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Live Confocal Analysis with Fluorescently Labeled Proteins

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1. Introduction

1.1. General

The development of laser scanning confocal microscopy provides a powerful means to observe structures and components within the cell. Equally important advances have also occurred in the development of reagents and techniques for generating functional fluorescently tagged proteins. When these labeled proteins are used in conjunction with confocal and other advanced fluorescence microscopes, the dynamics of a given protein within the living cell can be readily analyzed in real time. Live analysis provides an appreciation of the cellular and developmental dynamics that is virtually impossible to obtain through observation of fixed samples. In addition, tracking down the primary defect and determining causal relationships in mutant and drug-mediated phenotypes often can be accomplished only through live fluorescence analysis. Finally, live analysis has been used to confirm the existence of structures in the embryo that were previously contested as possible fixation artifacts ***(1)***.

Although live fluorescence analysis was rarely performed only a few years ago, it is becoming much more routine, in large part due to the widespread use of the green fluorescent protein (GFP; and *see* Chapter 15). Live fluorescence analysis has also benefitted from the commercial availability of excellent fluorescence reagents and increased access to advanced fluorescence microscopes, such as the confocal. The direct tagging of purified proteins with fluorescent moieties in vitro provides an alternative strategy to fusion with GFP for analyzing protein dynamics in living embryos. Direct fluorescent labeling of pro-

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teins may be the best approach in cases in which it is difficult to express the GFP fusion protein (e.g., during early stages of embryogenesis) or when fusion with GFP interferes with protein structure or function. In addition, direct fluorescent labeling allows the simultaneous visualization of multiple fluorescent probes.

In our experience, generating a functional fluorescently labeled protein and injecting it into the cell without damage are the most technically demanding aspects of this approach. Consequently, this chapter describes a specific protocol for directly attaching fluorescent probes to antibodies for live analysis and a section on applying this procedure more generally to purified and bacterially overexpressed proteins. We then describe general principles of microinjecting fluorescently labeled proteins, using *Drosophila* embryos as a specific example. Finally, we describe the successful application of these techniques in *Drosophila* and other model organisms.

1.2. Strategies for Creating Fluorescent Conjugates

Proteins can be covalently modified using several types of chemistry ***(2,3)***. Most commonly used are fluorophore derivatives that modify amino or sulfhydryl groups. Succinimidyl esters and isothiocyanates are two types of fluorophore derivatives that have been widely used to label primary amines of proteins (lysine residues and the N-terminal amino group). Succinimidyl esters are now more frequently used than isothiocyanates because they react more specifically, more quickly, at lower pH, and result in more stable products that do not deteriorate during storage. Sulfonyl chlorides, such as lissamine rhodamine B sulfonyl chloride and Texas red sulfonyl chloride are highly reactive and unstable in water, especially at the higher pH required to react with aliphatic amines, making reproducible conjugation difficult. Succinimidyl ester derivitives of these, which are more suited to the conditions required for reproducible conjugation reactions, have now been developed. The sulfhydryl groups of cysteine residues can be labeled using iodoacetic acid derivatives. Because cysteines are relatively rare in proteins, cysteine labeling can be more uniform than labeling of protein primary amines. In addition, cysteines can be engineered into proteins to provide a convenient target for labeling ***(4)***.

However, because cysteines are rare and often critical for protein function, random lysine labeling using succinimidyl esters of fluorophores has proven more generally applicable. Although preparing the optimal conjugates suitable for the most critical assays may require extensive experimentation, it is often relatively easy to conjugate fluorophores to proteins in a manner sufficient for visualization in vivo. Described below are two labeling protocols that we have successfuly employed to label antibodies and expressed fusion proteins with the succinimidyl esters of commonly used fluorophores.

2. Materials

1. 0.1*M* Na_2CO_3 pH 9.3
2. 2*M* potassium glutamate (pH 8.0). (This can be made by adjusting the pH of glutamic acid with potassium hydroxide.)
3. Small spin columns containing about 850–1000 µL of desalting gel (such as Bio-Gel P-6 from Bio-Rad) and *see* **Note 1**.
4. 25 m*M* stock of fluorophore dissolved in dimethyl sulfoxide (DMSO). For long-term storage of unreacted fluorophore at –80°C, the DMSO must be of high quality and dry. The fluorophore concentration should be determined by absorbance using the appropriate extinction coefficient because the values obtained by dissolving a weighed amount of dye are often inaccurate.
5. SM-2 polystyrene beads (Bio-Rad; cat no. 152-3920). Beads are prewashed in methanol, before equilibriating in the protein buffer.
6. Random IgG for pilot reactions (*see* **Subheading 3.1.**) can be purchased from many sources such as Jackson ImmunoResearch or Sigma.
7. Injection buffer: 50 m*M* K-glutamate (~pH 7.0), 0.5 m*M* $MgCl_2$, 1 m*M* glutathione.
8. Here are some useful sites on the Internet.
   a. The home page for Molecular Probes, provider of fluorescent reagents: http://www.probes.com/
   b. A very comprehensive listing of confocal microscopy resources, many movie and image links: http://www.cs.ubc.ca/spider/ladic/confocal.html
   c. Confocal images and movies of living cells from the Terasaki lab home page: http://www2.uchc.edu/htterasaki
   d. Integrated Microscopy Resource at University of Wisconsin, useful microscopy links, several images, and movies: http://www.bocklabs.wisc.edu/imr/home.htm
   e. Collection of images from the Biosciences Imaging group, University of Southampton: http://www.neuro.soton.ac.uk/research.info.html
   f. Images and movies from the Advanced Microscopy Imaging Laboratory, SUNY: http://corn.eng.buffalo.edu/www/confocalImages/readme.html
   g. Sedat lab home page including movies of nuclei and chromosomes: http://util.ucsf.edu/sedat/
   h. Bowerman lab home page with *Caenorhabditis elegans* confocal movies: http://eatworms.swmed.edu/Worm_labs/Bowerman/
   i. Home page of the Strome lab, with fluorescent *C. elegans* confocal movies: http://sunflower.bio.indiana.edu:80/~sstrome
   j. Home page of the Sullivan lab, with fluorescent *Drosophila* confocal movies: http://www-biology.ucsc.edu/people/billllab/main.html
   k. Home page of the Mitchison lab with a tour of cell motility: http://skye.med.harvard.edu/

l. The kinesin home page containing movies of cell motility: http://www.blocks.fhcrc.org/~kinesin/

