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COVALENT LABELING OF PROTEINS WITH FLUORESCENT COMPOUNDS FOR IMAGING APPLICATIONS

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Abstract

The labeling of proteins with fluorescent compounds for microscopy has allowed a greater understanding of biological processes. The preparation of fluorescent proteins is the first step in development of their use in microscopy. Methods are described to label and characterize a protein as an example of the general approach for other proteins. Skeletal muscle alpha-actinin was labeled with either fluorescein-5-maleimide or 5-iodoaceamidofluorescein and the reaction characterized. The maleimide reaction was much more rapid and efficient than the iodoacetamide reaction giving a coupling efficiency of 65% under the given ration conditions. The fluorescein-5-maleimide alpha-actinin was functionally characterized and there was essentially no influence on the fluorescein label on the F-actin binding properties of alpha-actinin. The fluorescein alpha-actinin was also shown to specifically bind to the Z-line of isolated myofibrils. A general outline and discussion are presented on how to label and characterize proteins for use in microscopy.

Key Words: fluorescence microscopy, covalent labeling, alpha-actinin, fluorescein alpha-actinin, myofibrils.

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Introduction

Covalent labeling of ligands for imaging applications is essentially an extension of classical histochemical staining methods in that it is a method which enhances the contrast of the specimen. The important exception is that covalent labeling allows for much higher resolution images and much more refined interpretations of a given image. An example of this is the detection of specific proteins using immunological techniques. One of the first applications of a covalently-labeled protein was the immunofluorescent detection of pneumococcal antigen in tissues using fluorescein-labeled primary antibodies (Coons *et al.*, 1942). Since that time there have been many developments in the methodology for preparing covalent adducts of various ligands for use in imaging applications (Haugland, 1992; Wang and Taylor, 1989). By far the most common use of labeled proteins is in immunofluorescence microscopy using fluorescently-labeled secondary antibodies. There are many excellent commercial suppliers of labeled secondary antibodies so the researcher need not spend valuable laboratory time preparing their own labeled secondary antibodies.

Another application of covalent labeling of proteins is in labeling of cytoskeletal proteins for cell biology applications. One of the first uses of this approach was the labeling of heavy meromyosin for the demonstration of actin involvement in cytokinesis (Aronson, 1965). Developments in the reaction chemistries (Hartig *et al.*, 1977; Haugland, 1992) allowed for use of much more efficient and milder reaction conditions which widened the spectrum of proteins which could be labeled. This, coupled with enhancements in light microscopy imaging technology, afforded the opportunity of following cytoskeletal dynamics in living cells using covalently labeled proteins (Wang and Taylor, 1980, 1989). Covalent labeling of proteins with undecagold resulted in high resolution electron microscopy applications of covalently labeled proteins (Safer *et al.*, 1986; Milligan *et al.*, 1990) and developments in fluorescence photo-oxidation have allowed for dual use of fluorescently labeled proteins at both the light and electron microscope level (Deerinck *et al.*, 1994). The recent development of molecular biological means to prepare fusion proteins

containing green fluorescent protein (Cubitt *et al.*, 1995) offers an alternative to covalent labeling of purified proteins. Thus, covalently labeling of proteins has wide usage in imaging applications both at the light and electron microscope level.

This paper is limited to discussion of methods for covalent labeling of proteins. This labeling employs a compound which is essentially bivalent in that one part of the molecule is reactive with the ligand of interest while the other part is a reporter molecule. The reactive moiety is a chemical reagent which reacts with the ligand to form a stable covalent linkage with the ligand. The reporter molecule is a chemical moiety which allows for detection of the ligand. Classical examples of the reporter molecules are fluorochromes, biotin, enzymes, and other selective haptens to which there are specific antibodies. The labeling of proteins takes advantage of reactive groups present on the proteins. These can be reactive amino acids present in the polypeptide such as primary amines (e.g., lysine; both intracellular and extracellular proteins) or sulfhydryls (e.g., cysteine; intracellular proteins). Most intracellular proteins have free sulfhydryl side chains (cysteines) while extracellular proteins have disulfide crosslinks (cystines) which are not readily reactive. Most all proteins have primary amines, either as the amino terminus or from lysine side chains. Although many other side chains or reactive groups on the proteins are potential sites for covalent modification, the reaction conditions are usually not desirable. A major concern for chemical modification of proteins is the maintenance of biological function. Thus, reactions which proceed at room temperature or lower and at neutral or slightly alkaline pH are desired and this in turn dictates to some extent what reaction chemistry one can utilize.

