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Imaging Gene Expression Using Antibody Probes

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1. Introduction

The merger of multiple immunoflurescent labeling and the laser scanning confocal microscope (LSCM) has greatly enhanced experimentation in many areas of biomedical research. Recently, genetic tools have become available for marking individual cells, or cells within a tissue of genetically mosaic animals ***(1,2)***. These tools have been primarily developed in *Drosophila* and utilize protein epitopes for which commercial antibodies are available, such as the *myc* epitope (***2***, ATCC and Oncogene Research) or an epitope in the CD-2 protein (***3***, Serotec). Similarly, cell lineage experiments have been conducted in *Drosophila* ***(4,5)***, as well as in other organisms such as *Caenorhabditis elegans* ***(6)*** or zebrafish ***(7,8)***, in which cells express either β-galactosidase or green fluorescent protein (GFP), are physically labeled with dextran coupled to a fluorophore ***(8)*** or are labeled with fluorescent lipophilic dyes, such as diI ***(9)***. Multiple labeling experiments are then performed comparing the expression of these cell markers with endogenous protein(s) of interest.

In this chapter I present methods for sample preparation, single- and multiple-antibody labeling of such samples, and subsequent analysis using the LSCM. I have focused primarily on using whole-mounted *Drosophila* imaginal discs as an example throughout. *Drosophila* imaginal discs are the larval precursors to adult tissues such as the eyes, antennae, wings, and legs. During larval life the imaginal discs are located internally, and therefore need to be dissected out for antibody staining. However, these techniques can be widely applied to a variety of other specimens, namely whole-mount embryos (invertebrate or vertebrate) or sectioned material. The recently published chapter by Patel ***(10)*** on imaging neuronal cell types in *Drosophila* embryos and larvae contains excellent details on preparing embryos for antibody staining. In addi-

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tion, it is quite likely that most immunohistochemical or immunofluorescent labeling techniques can be adapted for use with the LSCM.

Primary antibodies can be generated against a protein of interest in a variety of ways, and in several different animal species ***(11–13)***. Polyclonal antisera are most often purified in some manner (i.e., affinity purification), but may also work adequately in immunofluorescent labeling as crude whole sera ***(12)***. Monoclonal antibodies are produced as either tissue culture supernatants or ascites fluid ***(11,12)***. Even though antibody reagents may be quantitated to a given protein concentration (or may be distributed from commercial sources or other research laboratories with a suggested working dilution), the optimal working concentration of each primary antibody often needs to be determined experimentally for a particular application, such as the LSCM. Some antibody reagents can be used at lower concentrations (or higher dilutions) than recommended, simply because of the increased sensitivity of the confocal microscope. For multiple labeling experiments (**Subheading 4.**) determining the optimal antibody concentration for each antibody is especially important. As with all protein solutions, antibody reagents are susceptible to bacterial growth and degradation. If a reagent is not supplied with either sodium azide or thimerisol, add sodium azide to a final concentration of 0.02% as soon as possible after receiving an antibody in solution (or upon rehydrating a lyophilized antibody). If it is unclear whether these bacterial inhibitors are present, add azide as a precaution, as excess azide is preferable to none. Harlow and Lane ***(12)*** and Scopes ***(14)*** provide additional detailed methods for storing protein solutions, such as antibodies, to retain maximal protein activity.

Perhaps the single greatest advantage of employing the LSCM to antibody labeling experiments is the ability to analyze multiply labeled samples. This chapter details methods for multiple antibody labeling when primary antibodies are produced in different animal species (e.g., rabbit and mouse), as well as methods for multiple antibody labeling using primary antibodies raised in the same species (mouse). In addition, the use of fluorescent dyes that bind to particular subcellular structures are also described and incorporated into multiple labeling protocols. Specific examples will be provided for these types of labeling experiments that will also highlight the comparison of two coexpressed proteins in the *Drosophila* eye imaginal disc, and the analysis of protein expression within genetically marked (with the myc epitope) eye disc cells.

Samples for multiple antibody labeling are isolated and prepared as described in **Subheading 3.1.** Before carrying out multiple labeling experiments, working experimental conditions for each antibody reagent should be determined individually (*see* **Subheading 3.2.**). However, it may still be necessary to match antibody signals to each other in trial multiple labeling experiments. This is especially true if one protein is expressed more strongly than

another or if the titer of a primary antibody is much better than another. Be sure that individual antibody experiments are identical in such parameters as fixation, blocking and buffer conditions, increasing the odds that the multiple labeling will work well and making it easier to troubleshoot any problems that do arise. Potential problems such as incompatibility of epitope fixation or reagent crossreactivity are addressed in detail in **Subheading 4**.

Owing to the large number of antibodies made in either rabbits or mice, it is sometimes advantageous to do multiple labeling using two or more antibodies from the same host species (i.e., two monoclonals). This can sometimes be accomplished by doing antibody labeling steps sequentially. Although this technique is not successful for all combinations of antibodies, it is definitely worth trying. In additional, performing multiple antibody labeling sequentially, rather than simultaneously (**Subheading 3.4.**), can circumvent some reagent crossreactivity problems. An example of sequential labeling is provided in this section using two monoclonal antibodies, anti-myc (ATCC) and anti-22C10 (a *Drosophila* neuronal antigen, ***15***); both monoclonals are tissue culture supernatants. Sequential monoclonal antibody labeling has also been successfully utilized in standard immunohistochemical antibody labeling ***(16)***.