3. Methods

3.1. Generating Fluorescently Labeled Antibodies

The protocol described in this section utilizes commercially available succinimidyl esters of available fluorophores that provide a simple and efficient means of covalently attaching the fluorophores to proteins. Several fluorophores are available with excitation/emission maxima closely matching the krypton/argon laser emission lines (488 nm, 568 nm, 647 nm) and optical filters of commonly used confocal microscopes. The properties of several of these fluorophores are described in **Table 1**.

The required buffers and materials are listed in **Subheading 2**. Because the reactivity of the fluorophores varies from batch to batch (and may decline during storage), a pilot reaction with random IgG should first be performed to determine the appropriate labeling conditions. The protocol described below is for a small-scale labeling reaction (100 µL of antibody at concentrations between 0.5 and 20 mg/mL) and is performed at room temperature. This reaction can be scaled up as required (for **steps 1** and **5**, larger prepacked desalting columns are available (*see* **Subheading 2**).

1. Equilibrate a centrifuge desalting column (*see* **Subheading 2**) into coupling buffer (0.1*M* Na_2CO_3, pH 9.3). Apply 100 µL of the antibody solution (between 0.5 and 20 mg/mL) to the column and spin to elute the antibody into a microfuge tube. This procedure results in minimal dilution and excellent recovery of the antibody in coupling buffer.
2. Add 1 µL of fluorophore solution (in DMSO; *see* **Subheading 2**) to the antibody solution. Mix by gently flicking the tube (avoid bubbles) and *see* **Note 2**.
3. Incubate at room temperature for 30 min.
4. Add 10 µL of 2*M* potassium glutamate (pH 8.0). The free amino group on the glutamate quenches the reaction.
5. Apply the antibody to a second centrifuge column preequilibrated in injection buffer if the antibody will be used for live analysis or phosphate-buffered saline (PBS) if the antibody is to be used for immunofluorescence of fixed samples. This separates the labeled antibody from the uncoupled fluorophore and exchanges the antibody into a buffer suitable for injection or storage and *see* **Note 3**.
6. Remove a 10-µL aliquot from the labeled-antibody solution and store in the dark at 4˚C. As described in the next step, this will be used to determine the stoichiometry of labeling. For live analysis, divide the remainder of the labeled-antibody into 2-µL aliquots freeze in liquid nitrogen, and store at –80°C. If using for immunofluorescence of fixed samples, divide into 10 ul aliquots and snap freeze.
7. Dilute the 10 µL of labeled antibody to a final volume of 100–150 µL with injection buffer and take an absorption spectrum of this sample. **Figure 1** depicts

Table 1
Fluorophores Compatible with the Krypton/Argon Laser (Laser Lines at 488, 568, and 647 nm)

Fluorophore	Excitation	Emission	Extinction coefficient	$\varepsilon_{D280}/\varepsilon_{DM}$
Fluorescein[a]	495 nm	519 nm	74,000 $M^{-1}cm^{-1}$	0.19
Oregon green 488	495	521	76,000	0.19
Cy3	550	570	150,000	0.08
Tetramethylrhodamine[b]	546	576	95,000	0.21
Rhodamine red-X	560	580	129,000	0.17
X-Rhodamine[b]	574	602	78,000	0.20
Texas red-X	583	603	116,000	0.15
Cy5	649	670	250,000	0.05

All data was obtained from Molecular Probes ***(2)*** and from Amersham.
5- (and 6-) Carboxyfluorescein, succinimidyl ester (Molecular Probes, C-1311)
Oregon green 488 carboxylic acid, succinimidyl ester 5-isomer (Molecular Probes, O- 6147)
5- (and 6-) Carboxytetramethylrhodamine, succinimidyl ester (Molecular Probes, C-1171)
5- (and 6-) Carboxy-X-rhodamine, succinimidyl ester (Molecular Probes, C-1309)
Rhodamine red-X, succinimidyl ester, mixed isomers (Molecular Probes, R-6160)
Texas red-X, succinimidyl ester, mixed isomers (Molecular Probes, T-6134)
Cy3-OSu Monofunctional reactive fluorophore (Amersham PA13100).
Cy5 -OSu Monofunctional reactive fluorophore (Amersham PA13600)

[a]The absorption and fluorescence of fluorescein are pH dependent, for details see pp. 552,553 of the Molecular Probes Catalog ***(2)***. The values given here are at pH 9.0. Both absorption and emission are significantly reduced below pH 7.0.

[b]The values for these dyes given here are based on spectra taken in MeOH. The absorption and emission spectra in pH 8.0 buffer are red shifted ~8 nm and the extinction coefficients are ~ 10% lower ***(2)***.

Also *see* **Notes 10–13**.

absorption spectra for a protein labeled at two different stoichiometries. The stoichiometry of labeling is calculated by determining the ratio of the molar concentration of the fluorophore and the molar concentration of the protein (described in detail in **Fig. 2**).

