15

Live Imaging with Green Fluorescent Protein

Jim Haseloff, Emma-Louise Dormand, and Andrea H. Brand

1. Introduction

If developmental biologists were given the chance to design the perfect cell- or tissue-specific marker, they would ensure that it had several properties. First, it would function in living animals, eliminating the need for fixation and dehydration and their associated artefacts. Second, it would permit each stage of development to be studied in a single, intact embryo. Third, it would function in all cell types and would reveal their morphology, making it simple to identify different cells without compromising their viability.

In 1994, all cell and developmental biologists won the lottery when it was demonstrated that green fluorescent protein (GFP), a kind gift from the jellyfish *Aequorea victoria*, fulfilled all of these requirements ***(1)***. GFP is a naturally fluorescent, nontoxic, protein that functions in a wide variety of transgenic animals ***(1–5)***. The GFP protein is small (27 kDa) and can freely enter the nucleus, fill the cytoplasm of the cell, and diffuse into small cytoplasmic extensions. Most importantly, GFP requires no substrate, but fluoresces simply in response to ultraviolet (UV) or blue light. As the excitation and emission spectra of GFP are similar to those of fluorescein isothiocyatenate (FITC), the protein can be visualized with most conventional epifluorescence and confocal filter sets. And, as if we deserved a bonus, GFP is resistant to bleaching and is therefore ideal for long time-course analyses.

GFP is revolutionizing the study of developmental and cell biology in a wide variety of organisms from plants to mammals. Previously, cell labeling required the use of invasive techniques such as dye microinjection or immunohistochemistry. Using GFP as a cell marker, individual cells can be labeled in vivo and followed throughout development without harmful manipulation ***(1)***.

From: *Methods in Molecular Biology, vol. 122: Confocal Microscopy Methods and Protocols*
Edited by: S. Paddock © Humana Press Inc., Totowa, NJ

1.1. Properties of GFP

In *A. victoria*, GFP is concentrated in bioluminescent organs, called lumisomes, at the margin of the jellyfish umbrella. Light emanates from yellow tissue masses that consist of about 6000–7000 photogenic cells. The cytoplasm of these cells is densely packed with fine granules that contain the components necessary for bioluminescence ***(6,7)***. These include a Ca^{2+} activated photoprotein, aequorin, that emits blue-green light, and GFP, an accessory protein that accepts energy from aequorin and reemits it as green light ***(8)***. GFP is an extremely stable protein of 238 amino acids ***(3)***: its fluorescent properties are unaffected by prolonged treatment with 6 *M* guanidine-HCl, 8 *M* urea or 1% sodium dodecyl sulfate (SDS), and 2-d treatment with various proteases such as trypsin, chymotrypsin, papain, subtilisin, thermolysin, and pancreatin at concentrations up to 1 mg/mL ***(9)*** GFP is stable in neutral buffers up to 65°C, and displays a broad range of pH stability from 5.5 to 12.0.

Wild-type GFP has two excitation peaks, with maxima at 395nm (long wavelength UV) and 475 nm (blue light) ***(8,10)***, and emits green light with a peak wavelength of 508 nm. The intrinsic fluorescence of the protein is due to a unique chromophore that forms, posttranslationally and autocatalytically, by cyclization of amino acids 65–67, Ser-Tyr-Gly ***(3,11,12)***. The tyrosine residue is then oxidized ***(3,11,12)***. The crystal structure of GFP ***(13,14)*** shows an 11-strand β-barrel enclosing the central α helix that contains the chromophore. The barrel acts as a solvent cage to protect the chromophore from quenching and photochemical damage.

Several genomic and cDNA clones of GFP were isolated from a population of *A. victoria (3)*, and the coding sequence from one cDNA, pGFP10.1, has been used for protein expression, first in *Escherichia coli*, *Caenorhabditis elegans* ***(1,12,15)***, and *Drosophila melanogaster* [***(16)*** and now in many other organisms ***(2,4,5)***.

1.2. Imaging GFP

The development of GFP as a marker in many different biological systems has emphasised the need to image GFP at high resolution. Confocal microscopy has played a pivotal role in extending the potential of GFP as a tool in biological research. In this chapter, we describe protocols for imaging expression of GFP in *Arabidopsis* and *Drosophila* .

Although GFP can be detected by conventional epifluorescence microscopy (using most FITC filter sets or a Chroma GFP filter set), autofluorescence can be a problem when examining living whole-mount preparations. In *Drosophila* embryos, e.g., yolk autofluorescence can obscure the signal from GFP, while in larvae reflection from the cuticle can cause difficulties. Confocal micros-

copy generates optical sections of fluorescently labeled sample and is ideal for imaging cells labeled with GFP. When imaging the ventral side of a *Drosophila* embryo, the background fluorescence emanating from the more dorsally placed yolk can be completely eliminated by optical sectioning.

The resolution of confocal images is much greater than can be obtained by conventional epifluorescence techniques. Furthermore, after collecting a stack of optical sections (a Z-series) the sample can be reconstructed in three dimensions, then rotated or tilted to view cells or structures that would otherwise be obscured. Most confocal software enables time-lapse imaging in one focal plane, and 4D imaging, where Z series are collected over time. These series of images can then be played back as movies.

High resolution optical techniques can be used noninvasively to monitor the dynamic activities of living cells. Using coverslip-based culture vessels, specialized microscope objective lenses and the optical sectioning properties of the confocal microscope ***(17)***, it is possible to monitor simply and precisely both the three-dimensional arrangement of living cells, and their behavior through long time-lapse observations. For time-lapse studies, it is very important that GFP fluorescence be bright, to minimize high levels of illumination that can cause phototoxicity and photobleaching during observation (*see* **Subheading 7.2.**). Spectral variants of GFP are now available that allow double-labeling) ***(18,19)*** (and **Subheading 1.4.**). The precision with which cellular structures can be labeled with GFP, and the ease with which subcellular traffic can be monitored indicates that this approach will be invaluable for cell biological and physiological observations.

1.3. Mutant Forms of GFP

The expression of wild-type GFP was initially reported to be poor or variable in a number of heterologous systems. For example, the GFP mRNA sequence is mis-spliced in transgenic *Arabidopsis thaliana* plants, resulting in the removal of 84 nucleotides from within the coding sequence, between residues 380 and 463 ***(20)***. Alteration of the GFP coding sequence and mutation of the cryptic intron are required for proper expression of the gene in *Arabidopsis* and other plants ***(20,21)***. A number of groups have been attempting to improve expression of GFP in animal systems, and have produced GFP variants with altered codon usage. For example, GFP variants with "humanized" or other optimised codon usage lead to better translation efficiency. These also show improved levels of expression in plants ***(22–24)***, and it is likely that these altered forms confer some degree of immunity from aberrant RNA processing.