Below, I will describe methods for the labeling of skeletal muscle alpha-actinin with thiol-reactive fluorescein compounds as an example of how to covalently label a protein. This will be followed by assaying a biological function of the labeled protein and use of the protein in an imaging application.

Materials and Methods

Protein purification

Alpha-actinin was purified from rabbit skeletal muscle using modifications to the procedure described by Feramisco and Burridge (1980). The $MgCl_2$ precipitation step was omitted and the low ionic strength/37°C supernatant was cooled to 4°C and 21 g solid $(NH_4)_2SO_4$ was added per 100 ml supernatant and the solution stirred for 20 min. The $(NH_4)_2SO_4$ precipitate was collected by centrifugation at 5,000g for 30 min, resuspended in a minimal volume of dialysis buffer [1 M KCl, 10 mM KH_2PO_4 (pH 7.0), 1 mM EDTA, 0.1% 2-mercaptoethanol (2-ME), 0.1 mM phenylmethylsulfonyl fluoride (PMSF)] and dialyzed overnight against 10 vol of dialysis buffer relative to initial tissue mass. The dialyzed solution was clarified by centrifugation at 100,000g for 1.5 hour at 4°C and crude alpha-actinin in the supernatant was precipitated by adding 21 g solid $(NH_4)_2SO_4$ per 100 ml supernatant. The precipitate was collected by centrifugation and further purified by ion-exchange chromatography on diethylaminoethyl(DEAE)-cellulose (DE-52), hydroxylapatite, and size-exclusion chromatography on Sephacryl S-400. Alpha-actinin was stored as a suspension in 50% $(NH_4)_2SO_4$ at 4°C. Alpha-actinin retains it actin-binding properties for at least a year when stored under these conditions. Actin was purified from rabbit skeletal muscle as described by Pardee and Spudich (1982) and rabbit skeletal muscle myofibrils were purified as described by Swartz *et al.* (1990).

Protein labeling

Alpha-actinin was dialyzed at 4°C against 100 mM KCl, 10 mM HEPES (pH 8.0), 1 mM EGTA, 0.1% 2-ME to remove $(NH_4)_2SO_4$. The protein solution was made to 10 mM dithiothreitol (DTT) and incubated at room temperature for 30 min to reduce any oxidized thiols. The solution was cooled on ice and then desalted on a Sephadex G-25 column equilibrated with reaction buffer [100 mM KCl, 10 mM HEPES (pH 8.0), 1 mM EGTA] at 4°C. Elution of the protein fractions was monitored by ultra violet (UV) absorbance. To ensure separation of the reducing agents (2-ME and DTT) from the protein fractions, the level of thiol was monitored by the addition of 5,5'-dithiobis-(2-nitrobenzoic acid (DTNB) (Ellman, 1959) to fractions trailing the protein peak. The protein concentration was determined by absorbance at 280 nm using an extinction coefficient of 1.0 ml/mg.cm and a molecular weight of 100,000 per alpha-actinin monomer (Suzuki *et al.*, 1976). The dyes [fluorescein-5-maleimide (F-5M) and 5-iodoaceamido fluorescein (5-IAF), Molecular Probes, Eugene, OR] were prepared by dissolving about 1 mg dye/100 μl dimethylformamide, and the concentration of the dye was determined spectrophotometrically. A small aliquot of the stock solution was diluted in 10 mM Tris (pH 9.5), 1 mM NaN_3, 0.1% 2-ME and the absorbance read at 490 nM. The concentration was calculated using an extinction coefficient of 83,000 /M.cm (Haugland, 1992). For the reaction, the dye was first diluted in about 1/20 volume of reaction buffer then rapidly mixed with the protein solution and incubated at room temperature in the dark. The protein concentration was 1.5 mg/ml and 15 ml of protein was used for each dye giving 0.22 μmoles of alpha-actinin monomer per