Although the optimal stoichiometry will depend on the precise application and the fluorophore, we have found that for live analysis of *Drosophila* embryos or immunofluorescence of fixed samples, a fluorophore to antibody stoichiometry between 1 and 4 produces satisfactory results. If the stoichiometry is too high or low, vary the concentration of fluorophore added in **step 2** accordingly.

3.2. Generating Fluorescently Labeled Proteins

Fluorescent labeling of purified proteins can be accomplished using conditions similar to those described for antibody labeling. Because individual proteins behave differently, labeling protocols vary from protein to protein. A key issue in covalent labeling of proteins is to ensure that the labeled protein retains

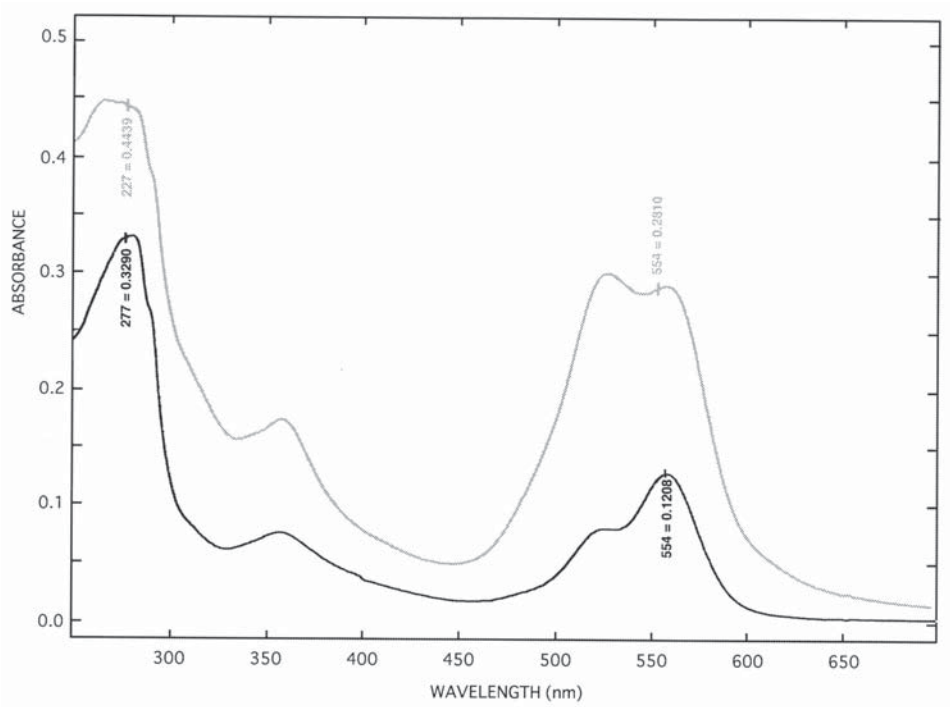

Fig.1. The absorption spectra of the same protein labeled at a fluorophore to protein molar ratio of 1 (**solid line**) and 5 (**stippled line**), respectively.

functionality (*see* **Note 5** on quality control). As many proteins are less stable than antibodies, the conditions described in the protocol below are milder than those used for antibody labeling.

1. We begin with protein in 50 m*M* Na phosphate pH 8.0, 250 m*M* NaCl, 1 m*M* 2-mercaptoethanol. The last step of our fusion protein purification was Superose-6 gel filtration chromatography (Pharmacia). This step served to buffer exchange the protein, to remove aggregated protein and proteolysis products, and was essential to obtain good localization of our fusion proteins. Other buffers and salt concentrations, optimized for your protein, can be used in the labeling reaction, but avoid buffers that contain primary amines (such as Tris) or strong reducing agents [such as dithiothreitol (DTT)]. In buffers below pH 8.0, the labeling reaction will proceed more slowly and it is also possible to get side reactions between the fluorophore and cysteine thiols.
2. To 75 µL of fusion protein (1–5 mg/mL) in 50 m*M* phosphate pH 8.0, 250 m*M* NaCl, 1 m*M* 2-mercaptoethanol, add 0.75 µL of 25 m*M* fluorophore dissolved in

Fig. 2. *(facing page)* Formulae for calculating fluorophore: protein labeling stoichiometry and extinction coefficients of novel proteins.

Figure 2

Calculation of Labeling Stoichiometry

Labeling stoichiometry is calculated by determination of the fluorophore to protein molar ratio. The value reflects the average number of fluorophore molecules conjugated to each protein molecule.

The fluorophore concentration in the sample is determined by dividing the absorbance of your sample at the absorbance maximum for the fuorophore by the extinction coefficent of the fuorophore at that wavelenght.

$$[\text{Fluorophore}] = A_{DM}/\varepsilon_{DM}$$

where A_{DM} is the absorbance of your sample at the absorption maximum for the labeling fluorophore (e.g., 495 nm for fluorescein) and ε_{DM} is the extinction coefficient of the fluorophore at its absorbance maximum (e.g., 74,000 $M^{-1}cm^{-1}$ for fluorescein; *see* **Table 1**).

The protein concentration in your sample is determined by taking the absorbance of your somaple at 280 nm, subtracting the absorbance of the fluorophore at 280 nm, and then dividing this by the extinction coefficient of your protein at 280 nm (details on calculating the extinction coefficient of your protein from its sequence will be given below).

$$[\text{Protein}] = [A_{280} - \text{ADM} \times (\varepsilon_{D280}/\varepsilon_{DM})]/\varepsilon_P$$

where A_{280} is the absorbance of your sample at 280 nm, ADM is the absorbance of your sample at the absorption maximum for the labeling fluorophore, ε_{D280} is the extinction coefficient of the fluorophore at 280 nm, ε_{DM} is the extinction coefficient of the fluorophore at its absorbance maximum, and ε_P is the extinction coefficient of your protein at 280 nm.