Wild-type GFP is temperature sensitive, and fluoresces poorly at temperatures above 25°C ***(25)***. Polymerase chain reaction (PCR)-based mutagenesis of

the GFP coding sequence has generated a thermotolerant mutant with improved fluorescence *(25)*. The mutant contains two altered amino acids (V163A, S175G) that greatly improve folding of the apoprotein at elevated temperatures. The V163A mutation has been isolated independently by several groups *(19,25–28)* and may play a pivotal role in folding. Further mutations originated in a screen by Cormack et al. *(29)*, who introduced large numbers of random amino acid substitutions into the 20 residues flanking the chromophore of GFP. They used fluorescence-activated cell sorting to select variants that fluoresced 20- to 35-fold more intensely than wild-type and showed that the folding of these mutant proteins in bacteria was more efficient. One of the variants [GFPmut1, *(29)*] changes two amino acids within the central α-helix of the protein, adjacent to the chromophore, F64L and S65T. Recombination of these mutations with V163A and S175G, which are found on the outer surface of the protein *(13,14,25)*, leads to markedly improved fluorescence *(30–32)*. The beneficial effect of both sets of mutations on protein folding, and their apparent additive effect, suggests that they may play separate roles in the folding or maturation process.

1.4. GFP Spectral Variants for Multichannel Confocal Microscopy

Although wild-type GFP can be excited by light of 400 nm and 475 nm wavelengths, the 400 nm excitation peak predominates. This is a useful property for simple detection of the protein using a long-wavelength UV source. However, efficient blue light excitation (approx 470–490 nm) is essential when using microscopes equipped with fluorescein filter sets, or confocal microscopes or cell sorters that use argon lasers.

The relative amplitudes of the excitation peaks of GFP can be altered by mutagenesis *(12,19,33,34)*. A GFP variant containing the I167T mutation *(12)* has dual excitation peaks of almost equal amplitude *(25)* and is highly fluorescent in vivo. This allows the efficient use of techniques that require either UV or blue light excitation of the protein, e.g., when screening GFP-expressing tissues with a UV lamp, or when using blue laser light excited confocal microscopy, respectively. GFP variants that contain the S65T mutation *(29,35)* are now widely used and provide optimized properties for blue light excitation, but are not useful for detection by long-wavelength UV light.

Different colored GFPs are particularly useful for distinguishing cells, organelles, or proteins in the same living organism. It is possible to alter the fluorescence spectra of GFP more extensively by introducing additional substitutions in and around the chromophore. For example, the Y66H substitution dramatically shifts both the excitation and emission spectra of GFP to give a "blue fluorescent protein" (BFP) *(12)* (excitation maximum = 382 nm, emission maximum = 448 nm). There is much interest in using these "color vari-

ants" for multichannel imaging with GFP. However, there is considerable spectral overlap between some of the mutants, and the choice of GFP variant and excitation source in an experiment must be carefully considered.

BFP is the variant most easily distinguished from GFP, thanks to its blue-shifted spectrum. However, the first generation of BFP variants ***(12,19)*** were weakly fluorescent and bleached rapidly compared to GFP. BFP variants with improved fluorescence, owing to the "folding"mutations V163A ***(25)*** and F64L ***(36,37)***, are now available. However, BFP must be excited using a UV light source, which is damaging to living cells, and to detect the protein by confocal microscopy requires the use of a separate UV laser.

An alternative approach is to use the cyan fluorescent variant (CFP) and the yellow fluorescent variant (YFP). CFP bears a tryptophan substitution within the chromophore (Y66W), which gives a broad excitation peak at 440–455 nm and an emission maximum at 483 nm. YFP (excitation maximum 514nm, emission maximum 527 nm) was generated by modifying residues that lie close to the chromophore (S65G, S72A, T203Y) ***(13,29)***. Whereas the first reported variants were sometimes poorly expressed, the introduction of altered codon usage and mutations to improve protein folding has improved fluorescence.

Multiline argon ion lasers are relatively inexpensive and emit light mainly at discrete wavelengths of 458 nm, 477 nm, 488 nm, and 514 nm (lasers with about 80 mW or better output are required for bright 458 nm emission). Each of the laser lines falls near to an excitation peak for one of the GFP variants [458 nm = CFP, 477 nm = wild-type GFP and mGFP5 ***(25)***, 488 nm = S65T variants of GFP, 514 nm = YFP]. Proteins can be tagged with the various fluorescent colors, and visualized in living cells using a confocal microscope equipped with an argon ion laser and a motorized excitation filter wheel containing laser line excitation filters. The different fluorescent tags can be excited in turn, using the appropriate laser lines, and the signals collected through specialized emission filter blocks.

More recently, it has been found that, on photoactivation by blue light, GFP is switched to a new green-absorbing and red-emitting state ***(38,39)***. This property of GFP has already been exploited to measure protein diffusion in the cytoplasm of living bacteria and is likely to have many further applications.

1.5. GFP-Protein Fusions

Wang and Hazelrigg demonstrated that proteins can be fused to either the C- or N- terminus of GFP without inhibiting fluorescence ***(16)***. This property has been exploited to fuse GFP to proteins of interest to examine their subcellular localization, or to target GFP to subcellular structures such as the cytoskeleton or organelles.