reaction. The conjugation reaction was done using 2 mole dye/mole alpha-actinin monomer thus, 0.44 μmole of each dye was added. After incubation at room temperature (22°C) for 16 hours, the reaction was quenched by addition of DTT to a final concentration of 1 mM. The protein was precipitated by mixing the solution with an equal volume of saturated $(NH_4)_2SO_4$, collected by centrifugation (a yellow-orange pellet was observed) and dissolved in a minimal volume of rigor buffer (RB: 75 mM KCl, 10 mM imidazole (pH 7.2), 2 mM MgCl2, 2 mM EGTA, 1 mM NaN_3) made to 1 mM DTT. Unconjugated dye and $(NH_4)_2SO_4$ were removed by desalting on a Sephadex G-25 column equilibrated with RB. The conjugated protein can be stored at this point for 1 week on ice or as an $(NH_4)_2SO_4$ pellet at 4°C in the dark for over a year and still maintain functionality.

The time course of the reaction was followed by removing aliquots at timed intervals and mixing with an equal volume reaction buffer containing 0.2% 2-ME to quench the reaction. Samples were then analyzed by SDS/PAGE (sodium dodecyl sulfate/polyacrylamide gel electrophoresis) followed by observation of the unstained gel using a UV light box. Images of the fluorescent protein in the gels were documented using Polaroid type 667 film with camera settings at f5.6 and exposure times of 5-15 sec.

Binding studies

The biological activity of F-5M alpha-actinin was compared to unlabeled alpha-actinin using an actin sedimentation assay similar to that described by Meyer and Aebi (1990). Different levels of alpha-actinin dimer (0.2-15 μM) were mixed with a fixed level of G-actin (18 μM final) in RB made to 1 mM adenosine triphosphate (ATP) and 1 mM DTT and incubated at room temperature for 1 hour. The mixtures were centrifuged at 13,000g for 20 min then the supernatant was carefully removed. The pellets were resuspended in SDS/PAGE sample buffer and aliquots of the supernatants were mixed with sample buffer. Both supernatants and pellets were analyzed for alpha-actinin content by densitometry of the alpha-actinin bands in SDS/PAGE. After electrophoresis, the gels were stained with Coomassie brilliant blue, and alpha-actinin content determined by densitometry of gels using unlabeled alpha-actinin as the protein standard. Binding isotherms were generated by plotting free [alpha-actinin] in the supernatant against bound [alpha-actinin] in the pellet.

Glycerinated rabbit skeletal muscle myofibrils were washed 3 times with 10 vol of RB containing 1 mg/ml bovine serum albumin (BSA) and 1 mM DTT (RB-BSA-DTT) then diluted to 1 mg/ml myofibrillar protein. Myofibrils (50 μl) were mixed with 150 μl RB-BSA-DTT containing 0.1 μM F-5M alpha-actinin (final) and incubated 1h at room temperature in the dark. The labeled myofibrils were collected by centrifugation for 15 sec in a microfuge at maximum speed (13,000g). The supernatant was removed and the myofibrils were resuspended in 200 μl RB-BSA-DTT; then 100 μl were plated on a coverslip and the myofbrils were fixed by pooling with 400 μl of 3% formaldehyde in RB for 15 min. The coverslips were drained, rinsed in RB, then mounted on 50 μl of mounting medium [75% glycerol (v/v), 75 mM KCl, 20 mM Tris (pH 8.5), 2 mM $MgCl_2$, 2 mM EGTA, 1 mM NaN_3, 1 mg phenylenediamine/ml] and sealed to slides with "Wet 'n' Wild" nail polish.

Microscopy

Myofibrils were imaged using a Zeiss axiovert microscope equipped with a 100X (NA 1.3) phase contrast objective, epifluorescence illumination with a 100W Hg lamp, a fluorescein narrow band pass filter and a CCD (Photometrics 200, KAF 1300 chip, Photometrics Inc., Tucson, AZ). The CCD was controlled from a matrix board in a Macintosh Quadra 840AV using IPLab Spectrum software (version 1.3, Signal Analytics, Vienna, VA). Images were obtained using 0.2-0.8 sec exposures giving a minimum dynamic range of 400 (max-min intensity). Images were processed using IPLab software by rotation to the horizontal using bilinear interpolation, cropping out a six sarcomere region of interest (ROI), converting the 16 bit image to 8 bit, and montaging the phase and fluorescence ROI's. Further text and graphic information was added to the images in Canvas (version 3.5, Deneba Software, Miami, FL).