Figure 1 depicts the spectra for the same protein labeled with rhodamine at stochiometries of approx 5 (**stipple**) and 1 (**solid**). Note the appearance of the characteristic absorption doublet of fluorophore in the overlabeled spectrum (**stipple**). The absorbance at 522 nm is caused by the fluorescence quenching due to noncovalent dimers of rhodamine bound to the same protein molecule. When labeling with rhodamine, this effect can occur even at relatively low labeling stoichiometries (less than 1) because this fluorophore is particularly prone to dimer formation, even in solution. The absorbance peak shoulder at about 355 nm is another rhodamine absorbance peak.

Determining the Extinction Coefficient of Your Protein at 280 nm

Gil and Von Hippel ***(24)*** have shown that it is possible to estimate the extinction coefficient of native proteins from their amino acid composition with a standard error of about 5% in most cases. For native proteins:

$$\varepsilon_{280}\ \text{nm} = [\text{no. of tryptophan molecules}) \times 5690 + (\text{no. of tyrosine molecules}) \times 1280 + (\text{no. of cysteine molecules}) \times 120] \times 1.05\ M^{-1}cm^{-1}$$

DMSO. As in the antibody labeling protocol, the amount of fluorophore added is varied to attain the desired labeling stoichiometry.
3. Incubate on ice for 5 min.
4. Add 7.5 µL of potassium glutamate (pH 8.0) and 0.75 µL of 0.5*M* DTT (optional) to stop the reaction.
5. Apply the labeling reaction to a centrifuge column equilibrated in 50 m*M* HEPES, 100 m*M* NaCl (pH 7.6), to remove free fluorophore and to transfer the protein to the buffer used for injection and *see* **Note 4**.
6. The stoichiometry of labeling is assayed by spectroscopy (**Figs. 1** and **2**). If the protein is over- or underlabeled, repeat the procedure varying the amount of fluorophore added accordingly. Proteins were labeled to a stoichiometry of approximately 0.5 fluorophore molecules per protein. We generally label proteins to lower stochiometries to our antibodies to prevent interference with function.
7. Freeze the labeled protein in 2-µL aliquots in liquid nitrogen and store at –80°C.

3.3. Preparing and Loading Injection Needles

1. Injection needles are constructed from 75-mm glass tubing with an outer and inner diameter of 1.21 mm and 0.90 mm, respectively (Drummond Scientific, Broomall, PA cat. no. N-51A).
2. A mechanical needle puller is used to draw the tubing to a fine closed point. Typically three or four needles are drawn and examined on a compound microscope. Those that form a smooth unbroken tip are choosen for injection. These are stored on a clay mount in a Petri dish maintained at 4°C.
3. The needle is backloaded using a 10-µL Hamilton syringe precooled to 4°C. As described in the previous section, the labeled proteins are stored in 2-µL aliquots at –80°C. Immediately prior to injection an aliquot is rapidly thawed and microfuged for 3 min at 10,000 rpm. This pellets denatured or aggregated protein and reduces the chance of the needle clogging. A Hamilton syringe is used to draw the labeled protein from the microfuge tube and backload it into the syringe. From this point, as much as possible, the loaded needle is maintained in the dark at 4°C.
4. The most technically demanding aspect of the injection procedure is generating a small hole, approximately 3–5 µm in diameter, at the tip of the needle. For reference syncytial embryonic nuclei are 5 µm in diameter. To achieve this the needle is mounted in the microinjection apparatus and pressurized. As shown in **Fig. 3A,B**, the needle is gently brought in contact with a glass slide covered with halocarbon oil (Series 77, CAS no. 9002-83-9, Halocarbon Products Corp.). A small break is readily detected because fluid immediately flows from the needle and is clearly visible in the oil.
5. Once this is achieved, the needle is removed from the injection apparatus and stored in the dark at 4°C. Kept in this manner, the loaded needle will suffice for a full day of microinjection.

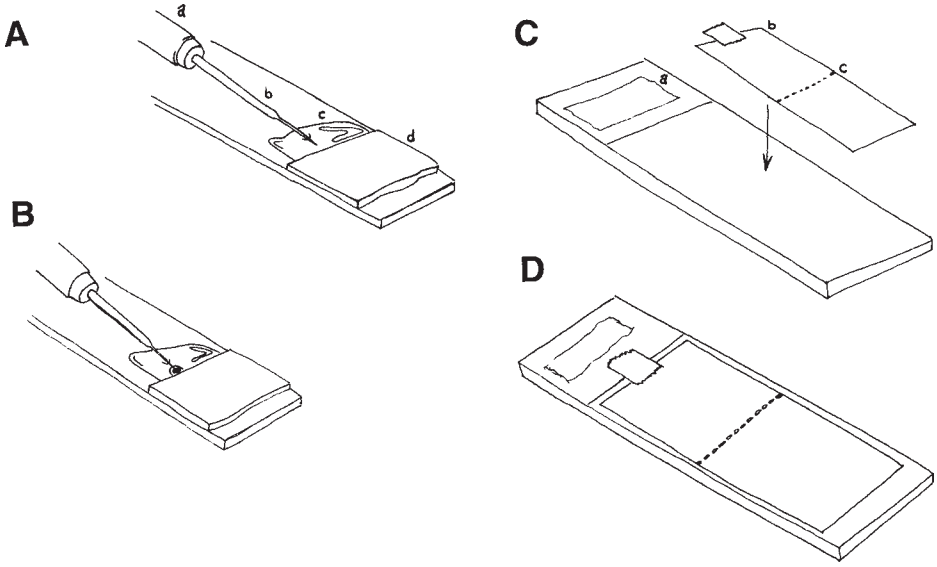

Fig. 3. Setup for breaking the tip of microinjection needles **(A,B)**. a: needle holder; b, microinjection needle; c, halocarbon oil. Preparation of samples for microinjection **(C,D)**. a, double-stick tape; b, coverslip; c, glue.

