutter Instrument Company)
http://www.universal-imaging.com/products/metamorph/ (MetaMorph)
http://www.zeiss.com/micro (Carl Zeiss Microimaging Inc.; microscopy and image analysis)
https://www.omegafilters.com/ (Omega Optical)

Live Cell Imaging

http://micro.magnet.fsu.edu/primer/techniques/livecellimaging/index.html (Molecular Expressions; optical microscopy primer, specialized microscopy techniques; introduction to live cell imaging techniques)
http://www.microscopyu.com/articles/livecellimaging/ (Nikon; MicroscopyU; introduction to live cell imaging techniques)

SPIM

http://www.cef-mc.de/fileadmin/user_upload/Seminars/20081002_Abstract_Stelzer.pdf (Light-sheet-based fluorescence microscopes [LSFM, SPIM, DSLM] reduce phototoxic effects by several orders of magnitude)
http://www.zeiss.com/C125716F004E0776/0/186443B05DA200CBC125717C003F7169/$File/Innovation_15_34.pdf (Zeiss; SPIM, a new microscope procedure)

STED

http://www.leica-microsystems.ru/website/products.nsf?open&language=english&path=/website/products.nsf/(allids)/d0af715211cfbe90c1257370002f68b0 (Leica Microsystems; Leica TCS STED [stimulated emission depletion microscope])

OMX

http://msg.ucsf.edu/sedat/omx/omxtechnology.html (OMX technology)
http://www.api.com/pdfs/lifescience/DeltaVisionOMX.pdf (Applied Precision; DeltaVision OMX)

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