2. Materials

1. 10× Phosphate-buffered saline (PBS) (for 1 L): 18.6 m*M* NaH_2PO_4 (2.56 g), 84.1 m*M* Na_2HPO_4 (11.94 g), 1.75 *M* NaCl (102.2 g). This stock solution is stable at room temperature. For 1× PBS, dilute 1:10 with deionized (d) H_20 and pH to 7.4 with NaOH or HCl. IX PBS is also stable at room temperature for long periods.
2. PEMF fixative: 0.1*M* PIPES (pH 6.9), 1 m*M* EGTA, 2 m*M* $MgSO_4$, 1.0% Nonidet p-40 (NP-40). These ingredients can be made as a stock solution and stored at room temperature. Add formaldehyde just prior to each experiment (final concentration 2–4% depending upon the antigen). Formaldehyde (37% w/v) from Sigma Chemical Co. (Catalog No. F1635) is commonly used but contains methanol. For methanol-sensitive epitopes, use formaldehyde from Polysciences, Inc. (Catalog No. 04018) that is methanol-free.
3. PBS–PFA fixative: 4.0% paraformaldehyde (PFA), 1× PBS. Weigh 4 g of PFA (Polysciences, Inc. Catalog No 00380) and add to 90 mL of dH_20 that has been heated to 50–60°C and is stirring as PFA is added (this is best done in a fume hood). Add 10 µL of a 10*N* NaOH solution as PFA does not go into solution unless it is basic. It may be necessary to wait 10–20 min for the solution to clear completely. When all of the PFA appears to be in solution, add 10 mL of 10× PBS stock solution. If a bit of insoluble material remains, filter the solution by pouring it through Whatman filter paper. Check the pH of the solution with litmus paper, rather than a pH meter, to avoid PFA contamination of the electrode. If necessary, adjust the pH to 7.4 with HCl. Store this solution at 4°C, in a glass container, protected from the light. This solution is good for up to a month.

4. PLP fixative: 2% PFA, 0.01*M* $NaIO_4$, 0.075*M* lysine, 0.037*M* $NaPO_4$ (pH 7.2). Make an 8% PFA solution as described for PBS-PFA fixative but omit the addition of PBS (make it up in just water). While the 8% PFA is cooling, dissolve 0.36 g of lysine in 10 mL of H_20, 7.5 mL of 0.1*M* $NaPO_4$ (pH 7.2), 2.5 mL of 0.1*M* Na_2HPO_4 on ice. Immediately before use, mix 15 mL of buffered lysine solution, 5 mL PFA, and add 50 mg of $NaIO_4$. This solution is made fresh each time, and chilled prior to sample addition.
5. Block buffer*: 50 m*M* Tris (pH 6.8), 150 m*M* NaCl, 0.5% NP-40, 5 mg/mL bovine serum albumin (BSA). Store at 4°C. This solution is good as long as no bacterial growth occurs. The shelf life can be extended to months by the addition of sodium azide to a final concentration of 0.02%.
6. Wash buffer*: 50 m*M* Tris (pH 6.8), 150 m*M* NaCl, 0.5% NP-40, 1 mg/mL of BSA. Store at 4°C. This solution is usable as long as no bacterial growth occurs. To prevent bacterial growth, thereby extending the shelf life add sodium azide to a final concentration of 0.02%.
7. Mounting buffer*: 50 m*M* Tris (pH 8.8); 150 m*M* NaCl; glycerol to desired percentage (10–90%). Store at room temperature. Glycerol can support bacterial growth, so take good care of glycerol stocks by autoclaving them or by dedicating a bottle to tissue mounting.
8. 5× *p*-Phenylenediamine (PDA) stock: 30 mg of PDA dissolved in 4 mL of dH_20; add 6 mL of 100% glycerol and mix well. Store aliquoted at –20°C and keep in the dark as much as possible. For 1× solution, make fresh for each use by diluting the 5× stock with 50 m*M* Tris (pH 8.8), 150 m*M* NaCl five-fold. PDA solutions (either 5× or 1×) are not stable over time (weeks to months), especially in glycerol solutions less than 80%. Therefore it is advisable to make fresh 5× PDA stocks often (weekly for 10% glycerol mounting). PDA can be obtained from Sigma (Catalog No. P1519).
9. *n*-Propyl gallate mounting solution: 0.5% *n*-propyl gallate, 70–80% glycerol in 1× PBS. Dissolve in PBS, then add glycerol. Mix well. Store aliquoted at –20°C. More stable than PDA solution (which discolors after several weeks to a month at 4°C or room temperature). Order from Sigma (Catalog No. P3130).
10. DABCO mounting solution: 2.5% DABCO, 70–80% glycerol in 1× PBS. Dissolve in PBS, then add glycerol. Mix well. Aliquot and store and –20°C. Also more stable than PDA. DABCO can be obtained from Sigma (Catalog No. D2522).
11. Suppliers
    a. American Type Culture Collection (ATCC), Maryland, USA
    b. Molecular Probes, Oregon, USA
    c. Amicon, Massachusetts, USA
    d. Oncogene Research Products, Massachusetts, USA

*These solutions could also be adapted to a PBS-based buffer system. Block buffer: 1× PBS, 0.5% NP-40, 5 mg/mL of BSA. Wash buffer: 1× PBS, 0.5% NP-40, 1 mg/mL of BSA. Mounting Buffer: 1× PBS/10% glycerol.