3.4. Preparing and Injecting Samples

Our experience has been primarily with *Drosophila* embryos studied with an inverted microscope. As many aspects of this procedure will more generally apply to other systems we describe it here (*see* **Note 5**).

1. So that the samples remain stably attached to the coverslip during injection and recording, we prepare a solution of clarified glue. From 5–10 mL of heptane and 12 or more inches of double-stick tape (3M) are added to a 50-mL conical tube and allowed to rotate overnight. The solution is aliquoted into 1.5-mL centrifuge tubes and microfuged to remove particulate matter. The consistency of the solution should be such that it can be easily micropipeted. Over time the heptane evaporates and the solution becomes viscous. Additional heptane is required to thin the solution.
2. To prepare embryos for injection, a slide is constructed with a small piece of double-stick tape at one end and a temporarily taped coverslip at the other end. A pipetman is used to trace 2 µL of glue solution along a thin line on the coverslip (**Fig. 3C,D**).
3. Twenty to thirty, 1–2-h-old *Drosophila* embryos are removed from a collection plate and placed on the piece of double-stick tape. These embryos are hand dechorionated and 10–15 embryos are placed in a line along the film of glue.
4. In order to prevent bleeding when the embryos are injected, they are placed in a drying chamber for 8–14 min (a sealed plastic dish containing Drierite serves as

an effective drying chamber). The drying time varies from 8 to 15 min owing to day-to-day variation in temperature and humidity. One must be careful to not to overdry the embryos because this will create recording artifacts. One sign of overdrying is stretch marks and indentations along the embryo.

5. Immediately after the embryos are dried, they are covered with oil and microinjected. The most critical aspect of manual microinjection is needle pressure. To obtain the correct needle pressure, the tip of the needle is placed in the oil near the embryos and pressure is applied until a small bubble of injection fluid is observed in the oil. Pressure is adjusted until one can observe a steady gradual increase in bubble volume. The needle is then pulled away from the bubble. If the pressure is correct no fluid flows from the needle. When the needle is reintroduced into the bubble fluid again flows and the bubble increases in volume. With this pressure, the injected embryos absorb enough fluid to replace that lost by dehydration. Using this procedure, we typically inject approximately 1–5% egg volume with fluorescently conjugated protein.
6. Once injected, the coverslip is removed from the slide and is ready for microscopy (*see* **Notes 6–9**).

4. Notes

1. Columns are sold prepacked (Bio-Rad; cat no. 732-6002) and used according to the manufacturer's instructions. Alternatively, you can buy the small Bio-Spin disposable plastic columns (Bio-Rad; cat no. 732-6008) and resin (Bio-Rad; cat no. 150-0738) separately and pack them yourself. A stock of desalting gel can be prepared by autoclaving 16 g of dry Bio-Gel P-6 resin in 200 mls of distilled water. This slurry may be stored at room temperature for extended periods of time. To pour a small centrifuge column load 1600–2000 µL of the autoclaved slurry (about 850–1100 µL of packed resin) into the column. During this process the columns can be supported by placing them in 13 × 100-mm glass test tubes. Equilibrate the column by pipetting 2 × 1.5 mL of the desired buffer onto the resin and allowing it to flow through by gravity. Put the column into a 17 × 100 mm plastic snap cap tube (with the cap removed) that contains a 1.5-mL microfuge tube. Remove excess buffer by spinning at top speed in a clinical centrifuge for 4 min. Load your sample onto the resin and centrifuge as before, collecting the flowthrough (your protein) in a fresh 1.5-mL microfuge tube.
   If the reactions in **steps 1–5** are to be scaled up for larger volumes, Econo-Pac 10G desalting columns (Bio-Rad, cat. no. 732-2010) may be used. These are convenient for sample volumes between 1 and 3 mL.
2. To determine the appropriate fluorophore concentration for the labeling reaction, we first set up three test reactions using a concentration of random IgG similar to that of the antibody to be labeled. To these reactions we add 1 µL of either 25 m*M*, 8 m*M*, or 3 m*M* fluorophore solution. We select a concentration that results in a 1:4 molar ratio of fluorophore to protein (*see* **Subheading 3.1.7**). If none of the initial fluorophore concentrations result in the desired stoichiometry, further test reactions are performed.