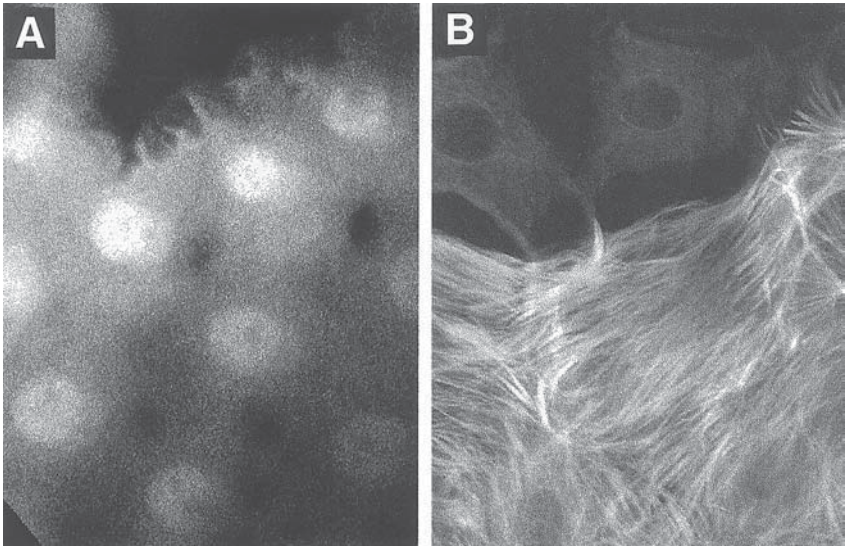


Fig. 1. Comparison of expression of unfused GFP **(A)** with Tau-GFP **(B)** in the epidermis of living *Drosophila* larvae. (A) GFP is detected at high levels in the cell nuclei and at lower levels in the cell cytoplasm. (B) Tau-GFP is excluded from the nucleus and instead highlights the cell cytoskeleton by binding to microtubules (***43***; *see* http://www.welc.cam.ac.uk/brand lab/).

1.5.1. GFP Targeted to the Cytoskeleton

The use of GFP to examine cytoskeletal dynamics, by fusion to cytoskeleton-associated proteins, has recently been reviewed ***(40)***. Cytoskeleton-associated protein fusions to GFP have also been useful for examining cell morphologies. For example, the microtubule-associated protein, τ ***(41,42)*** has been fused to GFP to target GFP to the microtubules ***(43–45)*** (**Fig. 1**). Because axons are particularly dense in microtubules, τ-GFP is an excellent marker for axonal morphology.

1.5.2. GFP Targeted to Organelles

Many organelle-targeted GFP fusions have been engineered by fusing the GFP protein to organelle-specific proteins (reviewed in **ref. *5***). GFP has been targeted to the nucleus ***(22,46–49)***, cell membrane ***(50)***, endoplasmic reticulum ***(20,51)***, mitochondria ***(18,48)***, and plastids ***(22)***. One of the cell membrane-targeted GFPs is a fusion to the NMDA receptor, a ligand-gated ion channel found in certain neurons ***(52)***. The GFP-NMDAR1 chimera is func-

tional and fluorescent, thus enabling studies of the expression, localization, and processing of the ion channel.

1.5.3. GFP-Protein Dynamics

Apart from being used as a marker of live cells or organelles, GFP can be fused to proteins to study their subcellular localization and redistribution during the cell cycle or in response to external stimuli (reviewed in **ref. *5***). For example, transport of a secreted protein, chromogranin B, has been visualised in HeLa cells by fusing GFP to the C-terminus of the protein ***(53)***. It is possible to visualize secretion of GFP, allowing analysis of transport through the secretory pathway in living cells. Endoplasmic reticulum-to-Golgi apparatus transport of a viral glycoprotein tagged with GFP has recently been visualized in living COS cells ***(54)***.

Although these studies used cultured cells, GFP fusions have also been used to examine protein expression in the whole organism. For example, in *C. elegans*, the expression and subcellular localization of Daf-7, a protein thought to be necessary for the functioning of a subset of chemosensory neurons, was investigated by examining the expression of a Daf-7-GFP fusion ***(55)***. Daf-7 is thought to regulate the formation of the dauer larva in response to pheromonal signals. Daf-7-GFP is found to be expressed in the cytoplasm of the appropriate chemosensory neurons to mediate this pheromone response.

The following protocols are for imaging expression of GFP in *Arabidopsis* and *Drosophila.*

2. Materials: Arabidopsis

2.1. Preparation of Arabidopsis *Seedlings*

1. Ethanol
2. Sterile water
3. Surface sterilizing solution (1% (w/v) sodium hypochlorite and 0.1% (v/v)
4. Nonidet P-40 (NP40) detergent)
5. GM medium:
   1× Murashige and Skoog basal medium with Gamborg's B_5 vitamins (Sigma)
   1% sucrose
   0.5 g/L 2-(*N*-morpholino)ethanesulfonic acid (MES)
   0.8% Agar (adjusted to pH 5.7 with 1*M* KOH)

2.2. Fluorescent Counterstaining of Arabidopsis Seedlings

1. 10 μg/mL aqueous solution of propidium iodide (Sigma)
2. FM 1-143 (Molecular Probes Inc.)

2.3. Double Labeling Arabidopsis Seedlings with GFP Variants (see Subheading 2.1.)

3. Methods for Visualizing GFP in Arabidopsis Seedlings

3.1. Mounting and Observing GFP-Expressing Arabidopsis Seedlings

1. Perform procedures in a laminar flow hood. Place 20–100 transgenic *Arabidopsis* seeds in a 1.5-mL microfuge tube and wash for about 1 min with 1 mL of ethanol.
2. Incubate the seeds with 1 mL of surface sterilizing solution for 15 min at room temperature.
3. Wash the seeds three times with 1 mL of sterile water, and transfer by pipet to agar plates containing GM medium (56). Add 25 mg/L kanamycin if antibiotic selection of transgenic seedlings is necessary.
4. Incubate sealed plates or vessels for 1–3 d in the dark at 4°C, and then transfer to an artificially lit growth room at 23°C for germination.
5. *Arabidopsis* seedlings germinate after 3 d, and can be used for microscopy for several weeks. Remove GFP-expressing *Arabidopsis* seedlings from agar media and mount in water under glass coverslips for microscopy.

We examine specimens using a Bio-Rad MRC-600 laser-scanning confocal microscope equipped with a 25 mW krypton/argon or argon ion laser and filter sets suitable for the detection of fluorescein and Texas red dyes (Bio-Rad filter blocks K1/K2 with krypton/argon ion laser, and A1/A2 with argon ion laser).

We routinely use a Nikon 60x PlanApo numerical aperture (NA) 1.2 water immersion objective lens to minimize loss of signal through spherical aberration at long working distances. For the collection of time-lapse images, the laser light source was attenuated by 99% using a neutral density filter, the confocal aperture was stopped down, and single scans were collected at 2-s intervals.

We transfer the large data files to an Apple® Macintosh computer, and the programs PicMerge, authored by Eric Sheldon, and 4DTurnaround, authored by Charles Thomas, are used with Adobe Photoshop® and Premiere® to produce QuickTime movies for display and analysis.

3.2. Fluorescent Counter-Staining of Arabidopsis *Seedlings*

Autofluorescent chloroplasts, normally present in the upper parts of the plant, and certain red fluorescent dyes can provide useful counterfluors for GFP.