e. Corning Costar Corporation, Massachusetts, USA
f. Polyscience Inc., Pennsylvania, USA
g. Fine Science Tools Inc., California, USA
h. Serotec Ltd., Oxfordshire, England
i. Jackson Immunoresearch Laboratories, Pennsylvania, USA
j. Sigma Chemical Company, Missouri, USA
k. Ted Pella Inc., California, USA
l. Vector Laboratories Inc., California, USA

3. Methods

3.1. Tissue Preparation

1. Third instar larvae or pupae are briefly washed in 1× PBS to remove any food matter that may be stuck to their outer cuticles.
2. Several larvae at a time are transferred to a depression well slide containing fresh PBS buffer. Individual larvae are bissected along the anterior/posterior axis using fine forceps (Dumont No. 5, Fine Science Tools, Catalog No. 11250-20) and the tail portion is discarded. The head portion is inverted inside out. Eye/antennal discs and four of the six leg discs are attached to the brain and central nervous system (CNS) by ganglia. The wing discs, haltere discs and the remaining two leg discs are attached both to the body wall and to the CNS by ganglia. Unwanted fat, digestive tract tissue, and salivary glands are carefully removed with the forceps. What remains are the imaginal discs, the CNS and the head cuticle. This complex is transferred to 1 mL of ice-cold fixative already present in a well of a 48-well tissue culture plate (Corning Costar Corp. Catalog No. 3548). Up to 20 disc complexes can be placed in one well, using the cuticle as a "handle" to grasp each complex with forceps.
3. Disc complexes are fixed for varying amounts of time depending upon the age of the animal dissected (20–30 min for larvae; up to 2 h for pupae) on ice (*see* **Notes 1–3**).
4. After fixation, tissues are incubated in a blocking buffer to prevent nonspecific binding of the primary antibody. Imaginal discs are blocked for at least 45 min either on ice or at 4°C. This is accomplished by transferring the disc complexes to another well of the 48-well plate containing 1 mL of blocking buffer (*see* **Notes 4–8**).

3.2. Single Immunofluorescent Labeling

1. After *Drosophila* imaginal disc complexes have been fixed and blocked, they are transferred to a well containing appropriately diluted primary antibody in wash buffer. Because primary antibodies are often of limited quantity, this step is done at a lower volume (400 µL) and overnight at 4°C. For antibodies that are used at very low concentrations, it may be advantageous to first make an intermediate dilution (in wash buffer). This intermediate dilution should be stable at 4°C for at least a few weeks, provided sodium azide (0.02%) has been added to it (*see* **Notes 9** and **10**).
2. Imaginal discs are washed 4 × 20 min by transferring them with forceps through four wells, each containing 1 mL of Wash Buffer. Washes are done at 4°C. Prior

to beginning these washes, recover the diluted primary antibody for reuse. Antibodies can often be used three or more times (one particular polyclonal antibody was used five times before a noticeable decrease in signal was observed). Store diluted primary antibodies for reuse in the same manner as intermediate dilutions (*see* **Notes 11** and **12**).

3. Transfer imaginal discs to a well containing the appropriate diluted secondary antibody in 400 µL of wash buffer. Incubate discs at 4°C for several hours (or overnight for large or thick samples). If the secondary antibody is conjugated to a fluorochrome, keep samples in the dark as much as possible by covering the plate or tube with foil (*see* **Notes 13–15**).
4. Discs are washed 4 × 15 min at 4°C.
5. If a directly conjugated secondary antibody is used, proceed to **Subheading 3.5.**, for equilibration of stained material in a mounting medium. If the secondary antibody is biotinylated or if a triple antibody sandwich is being used, transfer discs to a well containing the appropriately diluted (in wash buffer) tertiary reagent (*see* **Note 16**).
6. Incubate for 1–2 h at 4°C. Reduce the volume of the tertiary antibody solution as described for primary antibody incubation. Keep the samples in the dark as much as possible; this is most easily accomplished by wrapping the 48-well plate with foil.
7. Wash discs 4 × 15 min at 4°C also keeping the samples in the dark as much as possible. Proceed to **Subheading 3.5.** for equilibration of imaginal discs in mounting medium.

3.3. Double and Triple Antibody Labeling in Parallel

1. Two primary antibodies raised in different host animal species, such as rabbit polyclonal anti-*Drosophila* Atonal (***17*** and **Fig. 1A**) and mouse monoclonal anti-Daughterless (***18*** and **Fig. 1B**) are added simultaneously to their predetermined final dilutions in 400 µL of wash buffer, e.g., mouse anti-Daughterless to 1:50 dilution and rabbit anti-atonal to 1:5000. Imaginal discs are incubated in this solution at 4°C overnight (*see* **Notes 17–19**).
2. As with single immunofluorescent labeling, mixtures of primary antibodies can also be saved for reuse by adding sodium azide (0.02%) and storing at 4°C.
3. Imaginal discs are washed 4 × 20 min by transferring them through four wells each containing 1 mL of wash buffer at 4°C.
4. A mixture of secondary antibodies is made in 400 µL of wash buffer. In the double label experiment with anti-atonal and anti-daughterless, the secondary reagents are a 1:200 dilution of goat anti-rabbit biotin (Vector Labs) and a 1:200 dilution of rat anti-mouse IgG (Jackson Immunoresearch). Note that both immunofluorescent signals are being built up employing the two different methods (*see* **Notes 20–23**).
5. Incubate imaginal discs at 4°C for 1–2 h.
6. Discs are washed 4 × 15 min at 4°C.
7. Likewise a mixture of tertiary antibodies is made in 400 µL of wash buffer. For the given example, 1:200 streptavidin–rhodamine (Vector Labs) and 1:100 goat anti-rat-fluorescein isothiocyanate (FITC) (Jackson Immunoresearch) are used.