3. (Optional) If free flurophore has not been adequately removed for your application, the antibody can be incubated at 4°C for 1 h with an equal volume of SM-2 polystyrene beads that have been first washed in MeOH and then equilbrated in injection buffer. The labeled antibody can be tested for the presence of free fluorophore by addition of sodium dodecyl sulfate (SDS) to 0.5% followed by precipitation of the protein with five volumes of cold ethanol and analyzing the supernatant for the presence of flurophore (*see* determination of absorbance spectra below).
4. (Optional) If free fluorophore has not been adequately removed, the protein can be incubated at 4°C for 1 h with an equal volume of SM-2 polystyrene beads, as described in the previous section for separating antibody from uncoupled fluorophore.
5. Labeling damage to the protein and phototoxicity due to fluorescent excitation are two main concerns when using fluorescently labeled proteins in vivo. With well-characterized proteins, functional assays can often be used to determine if the activity or biochemical characteristics of a protein are perturbed by labeling. Often this is not possible with uncharacterized proteins, so we ensure that the labeling stoichiometry is less than one fluorophore per polypeptide. We routinely centrifuge the protein prior to microinjection (**step 3**, preparing and loading needles), to remove precipitated, denatured protein. A number of methods have been applied to ensure that only active protein is injected into the embryo and where applicable these assays have been employed to determine protein activity after labeling ***(5)***. In the case of cytoskeletal proteins, such as actin and tubulin, this has been accomplished by selecting active protein by multiple cycles of assembly and disassembly after labeling ***(6,7)***. The DNA binding proteins, histones and topoisomerase II, were labeled while bound to DNA cellulose to protect functionally important sites from modification ***(5,8)***.

   The concentration of injected protein is another concern; we have found that injecting proteins with concentrations greater than 10 mg/mL may perturb normal development. This concentration artefact and photo-induced damage can be detected by mitotic failures and aberrations in other cellular processes, most often centered around the injection area where protein concentration is the highest. To reduce the extent of photo-induced damage, one can reduce the frequency of image acquisition and attenuate the beam intensity by increasing the neutral density filter. Finally, we verify the results of our live recordings through fixed analysis, as these techniques complement one another ***(9)***. Although it is difficult to analyze dynamic processes using fixed material, large sample numbers are readily obtained. These images should correspond closely to the live studies. Applying fixed analysis in conjunction with live studies is especially important when studying mutant and drug-induced abnormal phenotypes.

   Live fluorescence analysis of the early embryonic divisions in the *Drosophila* embryo has proven a useful tool understand to unravel the primary defects that occur due to mutation mutants or drug treatments ***(9–12)***. Below we give three examples taken from our research that illustrate the application of this technique.

6. We injected rhodamine-labeled tubulin *(7)* into *Drosophila* embryos and captured confocal images every 10–30 s to study microtubule dynamics during the synchronous cortical embryonic divisions (**Fig. 4**). During prophase of nuclear cycle 11 bright asters of centrosome nucleated microtubules are observed either side of the embryonic nuclei, which appear as dark circles **(A)** due to the exclusion of tubulin monomers by the intact nuclear envelope. As the nuclear envelope breaks down during prophase, an influx of tubulin into the nuclear space occurs **(B)**. As metaphase proceeds, increased labeling of the mitotic spindle is observed, owing to the increase in density and length of microtubules between the pairs of sister centrosomes **(C–E)**. As chromosomes separate during anaphase the labeled spindles increase in length **(F)**. As the embryo enters telophase and the nuclear envelopes surrounding the sister nuclei reform, tubulin is again excluded from the nuclear space **(G)**. Strong tubulin labeling of prominent midbodies between sister nuclei and of duplicating centrosomes at the poles is observed. As each nucleus enters interphase the astral microtubule staining moves out the plane of focus as a consequence either of centrosomal migration or nuclear rotation **(H)**.
7. We have also used these techniques to examine the behaviour of chromosomes during the *Drosophila* cortical nuclear cycles, using rhodamine-labeled histones *(8,13,14)* as fluorescent probes (**Fig. 5**). During late prophase the DNA condenses **(A)** and the chromosomes begin to align on the metaphase plate **(B)**. During metaphase the chromosomes are maximally compacted **(C)**, before beginning to separate synchronously during anaphase **(D)**. During late anaphase sister chromosomes are completely separated and individual chromosome arms may be discerned **(E)**. The DNA decondenses during telophase as illustrated by the more diffuse histone labeling **(F)**. The DNA remains decondensed during the short intervening interphase **(G)**, before the chromosomes begin condensation once more, as the nuclei synchronously enter the next prophase **(H)**.
8. CP190 is a 1096 amino acid *Drosophila* centrosomal protein that oscillates in a cell-cycle-specific manner between the nucleus during interphase, and the centrosome during mitosis *(15–17)*. To characterize the regions of CP190 responsible for its nuclear and centrosomal localizations, we made a series of 6 X His fusion proteins spanning CP190. The purified fusion proteins were fluorescently labeled with rhodamine using the protocol described in this chapter, injected into syncytial *Drosophila* embryos, and followed using time-lapse fluorescence confocal microscopy *(18)*. Injection of labeled fusion proteins resulted in one of three localization patterns, examples of which are shown in **Fig. 6**. In each case, micrographs were selected from a time-lapse series taken of a portion of the embryo's surface. Fusion protein CP190 a (containing CP190 amino acids 167–321) localizes to nuclei during interphase and to the cytoplasm during mitosis (**Fig. 6**, leftmost panels). The fusion protein CP190 b (CP190 amino acids 266–608) localizes to centrosomes throughout the cell cycle (**Fig. 6**, middle panels) and a fusion protein spanning both of these regions (CP190 amino acids 167–608) localizes to nuclei during interphase and to centrosomes during mitosis (**Fig. 6**,