Assays

Protein concentration was measured with the bicinchoninic acid (BCA) assay as modified by Hill and Straka (1988) for use with reducing agents. BSA was used as the standard for myofibrils and unlabeled alpha-actinin was used as the standard for F-5M and 5-IAF alpha-actinin. SDS/PAGE was done on 10% gels as described in Fritz *et al.* (1989).

Results

Coupling reaction

The coupling of a dye to a protein is highly dependent upon the chemical reaction employed and the buffer conditions. Fig. 1 shows the dye structures and the chemistry of the reactions. Fig. 2 shows the time course of alpha-actinin conjugation with either F-5M (panel A, A') or 5-IAF (panel B, B'). At different times, the reaction was quenched with excess 2-ME followed by separation of labeled protein from unlabeled using SDS/

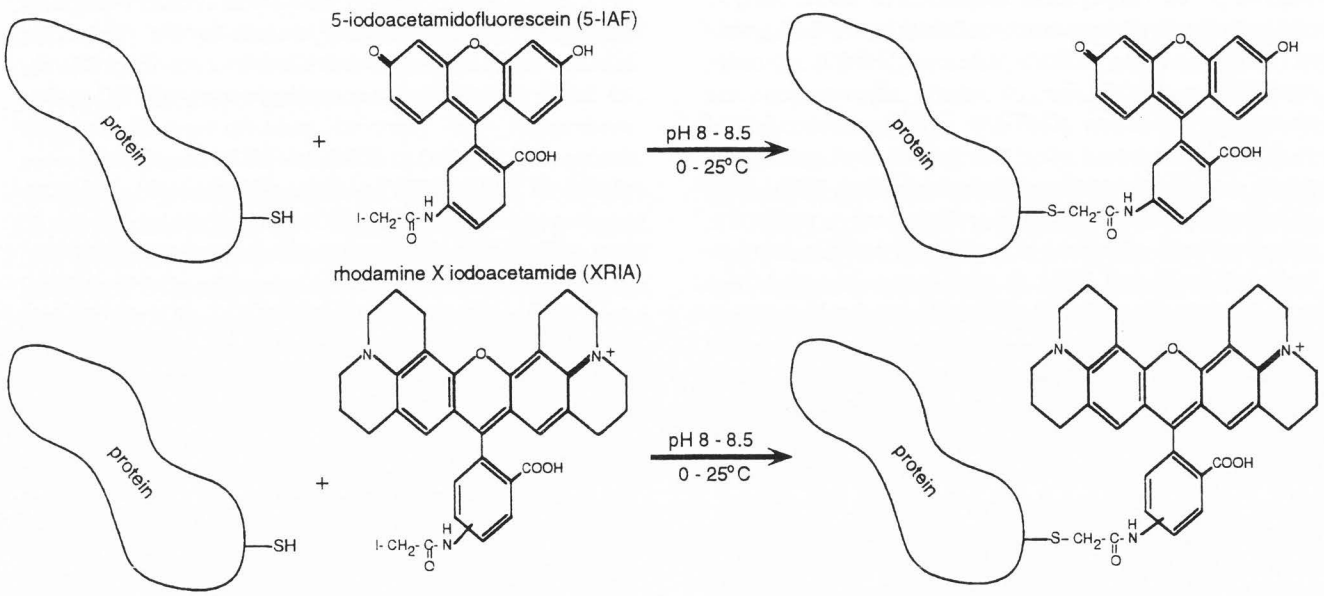

Figure 1. Diagram of covalent labeling proteins with thiol-specific dyes.