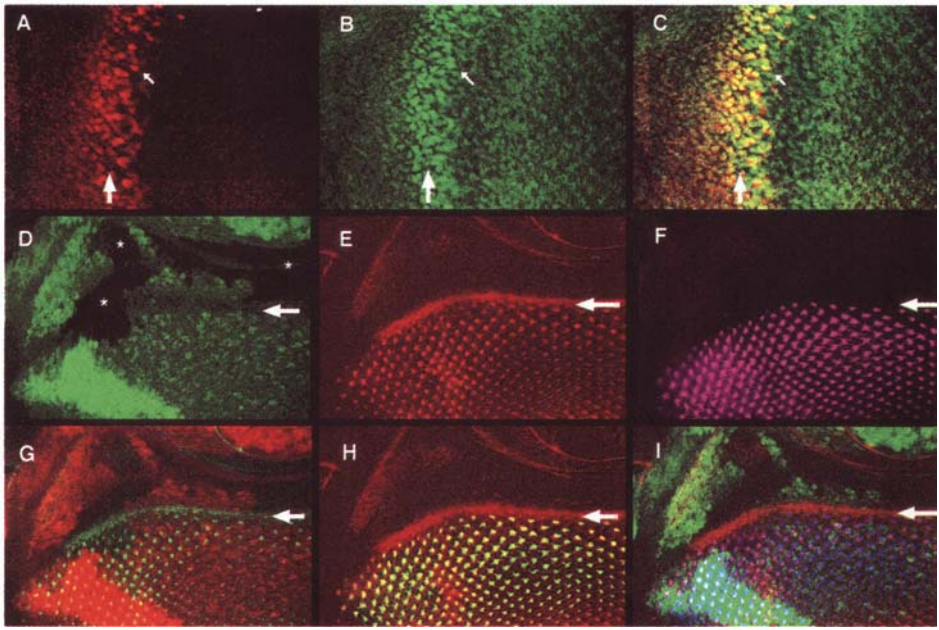

Fig. 1. Double and triple antibody labeling of *Drosophila* eye imaginal discs. **(A–C)** Confocal images of a wild-type eye disc stained with rabbit anti-Atonal (red in A,C) and mouse anti-Daughterless (green in B,C). Single images are shown in A,B and the confocal merge in C. The **small arrow** in all three panels points to a nucleus stained by both antibodies. **(D–I)** Confocal images of a third instar eye disc containing both mutant and mosaic *hairy* tissue. This disc was triple labeled with anti-myc (D,G,I), rhodamine–phalloidin (E,G,H,I), and 22C10 (F,H,I). Single label images are presented in D–F, two different merges of double labeling are shown in G,H, and the triple merger is in panel I. In D, three populations of cells are marked by the myc epitope. The brightest green cells, to the lower left, are homozygous wild-type and all lower level green cells are heterozygous for the *hairy* mutation. Those cells not expressing myc (black areas marked with asterisks) are homozygous mutant for *hairy*. Myc expression is green in D,I and red in G. Phalloidin staining is red in E,H,I and green in G. 22C10 expression is pink in F, green in H, and blue in I. In all panels, anterior is up and the **arrow** marks the position of the MF (*see* Color Plate II).

8. Incubate imaginal discs in this solution for 1 h at 4°C, keeping the 48-well plate wrapped in foil.
9. Wash imaginal discs 4 × 15 min at 4°C, keeping the plate wrapped in foil as much as possible. Proceed to **Subheading 3.5.** for preparation of labeled discs for LSCM analysis.
10. For triple-labeled samples simply add the third set of reagents to each antibody incubation step, as long as the primary antibodies have been made in three differ-

ent host species (e.g., rabbit, rat, mouse). If triple sandwiches are used, remember that the biotin–streptavidin system can be used only once and check for crossreaction between all secondary and tertiary reagents by performing all the pairwise double labelings in practice experiments. Multiple labeling experiments using more than one primary antibody from the same host species are addressed in **Subheading 3.4**.