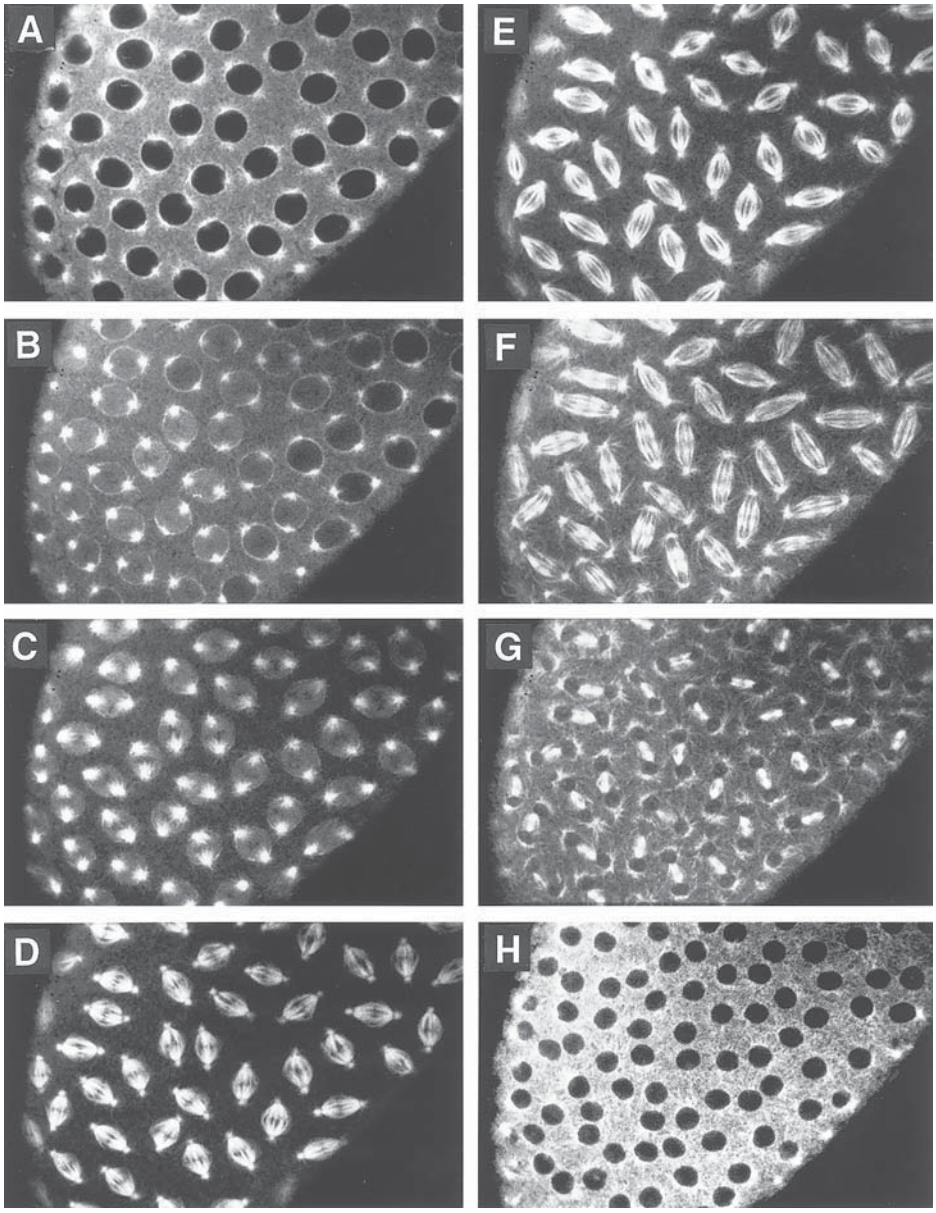

Fig. 4. Analysis of tubulin dynamics in living *Drosophila* embryos (Reprinted from *The Journal of Cell Biology*, by copyright permission of the The Rockerfeller University Press).

CP190 a+b, rightmost panels). Using this type of analysis, we were able to identify the 19 amino acid nuclear localization signal responsible for the nuclear