3.2.1. Labeling Root Meristem Cell Walls with Propidium Iodide

Propidium iodide is a cationic dye that does not readily cross intact membranes, and yet it penetrates throughout the meristem and binds to cell walls, forming an outline of the living cells. The dye is excluded by the Casparian strip present in older parts of the root and does not penetrate shoot tissue well, and thus is best suited for use in the root meristem (**Fig. 2**).

1. Grow *Arabidopsis* seedlings in sterile culture.
2. Remove seedlings from agar media and place in a well of a microtiter dish with 1 mL of staining solution for 10–20 min at room temperature.

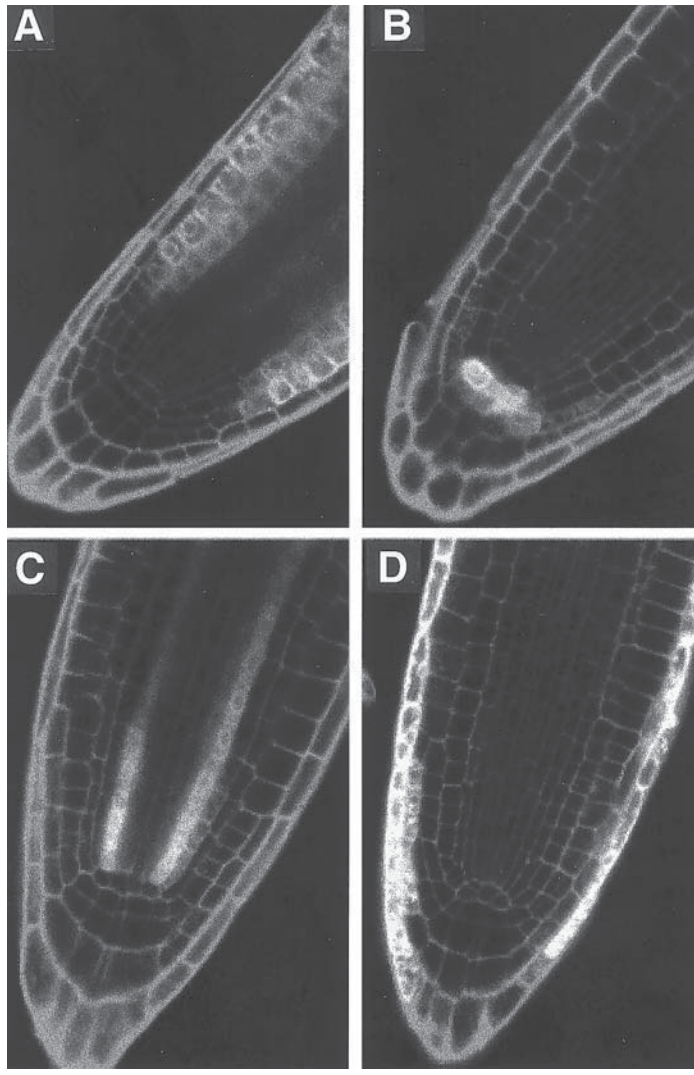


Fig. 2. GFP expression in *Arabidopsis* root tips double labeled with propidium iodide. Propidium iodide labels the root meristem cell walls. **(A–D)** Different patterns of GFP expression can be generated by enhancer detection. (J. P. H. and S. Hodge, unpublished; *see* http://brindabella.mrc-lmb.cam.ac.uk).

3. Mount seedlings in water under a coverslip for direct microscopic observation.
4. Propidium iodide is red fluorescent and can be detected using a filter set suitable for Texas red fluorescence, with little spillover between GFP (fluorescein) and propidium iodide channels.

3.2.2. Labeling Plasma Membranes with FM 1-43

The cationic styrylpyridinium dye FM 1-43 (Molecular Probes) provides a useful stain for the plasma membrane in root and shoot tissue of *Arabidopsis*. It is particularly useful for specifically labeling the plasma membrane of shoot epidermal cells, and we have been using this to characterize GFP expression patterns in *Arabidopsis* cotyledons and leaves.

1. Remove seedlings from sterile culture and place in 1 mL of 1µg/mL of FM 1-43 in water for 10 min at room temperature.
2. Mount seedlings in water under a coverslip for direct microscopic observation.
3. FM 1-43 emits a broad orange fluorescence, and signal can be detected in both red and green emission channels. We generally use 488 nm wavelength laser light to excite both GFP and FM 1-43, and to collect the emissions of both fluors in the same channel. This is possible because of the very localized distribution of FM 1-43 in shoot epidermal cells.

3.3. Double Labeling Arabidopsis Seedlings with GFP Variants

1. Grow plants expressing mCFP, mGFP5, and/or mYFP proteins in sterile culture, and mount in water for microscopy.
2. We use a Bio-Rad MRC-600 microscope equipped with an 80 mW argon ion laser and a motorized excitation filter wheel containing narrow band-pass filters to select laser lines at 458 nm for excitation of mCFP, 477 nm for excitation of mGFP5 (mGFP5), and 514 nm for excitation of mYFP. A multiline argon ion laser of higher power (>50 mW) is generally needed to provide 45 8nm illumination of useful intensity.
3. We use mCFP and mYFP, and mGFP5 and mYFP together for double-labeling experiments. The proteins are sequentially excited using the appropriate laser lines, and the signals collected through specialized emission filter blocks: mCFP/mYFP = 495 nm longpass dichroic mirror, 485±15 nm, 540±15 nm bandpass filters, or mGFP5/mYFP = 527 nm longpass dichroic mirror, 500 nm longpass, and 540 nm±15 nm bandpass filters (Omega Optical). The use of selective monochromatic excitation allows useful discrimination between mGFP5 and mYFP, which have overlapping fluorescent spectra. The greater spectral differences between mCFP and mYFP result in clean discrimination of the fluorescent signals.
4. Sequentially collected images may be merged and pseudocoloured using Adobe Photoshop.

4. Notes

4.1. Imaging Roots of Arabidopsis Seedlings

For extended time-lapse imaging of roots, sterile seeds can be sown in coverslip-based vessels (Nunc) which comprise four wells, each containing about 400 µL of low gelling temperature agarose with GM medium. The roots of these plants grow down through the media and then along the surface of the

coverslip. The roots are then ideally positioned for high resolution microscopic imaging through the base of the vessel.