3.4. Double and Triple Antibody Labeling in Series

1. Samples are isolated and prepared as described in **Subheading 3.1**. The first monoclonal (in this example, anti-myc) is diluted 1:5 in 400 µL of wash buffer and imaginal disc complexes are incubated in this solution overnight at 4°C.
2. Imaginal discs are washed 4 × 20 min by transferring them through four wells, each containing 1 mL of wash buffer. Washes are done at 4°C. The primary antibody can be saved for reuse.
3. Secondary antibody preparation and incubation are as described in **Subheading 3.2., step 3**. For mouse anti-myc, donkey anti-mouse biotin (Jackson Immunoresearch) at a dilution of 1:50 was used.
4. Disc complexes are washed 4 × 15 min at 4°C.
5. Tertiary antibody dilution and sample incubation were performed as described in **Subheading 3.2., step 5**. Streptavidin–fluoroscein (Jackson Immunoresearch) at a dilution of 1:200 was used. Rhodamine–phalloidin (Molecular Probes) was included with this last antibody incubation step at a concentration of 250 nM for a triple label. Incubation was extended for 3 h at 4°C, as this was previously determined to provide optimal rhodamine–phalloidin signal (*see* **Notes 24** and **25**).
6. Wash discs 4 × 20 min at 4°C, keeping the samples protected from the light by wrapping the 48-well plate in foil in this and all subsequent steps.
7. Imaginal disc complexes are taken from the last wash step into the second primary antibody dilution (in this case, 22C10 diluted 1:200 in a volume of 400 µL). Incubation is also done overnight at 4°C.
8. Discs are washed 4 × 20 min at 4°C.
9. Secondary antibody dilution and sample incubation are performed as described in **Subheading 3.2., step 5**. For detecting 22C10 labeling, goat anti-mouse IgG-Cy5 (Jackson Immunoresearch) was used at a dilution of 1:50.
10. Wash discs 4 × 20 min at 4°C.
11. Proceed to **Subheading 3.5.** for mounting imaginal discs and **Subheading 3.6.** for merging confocal images. The imaginal discs in the provided example are triply labeled for myc epitope expression using fluorescein (**Fig. 1D**), 22C10 expression using Cy5 (**Fig. 1F**), and phalloidin conjugated to rhodamine (**Fig. 1E**), which labels the actin cytoskeleton, thereby highlighting the morphology of eye imaginal disc cells (**Fig. 1I**).

3.5. Preparing Labeled Samples for LSCM Analysis

1. After the last washing step in each of the labeling protocols, transfer imaginal disc complexes to 1 mL of Tris/glycerol solution (*see* **Subheading 2.1.**). For

very flat mounted preparations of imaginal discs (especially the eye disc) use 10% glycerol. For other specimens, e.g., embryos, use 50–90% glycerol (*see* **Note 26**).

2. Allow samples to equilibrate in glycerol solutions for at least 1 h at room temperature (for immediate mounting) or at 4°C overnight. Keep samples protected from the light by wrapping them in foil.
3. Glycerol-equilibrated samples can be stored at 4°C for several weeks, or at –20°C for months protected from the light. For –20°C storage, the glycerol content must be greater than 10% to prevent samples from freezing. Do not conduct longer term sample storage in the presence of anti-photobleaching agents (*see below*) as they can be unstable.
4. Free radical scavengers reduce or eliminate rapid fluorescent signal fading or photobleaching, which results from either epifluorescent or laser light illumination. Therefore, just prior to LSCM viewing and analysis (within a day or so), incubate imaginal disc complexes in an anti-photobleaching agent for 30–60 min at either room temperature or 4°C, wrapping them in foil.
5. Imaginal disc complexes are dissected apart and arranged for final mounting on a microscope slide. To accomplish this, one well of imaginal discs at a time is transferred to a clean microscope slide in a puddle of mounting buffer. If the puddle of mounting buffer begins to dry up during the mounting procedure, keep adding small amounts to discs to ensure that they do not dry out.
6. Using small tungsten wire dissection needles [Ted Pella Inc. Catalog No. 27-11; see Patel ***(10)*** for a detailed protocol of needle preparation] dissect imaginal discs apart from the remaining cuticle, brain and other unwanted larval body parts. If necessary, dissect imaginal discs apart from each other.
7. For flat mount preparations (preferable with eye imaginal discs) remove with forceps all unwanted body cuticle (as it is much thicker than the discs) and place all other debris off to one side (this will act to "trap" unwanted air bubbles; *see below*). Arrange discs in their final positions on the slide.
8. Carefully lower a 22 × 22 No. 1 coverslip (thickness should be matched to the objective lens used) onto the imaginal discs. To avoid having the discs move around during this process, use only a small amount of mounting buffer.
9. Once the coverslip is in place, add mounting buffer dropwise from one side of the coverslip, if there is too little buffer. Excess buffer can be carefully wicked away, using a tissue paper labwipe gently touching one side of the coverslip. Coverslips should be lowered at an angle to avoid trapping air bubbles around the discs. Often bubbles that do appear will congregate around debris left to one side of the discs. Bubbles trapped adjacent to specimens can sometimes be freed by gently tapping on the coverslip.
10. Coverslipped slides should be sealed to avoid evaporation of mounting media. Fingernail polish works well for this, but it should be colorless (clear) as many pigmented nail polishes (auto)fluoresce.
11. Slides are ready for microscope/LSCM analysis when the nail polish is dry or can be stored at 4°C protected from the light.