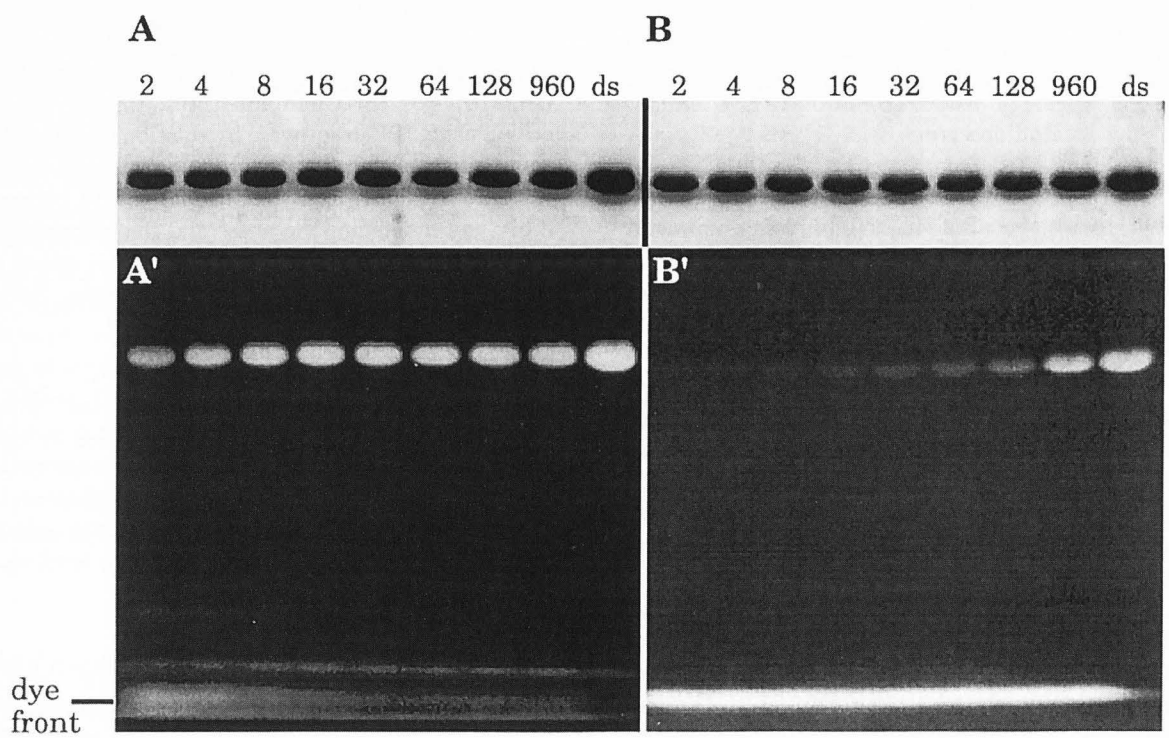

Figure 2. Time course of alpha-actinin labeling with thiol-specific dyes followed with SDS/PAGE. Time course of F-5M (panel A, A') and 5-IAF (panel B, B') labeling of alpha actinin. The dyes were mixed with the protein in 100 mM KCl, 10 mM HEPES (pH 8.0), 1 mM EGTA at 20°C. At specified times, aliquots were removed, and the reaction quenched with 2-ME. Samples were separated from unconjugated dye by SDS/PAGE followed by imaging under UV illumination (panels A' and B') and white light after Coomassie staining (panels A and B). Text above lanes designates incubation time in min and ds is de-salted protein after 960 min incubation. The fluorescence at the bottom of gels (A' and B') was the unconjugated dye.

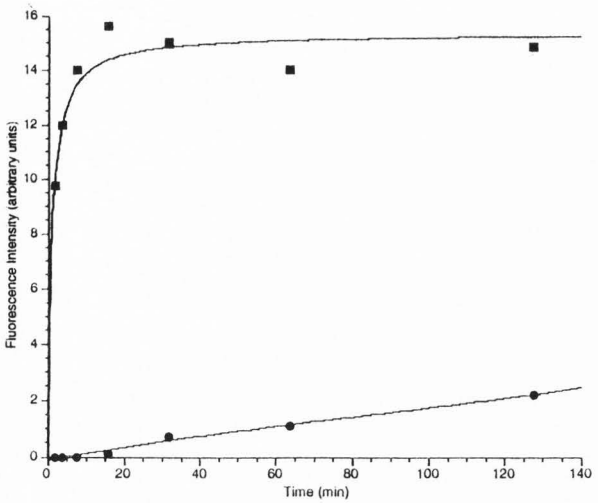

Figure 3. Kinetics of covalent labeling of alpha-actinin with thiol-specific dyes. Photographs of the UV gels were digitized using a calibrated flatbed scanner and light-intensity measured from the digital images. The integrated intensity was determined for each band after correction for background intensity and plotted as a function of time. Note that reaction of alpha-actinin with F-5M (filled squares) was much more rapid than with 5-IAF (filled circles).