5. Materials: *Drosophila*

5.1. Live Drosophila *Samples*

Home-made sieves: Cut the top off a 15 mL polypropylene Falcon tube, about 4 cm from the screw-on cap. Cut a wide hole in the cap. Place fine gauze over the end of the Falcon tube and hold it in place by screwing on the cap.
18 × 18 mm Coverslips (Menzel-Glaser)
22 × 40 mm Coverslips (Menzel-Glaser)
50% Clorox
Glue (double-sided Scotch tape glue dissolved in heptane)
Voltalef oil: 10S
Halocarbon oil: 50% Halocarbon 27 and 50% Halocarbon 700 (Sigma)
Air-permeable Teflon membrane mounted on Perspex frame
Parafilm

5.2. Fixed Drosophila *Samples*

PBT [phosphate-buffered saline (PBS), 0.1% Triton X-100 (Sigma)]
50% Clorox
4% Formaldehyde (BDH) in PBT (make up fresh)
Heptane (Sigma-Aldrich)
Methanol (Fisher Scientific International)
Vectashield (Vector Labs)

6. Methods for Visualizing GFP in *Drosophila*

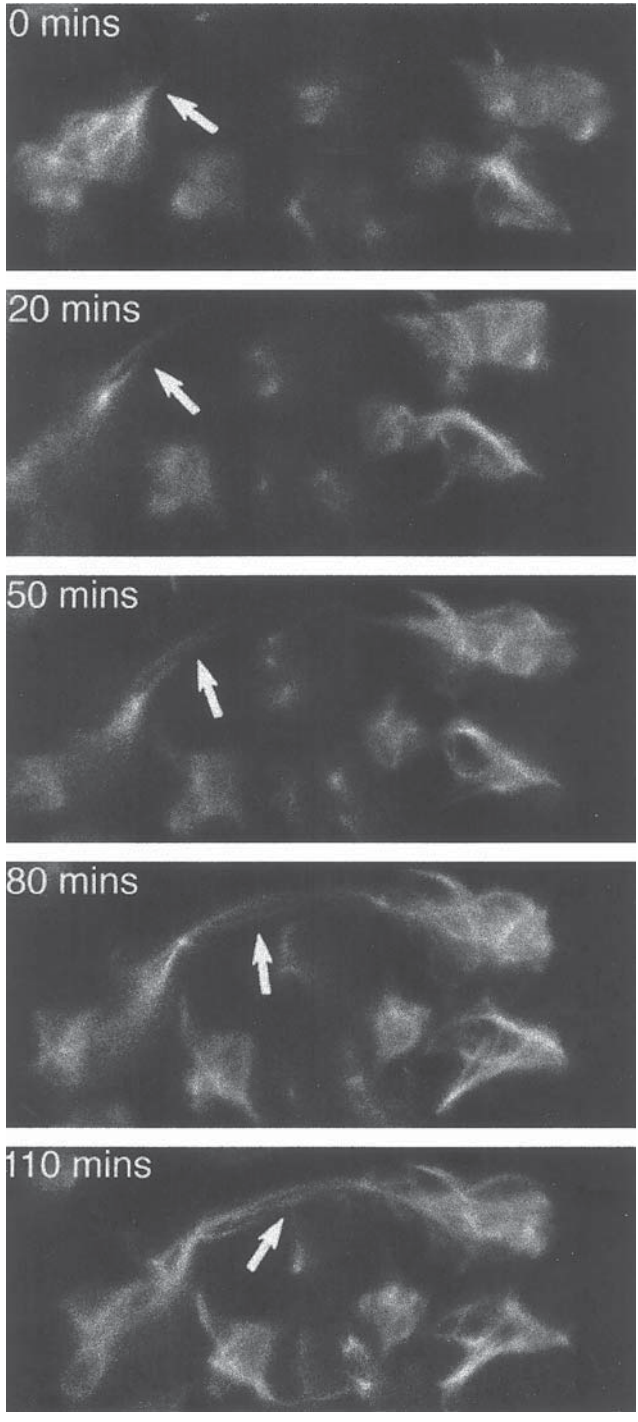
The following protocols are for examining GFP fluorescence in either live (**Fig. 3**) or fixed whole-mount embryos (**Fig. 4**). We have also stained embryos with rabbit anti-GFP antibodies (Clontech) with excellent results, although we have had less success using monoclonal anti-GFP antibodies (Clontech).

6.1. Live Drosophila *Samples*

1. Collect embryos in a vial, or on an apple juice agar plate. To ensure that most embryos are at the appropriate stage, it is helpful to do short collections (2–4 h) and then age them until the appropriate stage of development.
2. Wash eggs into a sieve placed on a plastic tray, with water and a paintbrush.
3. Dechorionate in 50% Chlorox for 3 min, then wash thoroughly with water.

4a. For an upright microscope, transfer the embryos with a paintbrush into a drop of Voltalef or Halocarbon oil placed in the middle of an air-permeable Teflon membrane stretched over a Perspex frame (designed by E. Wieschaus).

Place an 18 × 18 mm coverslip on either side of the embryos, 2–3 cm apart, to prevent them being squashed, and cover with a 22 × 40 mm coverslip. The embryos will develop normally through embryogenesis in most cases.