3.6. LSCM Collection, Merging and Analysis of Antibody Labeled Samples

1. Fluorescent images shown in **Fig. 1** were collected using a Bio-Rad MRC600 Laser Scanning Confocal Microscope (Bio-Rad Microscience, Hercules, CA) equipped with a krypton/argon laser (American Laser Co., Ltd., Salt Lake City, UT), which has three major lines at wavelengths 488 nm (fluorescein), 568 nm (rhodamine), and 647 nm (Cy5).
2. Eight to ten single optical planes were Kalman averaged, normalized, and collected at a resolution of 8 bits.
3. The images were transferred to a Macintosh Quadra computer with a 24-bit color card, assigned specific colors, then merged into a single image using the Adobe® Photoshop 3.0 (Adobe Software) program. Details of image collection and storage will depend upon the microscope, LSCM, and computer(s) used.
4. **Figure 1** contains two examples of *Drosophila* eye imaginal disc multiple antibody labeling. In the first example, double labeling with two nuclear antibody proteins, rabbit anti-Atonal (**Fig. 1A**) and mouse anti-Daughterless (**Fig. 1B**) was used to demonstrate a stripe of cells (the merging of red and green creates yellow) in **Fig. 1C** that coexpress both proteins. This type of data presentation is not different from standard immunofluorescence with one important exception. The cells coexpressing both proteins are located within a physical depression of the eye imaginal disc known as the morphogenetic furrow (MF). In addition, because some cells within the MF undergo differentiation into neuronal photoreceptor cells, their nuclei are located in different focal planes from each other and/or from nuclei of undifferentiated cells. By analyzing this double label preparation with the LSCM, it was possible to optically section through the MF region of the eye disc at different depths and determine unambiguously that all MF cells expressing Atonal (**Fig. 1A**) also express high levels of Daughterless (**Fig. 1B**).
5. The second multiply labeled eye disc (**Fig. 1D–1I**) is a mixture of homozygous wild-type cells (brightest green cells in **Fig. 1D**), cells heterozygous for a mutation in the *Drosophila hairy* gene (intermediate green cells in **Fig. 1D**), and homozygous mutant (*hairy*–) cells (those cells not fluorescing, asterisks in **Fig. 1D**). The green fluorescence indicates the presence of a myc epitope, whose expression in this larva marks cells containing one or two copies of the normal *hairy* gene product. This mosaic eye disc was also assayed for expression of two commonly used eye development markers, monoclonal antibody 22C10, which labels differentiating photoreceptor cells (pink cells in **Fig. 1F**), and rhodamine–phalloidin (red labeling in **Fig. 1E**), which binds to actin in all eye disc cells and highlights the position of the MF (arrow in **Fig. 1C–1I**). Two combinations of these labeled images are presented in **Fig. 1G,H** and the triple merger of all three images is shown in **Fig. 1I**. Note the color substitution in these panels.
6. Different image combinations can be built using the Photoshop program to facilitate analysis of the overlap of different antibody signals (**Fig. 1G,H**) or to consolidate this information into a single panel, conserving space for journal presentation (**Fig. 1I**). The final color used for each image is a matter of choice in

the Photoshop program. The myc epitope is green in **Fig. 1D**, accurately reflecting its labeling with fluorescein, but red in **Fig. 1G**. Likewise, 22C10, which was labeled with Cy5, is pink in **Fig. 1F** but green in **Fig. 1H** and blue in **Fig. 1I**.

4. Notes

4.1. Tissue Preparation

1. The choice of fixative also contributes substantially to proper preservation of the antigenic site(s) of many proteins. Several different fixatives can be used for preparing LSCM samples, including PEMF, often used for nuclear proteins; PLP, often used for cell surface or extracellular proteins; and PBS-PFA, which I have used with some success for nuclear, cytoplasmic, or extracellular proteins (*see* **Subheading 2.2.** for detailed buffer recipes). These choices of fixatives are provided only as rough guidelines. Some extracellular proteins are fixed quite well in PEMF and some nuclear antibody experiments work adequately using PLP. Exact fixation conditions often need to be determined by trial and error for each antigen of interest. A good rule of thumb is to choose one fixative that works well for preserving the majority of epitopes of interest and try this protocol first whenever testing new antibodies (e.g., fix *Drosophila* imaginal discs in PEMF for 20 min on ice), then vary this experimental parameter only as needed. This is especially important for multiple labeling experiments.
2. An important consideration in determining the time of fixation is the epitope itself. Tissue or cellular morphology may not be preserved adequately by short fixation times; alternatively epitopes can be destroyed if the fixation is too long. Therefore, the time of fixation may need to be determined empirically. This and subsequent steps could also be performed on embryos or tissues in 1.5-mL microcentrifuge tubes or, by reducing the volumes, could be used to stain sectioned materials on slides.
3. Underfixation can cause high background and overfixation can result in low or no signal. The type of fixative, the concentration of fixative, and the length of time samples are fixed can all be varied to correct this problem.
4. The blocking step can be extended to several hours (I have blocked tissues for up to 8 h with no adverse effect), but this is done only as a matter of convenience. No improvement in the quality of antibody staining has been noted when extended blocking steps were performed. Either BSA or whole animal sera can be used to block nonspecific antibody binding, and some antibody protocols use a combination of both. From 1 to 5 mg/mL of good quality BSA (fraction V, Sigma Chemical Co.) works as well as animal sera (1–10% is often used), but either is adequate
5. If animal sera is used as a blocking agent, it is commonly from the same species that was used to make the secondary antibody. For example, use normal goat serum if the secondary antibody is goat anti-mouse IgG (in this instance the primary antibody would be a mouse monoclonal antibody made against a *Drosophila* protein). Although it is probably not critical that the blocking sera be species matched to the secondary antibody host animal, this can be a source of nonspecific background if the blocking sera contains an IgG molecule that the secondary

antibody can recognize. Heat sera at 56°C for 30 min to inactivate complement. Filter warm serum through a 0.22-µm filter and store aliquoted at –20°C in a nondefrosting freezer.