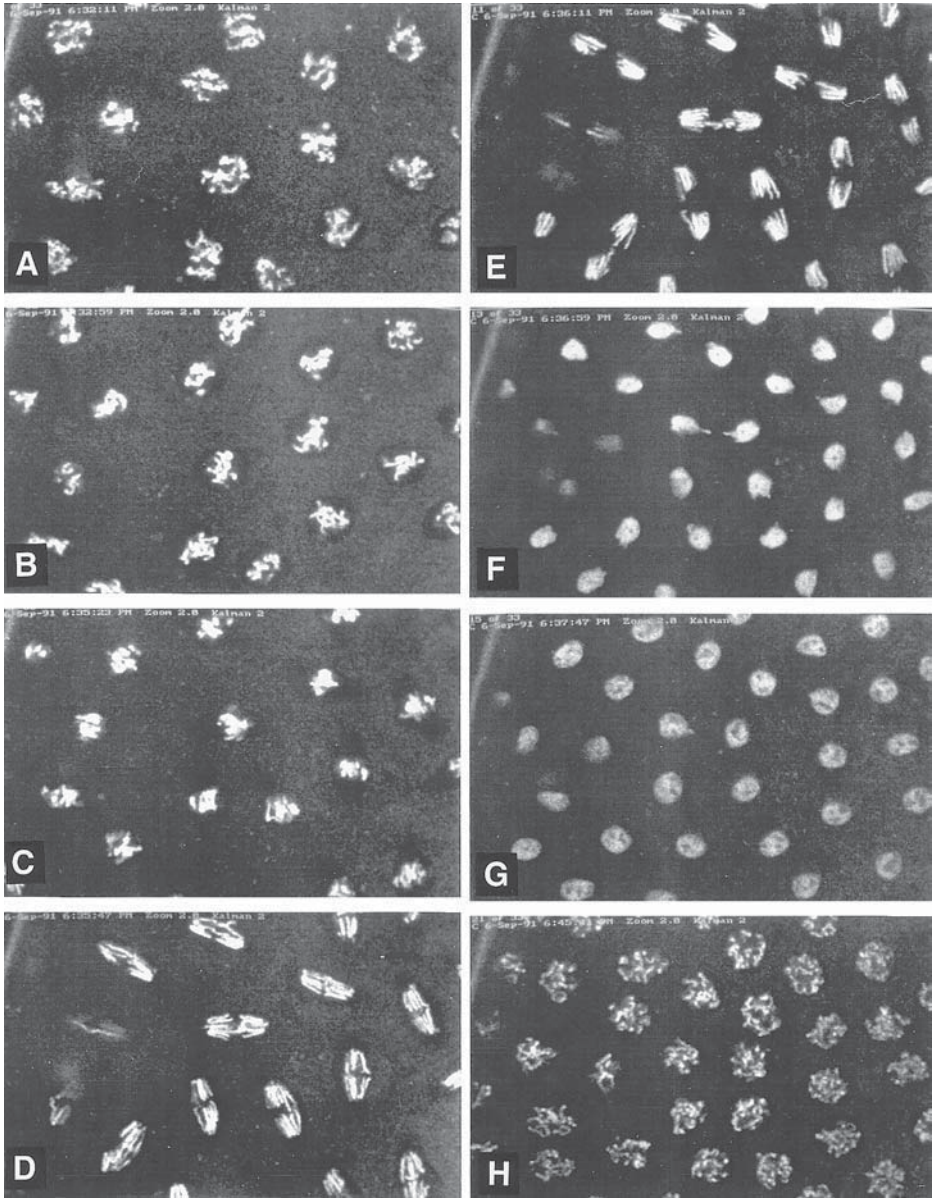

Fig. 5. Analysis of chromosome behaviour using labeled histones in living *Drosophila* embryos. (Reproduced from *Molecular Biology of the Cell* [1993], Vol.4, pp. 885–8960, with permission of the American Society for Cell Biology.)

localization of our fusion proteins and a region of 124 amino acids near the center of the protein that is sufficient to confer robust centrosomal localization.

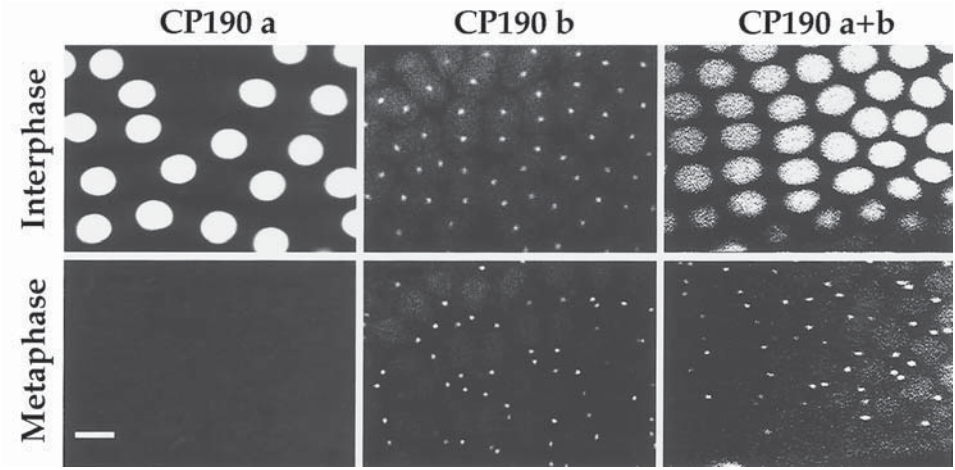

Fig. 6. Domain analysis of the centrosomal protein, CP190 in living *Drosophila* embryos.

9. The technique of microinjecting fluorescently labeled proteins into living samples and following their dynamics by confocal microscopy has also been performed in other model systems. The reorganization of the cytoskeleton during meiosis and premature cytoplasmic streaming has been studied by microinjection of *Drosophila* oocytes ***(19,20)***. The behavior of cortical actin ***(1)*** and the segregation of germ granules ***(21)*** have been examined in living *C. elegans* embryos by microinjection of oocytes with labeled actin and an anti-P granule antibodies, respectively. In the *Xenopus* embryo, live confocal analysis of eggs injected with rhodamine tubulin was used to examine the relationship between microtubules and cortical rotation ***(22)***. Labeled tubulin has also been injected into the zebrafish zygote and used to monitor cytokinesis of early blastomeres using live confocal microscopy (S. Jesuthasan, personal communication), and into the giant squid axon to monitor slow axonal transport ***(23)***.
10. Oregon green 488 is a new fluorophore that has the same absorption and emission spectra as fluorescein, but is more photostable, has a greater resistance to photobleaching and is less pH dependent.
11. Other fluorophores such as the Molecular Probes series of BODIPY™ fluorophores, have been developed but may be significantly quenched upon conjugation to proteins. Derivitives of these fluorophores containing spacer arms may prove valuable for preparing conjugates with proteins in the future.
12. For single labels, we prefer Oregon green, Cy3, and Cy5. Tetramethylrhodamine is a less expensive alternative to Cy3 that is also commonly used. For double labels, we prefer Oregon green with Texas red, X-rhodamine, or Cy5. The emission spectra of Cy5 and fluorescein are better separated, but Cy5 is barely visible to the naked eye so for many applications Texas red or X-rhodamine is preferable. Tetramethylrhodamine or Cy3 can also be used for double labeling with

fluorescein or Oregon green, but the emission spectra of these dyes overlap more significantly, making signal separation difficult.

13. Although very useful because of their spectral properties, we have found Texas red-X and X-rhodamine the most difficult to use for antibody labeling because of their hydrophobicity. Texas red-X appears to work better than X-rhodamine and is fine if used at labeling stoichiometries less than 1.2 (dye/ antibody).

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