0 mins
20 mins
50 mins
80 mins
110 mins

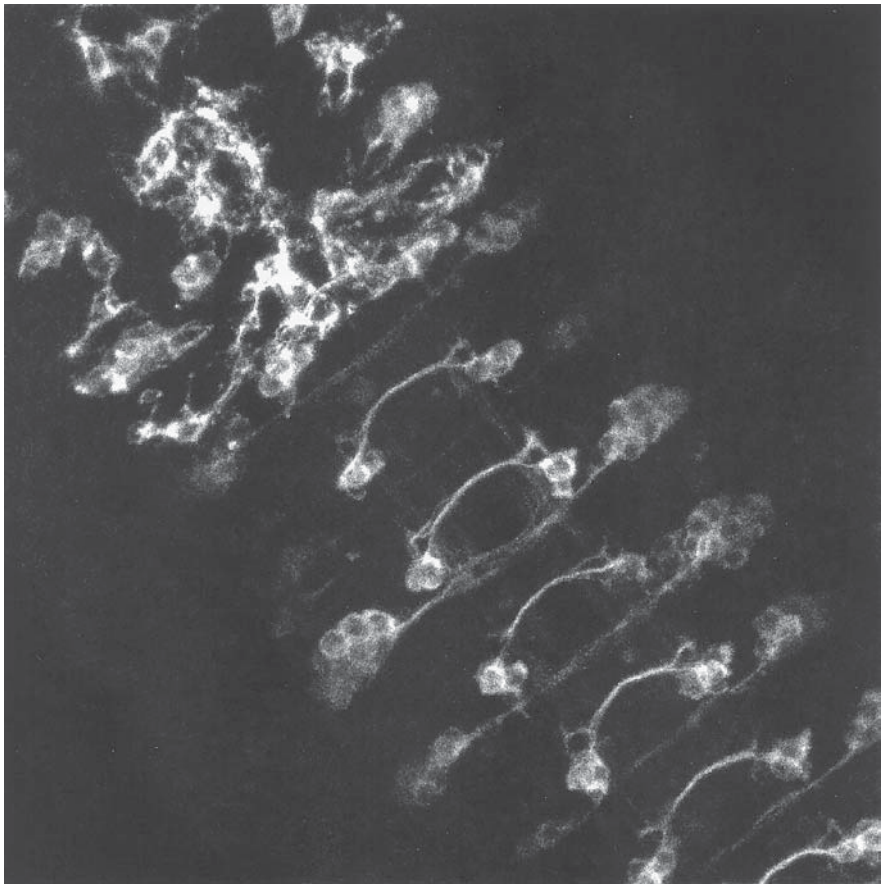


Fig. 4. GFP in neurons and glia of a fixed *Drosophila* embryo. Tau-GFP continues to fluoresce after formaldehyde fixation. In this transgenic line, Tau-GFP labels both neurons and glia in the thorax (**top**), but only neurons in the abdomen (**bottom**) ***(45)***.

We do not seal the coverslips as it is often useful to roll the embryos to obtain a dorsal, ventral, or lateral view. In addition, the solvents in many nail varnishes have been reported to inhibit GFP fluorescence ***(1)***.

4b. When using an inverted microscope, the embryos can be mounted simply by gluing them to a coverslip. Cut a square hole in the middle of a small piece of parafilm, and place this "frame" around the embryos to prevent oil running off.

Fig. 3. *(facing page)* Time-course of axon outgrowth in a *Drosophila* embryo. Neurons expressing Tau-GFP can be followed in living embryos. Tau-GFP labels cell bodies and is transported into axons ***(43)***. The axon projections grow toward and then across the ventral midline in the embryonic central nervous system.

6.2. *Fixed* Drosophila *Samples*

GFP remains fluorescent after fixation and, although real-time analysis is sacrificed, GFP expression can be correlated with endogenous gene expression. GFP fluorescence is preserved, if somewhat diminished, after formaldehyde fixation.

1. Transfer dechorionated embryos to an Eppendorf tube, and half fill with heptane.
2. Remove residual PBT and top up with 4% formaldehyde in PBT. The embryos should float at the interface between the formaldehyde and heptane. Fix for 30 min with gentle rolling.
3. Replace the formaldehyde with methanol and remove the vitelline membranes by shaking vigorously for 30 s.
4. Wash in methanol for 10 s, then replace with PBT to rehydrate the embryos. Do not leave the embryos in methanol longer than necessary as it has been reported to lead to a rapid and irreversible loss of GFP fluorescence ***(10)***.
5. Proceed with antibody staining (e.g., *see* **ref. *57***).
6. Mount embryos in Vectashield. Use two 18 × 18 mm coverslips flanking the embryos as supports, then cover with a 22 × 40 mm coverslip. We do not seal the coverslips (*see* **Subheading 6.1., step 4a**).

In our hands, GFP fluorescence does not survive *in situ* hybridization protocols, which include an overnight incubation in formamide.

6.3. Confocal Microscopy

We collect our images using a Bio-Rad MRC1024 confocal scan head and krypton/argon mixed gas laser, on a Nikon E800 upright microscope. GFP is excited by the 488nm laser line. We use a laser power between 1% and 30%, and the standard setup for imaging FITC, which uses a 522/32 emission filter (i.e., wavelengths between 506–538 nm are transmitted). The Chroma HQ500LP emission filter, which transmits light of wavelengths greater than 500 nm, gives a brighter GFP signal. We have used GFP labeling in conjunction with other vital dyes, such as DiI for cell lineage tracing and acridine orange to detect dying cells.

Embryos can be viewed with a 60× planapochromat, 1.4 NA oil immersion objective lens. To reduce spherical aberration when focusing deep into an aqueous sample, embryos are mounted in water, or another aqueous mounting medium, and viewed with a long-working distance, cover slip corrected, water immersion objective (e.g., a Nikon 60× planapochromat, 1.2 NA, working distance 220 μm) ***(17)***. Unfortunately, these objectives are extremely expensive ($7000–8000, £5000–£6000).When imaging more than 60–80 μm deep in the tissue, even under the conditions described previously, image quality degrades rapidly. To reduce noise and sharpen the image, we Kalman average between 2 and 15 frames whenever possible.

7. Notes

7.1. Movement

When movement is rapid, it is not possible to Kalman average several scans, as this blurs rather than sharpens the image. Similarly, it may not be possible to project a Z-series, as each optical section will not be aligned with its neighbors. For example, when imaging the synchronous nuclear divisions in precellular embryos, we scan a single focal plane continuously on a slow setting. When observing macrophage movements, we collect an image every 15–20 s on a slow setting, and Kalman average 2–3 frames.

As mentioned in above, we do not seal our coverslips. For this reason, we collect Z-series from the top of the sample to the bottom, thereby forcing the coverslip against the embryo. If the focus motor is set in the opposite direction, the objective can lift the coverslip, and the embryo with it. It is possible to glue the embryos to the coverslip in an appropriate orientation, using a nontoxic glue such as Scotch tape dissolved in heptane, although this may impair image quality.

7.2. Bleaching

GFP is an ideal marker for time-lapse studies because of its resistance to photo-bleaching. When examining intact embryos in their vitelline membranes, we can collect images in a single focal plane, every 15 s, over a 2-h period without a major loss of signal. When collecting a 4D series, it has been possible to collect a Z stack of optical sections every 5 min over 5 h. To limit bleaching and photo damage to the specimen we try to keep the number of scans for Kalman averaging and the number of sections per Z-series to a minimum. In living samples, cells often continue to express GFP during the period of the time course, thereby replenishing the fluorescent protein.

We do almost all of our imaging on whole-mount embryos within their vitelline membranes. In our experience, animals that have been dissected prior to viewing (e.g., as “flat preps”) tend to bleach more readily.

7.3. Making Movies

The Bio-Rad Laser Sharp confocal software allows confocal images to be converted to PICT or TIFF format. When converting large Z- or time-series, it is faster to use Confocal Assistant to generate TIFF files. These can then be exported to a Macintosh computer and assembled using Adobe Photoshop or Illustrator. To make movies that can be transferred to videotape, we assemble Z- or time-series in Adobe Premiere.