6. In these *Drosophila* experiments, washing steps between fixation and blocking have not been necessary, most likely because imaginal discs are only several cell diameters thick, facilitating rapid equilibration when discs are transferred between solutions. However, thicker and/or larger tissues may require several (two to three) brief (5–15 min) washing steps in between fixation and blocking. Likewise, imaginal discs do not need agitation in any of the steps described, but good quality antibody labeling of larger tissues and/or embryos may require gentle agitation on a shaking or rocking table or end-over-end rotation within a closed tube.
7. *Drosophila* imaginal discs are prepared (dissected, fixed, and blocked) fresh for each labeling experiment, but other samples are very amenable to being prepared ahead of time and stored. For example, both *Drosophila* ***(10)*** and zebrafish embryos ***(19)*** can be fixed and stored in a nondefrosting –20°C freezer in ethanol or methanol for several weeks to months (I have successfully used both of these type of embryos in antibody staining experiments after storage of longer than 1 year at –20°C). For those epitopes that are sensitive to alcohol incubations and insensitive to long fixation times, it may also be possible to store fixed material in fixative at 4°C. All stored samples are rehydrated, washed, and blocked just prior to primary antibody application. In addition, sectioned material is often prepared ahead of time and stored at either room temperature (e.g., paraffin embedded) or at –20°C (e.g., cryosectioned material) prior to antibody labeling. As for fixation conditions, the ability to use tissues and samples prepared in advance will need to be determined experimentally for each antibody of interest.
8. Larger tissues or embryos may require the addition of 1% dimethyl sulfoxide (DMSO) (Sigma Catalog No. D5879) to fixation, blocking, and all antibody incubation steps. DMSO facilitates large molecules passing through the cell membrane.

4.2. Single Immunofluorescent Labeling

9. The concentration of primary antibody used is also an important factor for the success of antibody labeling experiments. To determine empirically the optimal concentration to use, pick a broad dilution range for initial experiments. Polyclonal antibodies (and ascites) are often used in the dilution range of 1:100 to 1:50,000. Tissue culture supernatants from hybridoma cell lines typically are used in the range of undiluted to 1:500. If a hybridoma supernatant is still weak when it is used undiluted, concentrate the supernatant by using Centricon protein microconcentrators (Amicon, Catalog No. 42409). At least a 50-fold concentration can be achieved in this manner.
10. The length of time that samples are incubated with primary antibodies can also be widely varied. If the signal is low, try lengthening the incubation time to up to 24 h. If the background is a problem, antibody binding can be shortened to as little as 1 hour at either 4°C or room temperature.

11. Nonspecific antibody signal may also be reduced by preabsorption of primary antibodies. This may particularly help polyclonal antibodies. Dilute an antibody four- or five-fold in wash buffer, add fixed experimental samples, and incubate for 1 hour up to overnight with gentle mixing. For *Drosophila* imaginal discs, this could be either an extra well of fixed disc complexes or fixed embryos. After preabsorption, remove the antibody solution and dilute to the desired final concentration or store at 4°C with 0.02% azide added. Discard the material used for preabsorption.
12. Background signal may also be lowered by the addition of a second blocking step after excess primary antibody has been washed away and just prior to the addition of a secondary antibody reagent.
13. Secondary antibodies are made by immunizing host animals against either whole immunoglobulin proteins or portions of these proteins [e.g., $F(ab')_2$ fragments]. If it is known whether a primary antibody is IgG or IgM, be sure to select a secondary antibody that is appropriate. If this information is unknown, many vendors offer secondary reagents that recognize both. For most purposes, reagents made against whole immunoglobulin molecules work well, but those antibodies made against the variable $F(ab')_2$ fragment sometimes lower nonspecific background.
14. The optimal concentration of a secondary reagent may need to be determined empirically, using the manufacturer's recommended dilution as a starting point. Many antibody reagents arrive lyophilized and can be stored indefinitely in this form at 4°C. Reconstitute them according to the accompanying instructions, centrifuge the solution (to remove insoluble particles), and transfer the supernatant to a fresh tube. Antibody reagents should be stored according to the manufacturer's recommendations, which often suggest aliquoting a reagent and storing all but one working aliquot at either –80°C or –20°C in a nondefrosting freezer. The working aliquot is kept at 4°C and is stable for weeks to months. In addition, some researchers store their antibody reagents solely at 4°C with no apparent loss of activity. The most important aspect of reagent storage is to avoid repeated freeze-thawing, which can rapidly lower the titer of antibodies.
15. Many antibody labeling experiments work well using a secondary antibody directly conjugated to a fluorochrome. The fluorochrome used is usually a matter of choice, or based upon the filters available on fluorescent microscopes and/or LSCMs. Commonly used fluorochromes are FITC, lissamine–rhodamine, Texas red, and more recently Cy5 and Cy3.
16. A weak fluorescent signal can often be improved by building up the signal through the subsequent addition of another antibody reagent that specifically recognizes the secondary antibody. In this scenario, the secondary antibody would be unconjugated and the tertiary antibody would contain the fluorophore of interest. An example of such signal building is: primary antibody = mouse anti-*Drosophila* antigen; secondary antibody = rabbit anti-mouse IgG (Jackson Immunoresearch); tertiary antibody = donkey anti-rabbit-FITC (Jackson Immunoresearch). Another antibody labeling system that can also amplify a signal employs a secondary antibody that is biotinylated and a fluorophore conju-

gated to either avidin or streptavidin. Biotin and streptavidin/avidin have a very specific affinity for one another, in which multiple biotin molecules bind to each avidin or streptavidin. The high specificity of this binding can lower or prevent background fluorescence due to secondary reagents binding nonspecifically. These amplification reagents may facilitate multiple labeling experiments when employed to match the signal strength of different antibody reagents.