References

1. Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W., and Prasher, D. C. (1994) Green fluorescent protein as a marker for gene expression. *Science* **263,** 802–805.

2. Cubitt, A. B., Heim, R., Adams, S. R., Boyd, A. E., Gross, L. A., and Tsien, R. Y. (1995) Understanding, improving and using green fluorescent proteins. *Trends Biochem. Sci.* **20,** 448–455.
3. Prasher, D. C., Eckenrode, V. K., Ward, W. W., Prendergast, F. G., and Cormier, M. J. (1992) Primary structure of the aequorea victoria green fluorescent protein. *Gene* **111,** 229–233.
4. Prasher, D. C. (1995) Using GFP to see the light. *Trends Genet.* **11,** 320–323.
5. Gerdes, H. and Kaether, C. (1996) Green fluorescent protein: applications in cell biology. *FEBS Lett.* **389,** 44–47.
6. Davenport, D. and Nichol, J. A. C. (1955) Luminsecence in *Hydromedusae*. *Proc. Royal Society* **Series B, 144,** 399–411.
7. Morin, J. G. and Hastings, J. W. (1971) Energy transfer in a bioluminescent system. *J. Cellular Physiol.* **77,** 313–318.
8. Morise, H., Shimomura, O., Johnson, F. H., and Winant, J. (1974) Intermolecular energy transfer in the bioluminescent system of *Aequorea*. *Biochemistry* **13,** 2656–2662.
9. Bokman, S. H. and Ward, W. W. (1981) Renaturation of *Aequorea* green fluorescent protein. *Biochem. Biophys. Res. Comm.* **101,** 1372–1380.
10. Ward, W. W., Cody, C. W., Hart, R. C., and Cormier, M. J. (1980) Spectrophotomeric identity of the energy transfer chromophores in *Renilla* and *Aequorea* green fluorescent proteins. *Photochem. Photobiol.* **31,** 611–615.
11. Cody, C. W., Prasher, D. C., Westler, W. M., Prendergast, F. H., and Ward, W. W. (1993) Chemical structure of the hexapeptide chromophore of the *Aequorea* green fluorescent protein. *Biochemistry* **32,** 1212–1218.
12. Heim, R., Prasher, D. C., and Tsien, R. Y. (1994) Wavelength mutations and post-translational autoxidation of green fluorescent protein. *Proc. Natl. Acad. Sci. USA* **9,** 12,501–12,504.
13. Ormo, M., Cubitt, A. B., Kallio, K., Gross, L. A., Tsien, R. Y., and Remington, S. J. (1996) Crystal structure of the *Aequorea victoria* green fluorescent protein. *Science* **273,** 1392–1395.
14. Yang, F., Moss, L. G., and Phillips, G. N. (1996) The molecular structure of green fluorescent protein. *Nature Biotechnol.* **14,** 1246–1251.
15. Inouye, S. and Tsuji, F. I. (1994) *Aequorea* green fluorescent protein - expression of the gene and fluorescence characteristics of the recombinant protein. *FEBS Lett.* **341,** 277–280.
16. Wang, S. and Hazelrigg, T. (1994) Implications for *bcd* messenger RNA localization from spatial distribution of exu protein in *Drosophila* oogenesis. *Nature* **369,** 400–403.
17. Haseloff, J. and Amos, B. (1995) GFP in plants. *Trends Genet.* **11,** 328,329.
18. Rizzuto, R., Brini, M., De Giorgi, F., Rossi, R., Heim, R., Tsien, R. Y., and Pozzan, T. (1996) Double labelling of subcellular structures with organelle-targeted GFP mutants *in vivo*. *Curr. Biol.* **6,** 183–188.
19. Heim, R. and Tsien, R. Y. (1996) Engineering green fluorescent protein for improved brightness, longer wavelengths and fluorescence resonance energy transfer. *Curr. Biol.* **6,** 178–182.

20. Haseloff, J., Siemering, D. R., Prasher, D. C., and Hodge, S. (1997) Removal of a cryptic intron and subcellular localisation of green fluorescent protein are required to mark transgenic *Arabidopsis* plants brightly. *Proc. Natl. Acad. Sci. USA* **94,** 2122–2127.
21. Reichel, C., Mathur, J., Eckes, P., Langenkemper, K., Reiss, B., Koncz, C., Schell, J., and Maas, C. (1996) Enhanced green fluorescence by the expression of an *Aequorea victoria* green fluorescent protein mutant in mono- and dicotyledonous plant cells. *Proc. Natl. Acad. Sci. USA* **93,** 5888–5893.
22. Chiu, W., Niwa, Y., Zeng, W., Hirano, T., Kobayashi, H., and Sheen, J. (1996) Engineered GFP as a vital reporter in plants. *Curr. Biol.* **6,** 325–330.
23. Haas, J., Park, E. C., and Seed, B. (1996) Codon usage limitation in the expression of HIV-1 envelope glycoprotein. *Curr. Biol.* **6,** 315–324.
24. Pang, S. Z., DeBoer, D. L., Wan, Y., Ye, G., Layton, J. G., Neher, M. K., Armstrong, C. L., Fry, J. E., Hinchee, M. A. W., and Fromm, M. E. (1996) An improved green fluorescent protein gene as a vital marker in plants. *Plant Physiol.* **112,** 893–900.
25. Siemering, K. R., Golbik, R., Sever, R., and Haseloff, J. (1996) Mutations that suppress the thermosensitivity of green fluorescent protein. *Curr. Biol.* **6,** 1653–1663.
26. Crameri, A., Whitehorn, E. A., Tate, E., and Stemmer, W. P. C. (1996) Improved green fluorescent protein by molecular evolution using DNA shuffling. *Nature Biotechnol.* **14,** 315–319.
27. Davis, S. J. and Viestra, R. D. (1998) Soluble, highly fluorescent variants of green fluorescent protein (GFP) for use in higher plants. *Plant Mol. Biol.* **36,** 521–528.
28. Kohler, R. H., Zipfel, W. R., Webb, W. W., and Hanson, M. R. (1997) The green fluorescent protein as a marker to visualize plant mitochondria *in vivo*. *Plant J.* **11,** 613–621.
29. Cormack, B. P., Valdivia, R. H., and Falkow, S. (1996) FACS-optimized mutants of the green fluorescent protein (GFP). *Gene* **173,** 33–38.
30. Zernicka-Goetz, M., Pines, J., Ryan, K., Siemering, K. R., Haseloff, J., Evans, M. J., and Gurdon, J. B. (1996) An indelible lineage marker for *Xenopus* using a mutated green fluorescent protein. *Development* **122,** 3719–3724.
31. Zernicka-Goetz, M., Pines, J., Siemering, K. R., Haseloff, J., and Evans, M. J. (1997) Following cell fate in the living mouse embryo. *Development* **124,** 1133–1137.
32. Schuldt, A., Adams, J. H. J., Davidson, C. M., Micklem, D. R., Haseloff, J., St Johnston, D., and Brand, A. H. (1998) Miranda mediates asymmetric protein and RNA localisation in the developing nervous system. *Genes Develop.* **12,** 1847–1857.
33. Delagrave, S., Hawtin, R. E., Silva, C. M., Yang, M. M., and Youvan, D. C. (1995) Red-shifted excitation mutants of the green fluorescent protein. *Bio-Technology* **13,** 151–154.
34. Ehrig, T., O'Kane, D. J., and Predergast, F. G. (1995) Green fluorescent protein mutants with altered fluorescence excitation spectra. *FEBS Lett.* **367,** 163–166.
35. Heim, R., Cubitt, A. B. and Tsien, R. Y. (1995) Improved green fluorescence. *Nature* **373,** 663,664.
36. Stauber, R. H., Horie, K., Carney, P., Hudson, E. A., Tarasova, N. I., Gaitanaris, G. A., and Pavlakis, G. N. (1998) Development and applications of enhanced green fluorescent protein mutants. *Biotechniques* **24,** 462–471.

37. Yang, T. T., Sinai, P., Green, G., Kitts, P. A., Chen, Y. T., Lybarger, L., Chervenak, R., Patterson, G. H., Piston, D. W., and Kain, S. R. (1998) Improved fluorescence and dual colour detection with enhanced blue and green variants of the green fluorescent protein. *J. Biol. Chem.* **273,** 8212–8216.
38. Elowitz, M. B., Surette, M. G., Wolf, P. E., Stock, J., and Leibler, S. (1997) Photoactivation turns green fluorescent protein red. *Curr. Biol.* **7,** 809–812.
39. Sawin, K. E. and Nurse, P. (1997) Photoactivation of green fluorescent protein. *Curr. Biol.* **7,** 606,607.
40. Ludin, B. and Matus, A. (1998) GFP illuminates the cytoskeleton. *Trends Cell Biol.* **8,** 72–77.
41. Butner, K. A. and Kirschner, M. W. (1991) Tau protein binds to microtubules through a flexible array of distributed weak sites. *J. Cell Biol.* **115,** 717–730.
42. Callahan, C. A. and Thomas, J. B. (1994) Tau-b-galactosidase, an axon-targeted fusion protein. *Proc. Natl. Acad. Sci. USA* **91,** 5972–5976.
43. Brand, A. (1995) GFP in *Drosophila*. *Trends Genet.* **11**, 324,325.
44. Micklem, D. R., Dasgupta, R., Elliott, H., Gergely, F., Davidson, C., Brand, A., Gonzalez-Reyes, A., and St Johnston, D. (1997) *mago nashi* is required for the polarisation of the oocyte and the formation of perpendicular axes in *Drosophila*. *Curr. Biol.* **7,** 468–478.
45. Dormand, E. L. and Brand, A. H. (1998) Runt determines cell fates in the *Drosophila* embryonic CNS. *Development* **125,** 1659–1667.
46. Davis, I., Girdham, C. H., and O'Farrell, P. H. (1995) A nuclear GFP that marks nuclei in living *Drosophila* embryos; maternal supply overcomes a delay in the appearance of zygotic fluorescence. *Develop. Biol.* **170,** 726–729.
47. Ogawa, H., Inouye, S., Tsuji, F. I., Yasuda, K., and Umesono, K. (1995) Localization, trafficking and temperature-dependence of the *Aequorea* green fluorescent protein in cultured vertebrate cells. *Proc. Natl. Acad. Sci. USA* **92,** 11,899–11,903.
48. Rizzuto, R., Brini, M., Pizzo, P., Murgia, M., and Pozzan, T. (1995) Chimeric green fluorescent protein as a tool for visualizing subcellular organelles in living cells. *Curr. Biol.* **5,** 635–642.
49. Shiga, Y., Tanakamatakatsu, M., and Hayashi, S. (1996) A nuclear GFP beta-galactosidase fusion protein as a marker for morphogenesis in living *Drosophila*. *Dev. Growth Differ.* **38,** 99–106.
50. Moriyoshi, K., Richards, L. J., Akazawa, C., O'Leary, D. D. M., and Naanishi, S. (1996) Labeling neural cells using adenoviral gene transfer of membrane-targeted GFP. *Neuron* **116,** 255–260.
51. Terasaki, M., Jaffe, L. A., Hunnicutt, G. R., and Hammer, J. A. (1996) Structural change of the endoplasmic reticulum during fertilization: evidence for loss of membrane continuity using the green fluorescent protein. *Develop. Biol.* **179,** 320–328.
52. Marshall, J., Molloy, R., Moss, G., Howe, J., and Hughes, T. (1996) The jellyfish green fluorescent protein: a new tool for studying ion channel expression and function. *Neuron* **14,** 211–215.

53. Kaether, C. and Gerdes, H. (1995) Visualization of protein transport along the secretory pathway using green fluorescent protein. *FEBS Lett.* **396,** 267–271.
54. Presley, J. F., Cole, N. B., Schroer, T. A., Hirschberg, K., Zaal, K. J. M., and Lippincott-Schwartz, J. (1997) ER-to-Golgi transport visualized in living cells. *Nature* **389,** 81–85.
55. Schackwitz, W. S., Inoue, T., and Thomas, J. H. (1996) Chemosensory neurons function in parallel to mediate a pheromone response in *C. elegans*. *Neuron* **17,** 719–728.
56. Valvekens, D., Van Montagu, M., and Van Lijsebettens, M. (1988) *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. *Proc. Natl. Acad. Sci. USA* **85,** 5536–5540.
57. Patel, N. H. (1994) Imaging neuronal subsets and other cell types in whole-mount *Drosophila* embryos and larvae using antibody probes, in Drosophila melanogaster: *Practical Uses in Cell and Molecular Biology* (Goldstein, L. S. B. and Fyrberg, E. A., eds.), Academic Press, San Diego, pp. 446–485.