4.3. Double and Triple Antibody Labeling in Parallell

17. Crossreactivity occurs when part or all of another antibody signal is added to a known labeling pattern. This is caused primarily by secondary or tertiary antibody reagents not being specific enough, usually by recognizing more than one species of immunoglobulin molecule. The more evolutionarily related the host species are to each other (primary and secondary antibodies), the more likely that crossreaction problems will arise with the addition of the next set of reagents (secondary or tertiary antibodies). To help minimize such problems try using secondary or tertiary reagents that have been crossreacted with IgG fractions from multiple animal species. For example, in a double label experiment with primary antibodies made in both rat and mouse, use reagents such as goat anti-rat IgG and donkey anti-mouse biotin from Jackson Immunoresearch that have been specifically immunodepleted of mouse or rat IgG reactivity, respectively. The drawback to using these types of reagents is that they often work at a lower titer than equivalent reagents that have not been cross-species purified.
18. More rarely, antibody crossreaction occurs when primary antibodies against different members of the same gene family are used. In this circumstance, one primary antibody recognizes more than one protein of interest. In the example given in **Subheading 4.1.2.**, both Atonal and Daughterless are transcription factors containing highly related basic helix–loop–helix domains ***(20–22)***. But because both of these antibody reagents were developed against a less conserved region (that does not contain the basic helix–loop–helix domain) of these proteins ***(17,23)***, cross-reactivity is not an issue here. To determine if this is a problem, it is necessary to know the immunogen used to make primary antibody reagents in question.
19. Optimizing all primary antibody and secondary and/or tertiary reagent concentrations helps to minimize crossreactivity as well.
20. Another problem in multiple labeling experiments that looks very much like a crossreactivity problem occurs when one very strong fluorescent signal bleeds through to another wavelength channel on the LSCM. In this case, an image also appears additive for labeling patterns and signals. This is particularly a problem when two fluorochromes emit close together. For instance, strong rhodamine–phalloidin signal can sometimes be detected on the fluorescein channel even though the emission spectra are 80 nm apart. To distinguish between bleedthrough and reagent crossreaction, switch the first fluorochrome protein pattern farther away from the emission wavelength of the other label(s), e.g., substituting Cy5 (emits at 647 nm) for rhodamine.

21. Perhaps the hardest problem to solve occurs when two or more proteins of interest are found to require different fixation conditions. If one signal is optimal but the other is marginal in a particular set of conditions, try boosting the marginal signal by building a triple (or even quadruple) sandwich. It may also be possible to titer back the better signal (by dropping primary or secondary antibody concentrations) to even out signaling intensity. This may help equalize images at the LSCM. If one set of fixation conditions appears harsher than another, do the antibody labeling sequentially (**Subheading 4.2.**) with the addition of a harsher fixation step and a second blocking step prior to addition of the second antibody. Lastly, if the choice of fixative itself it not at issue, try intermediate concentrations of fix or detergent to optimize both signals.
22. Multiple labeling of samples can also take advantage of fluorescing subcellular dyes (e.g., rhodamine–phalloidin (Molecular Probes), which binds to F-actin filaments within the cytoplasm of cells). Detailed descriptions of such dyes, including their optimal binding conditions and excitation and emitting wavelengths, can be found in the Molecular Probes, Inc. *Handbook of Fluorescent Probes and Research Chemicals* (Eugene, OR). The use of these compounds is most simply incorporated by adding them to a predetermined final concentration to the last antibody labeling step (either secondary or tertiary antibody). The excess is then washed away during the last set of sample washes. Strongly staining dyes like propidium iodide, which binds to nucleic acids, should be added instead to the first wash of the last set of washing steps. A specific example is given in **Subheading 3.4.**
23. Some subcellular dyes emit in the low ultraviolet range and are not detected by many LSCMs. Also other dyes, such as propidium iodide bind to both RNA and DNA, requiring additional sample preparation steps such as RNase treatment and the presence of detergent to achieve specific nuclear DNA labeling ***(24)***. Therefore, working out compatible multiple labeling conditions for these reagents and antibody reagents may need to be done by trial and error.

4.4. Double and Triple Antibody Labeling in Series

24. A blocking step (**Step 3.4**) could be used prior to the addition of the second primary antibody, but did not prove necessary in the given example.
25. For multiple primary antibodies from the same host species (or to minimize cross-reaction problems), try reversing the order of primary antibody addition. For example, in multiple labels with the anti-myc monoclonal and the 22C10 monoclonal, if the myc antibody was used first, no cross-reaction of signals occurred, but if the order was reversed, the 22C10 expression pattern became additive with the myc expression pattern.

4.5. Preparing Labeled Samples for LSCM Analysis

26. Compounds that prevent rapid fading of fluorophores are *p*-phenylenediamine (PDA), *n*-propyl gallate, and DABCO (1,4-diazabicyclo[2.2.2] octane). Several commercially prepared anti-photobleaching agents are also available (e.g., Slowfade, Molecular Probes, Catalog No. S828). Among standard fluorophores,

fluorescein (FITC) is the most susceptible to photobleaching. However, due to the intensity of the laser of the LSCM, it is a good idea to use an antifading agent with all fluorophores.

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