PAGE. The progress of the conjugation reaction was determined by photographing the gels under UV illumination (panels A' and B') and analysis of the pictures for intensity using a flatbed scanner. After UV photography, the gels were stained with Coomassie, destained and imaged using a flatbed scanner (panels A and B). The UV gels show that the conjugation with F-5M was very rapid and that there was very little dye at the dye front after about 32 min incubation. Conversely, the reaction with 5-IAF proceeded very slowly with protein conjugate bands only being easily visible after 32-64 min of reaction while there was substantial fluorescence at the dye front even after 960 min. The continual slow reaction of 5-IAF with protein suggests that the reagent was not exhausted by hydrolysis. The last lane of each gel (ds) shows the labeled protein after 960 min of the reaction followed by desalting to remove unconjugated dye. Note that there was essentially no fluorescence at the dye front for either the F-5M or 5-IAF conjugate. The progress of the reaction was also monitored by measuring the integrated intensity of the bands from the photographs using a flatbed scanner and image analysis using IPLab Spectrum. This is shown graphically in Fig. 3. The reaction with F-5M is more than halfway done after 2 min incubation and is essentially complete at 32 min while the reaction with 5-IAF was much less complete even at 128 min.

Estimation of the dye to protein ratio by spectrophotometry showed that the F-5M conjugate had 1.3 mole fluorescein/mole alpha-actinin monomer while the 5-IAF conjugate had 0.34 mole fluorescein/mole protein. Thus, under the buffer and temperature conditions used for the conjugation reaction, the F-5M reaction was much more efficient giving 0.65 coupling efficiency (1.3 mole coupled/2 mole added) while the 5-IAF gave only a 0.17 coupling efficiency. Only the F-5M conjugate was used in further studies because of its higher labeling ratio.

Functionality test

Alpha-actinin is characterized as an actin bundling protein; thus, one can characterize the ability of alpha-actinin to induce bundling using a sedimentation assay which pellets the alpha-actinin/actin complex. Comparing the bundling ability of unlabeled alpha-actinin with labeled allows for determination of whether the labeling influences a biological property of the molecule. Fig. 4 shows gels which contain the supernatant and pellet fractions of samples with different levels of alpha-actinin but constant actin. The unlabeled alpha-actinin samples are in panel A while the F-5M labeled alpha-actinin samples are in panel B. The unlabeled panel shows that at 0.4 μM alpha-actinin, most of the actin and alpha-actinin was in the pellet. As alpha-actinin was increased, there was an increase in the amount of alpha-actinin and actin in the pellets suggesting that the alpha-actinin bound to and bundled the actin into aggregates. Neither actin or alpha-actinin was found in the pellet when incubated in buffer alone (data not shown). There was very little difference in the appearance of the gels between the unlabeled and labeled alpha-actinin.

Quantitative analysis of the bound and free alpha-actinin by densitometry of the gels is shown in Fig. 5. The curves were the best fit to the hyperbolic equation:

$$B=(B_{max}*free)/(K_d + free) \quad (1)$$

The unlabeled alpha-actinin gave a B_{max} of 2.7 μM and a K_d of 1.2 μM while the F-5M alpha-actinin gave a B_{max} of 3.7 μM and a K_d of 2.5 μM for the alpha-actinin dimer. The B_{max} value corresponds to saturation at 1 alpha-actinin dimer per 5-7 actin monomers (18 μM actin for the assay). These values were close considering the scatter of the data suggesting that the labeling did not dramatically alter a biological property of the alpha-actinin. A more heavily labeled alpha-actinin is likely to result in changes in biological function.

Myofibril binding

Previous studies have shown that labeled alpha-actinin (smooth or skeletal) will bind to the Z-line of skeletal myofibrils (Sanger *et al.*, 1984; Swartz *et al.*, 1993). Thus, rigor myofibrils were incubated with F-5M labeled alpha-actinin to demonstrate this property of

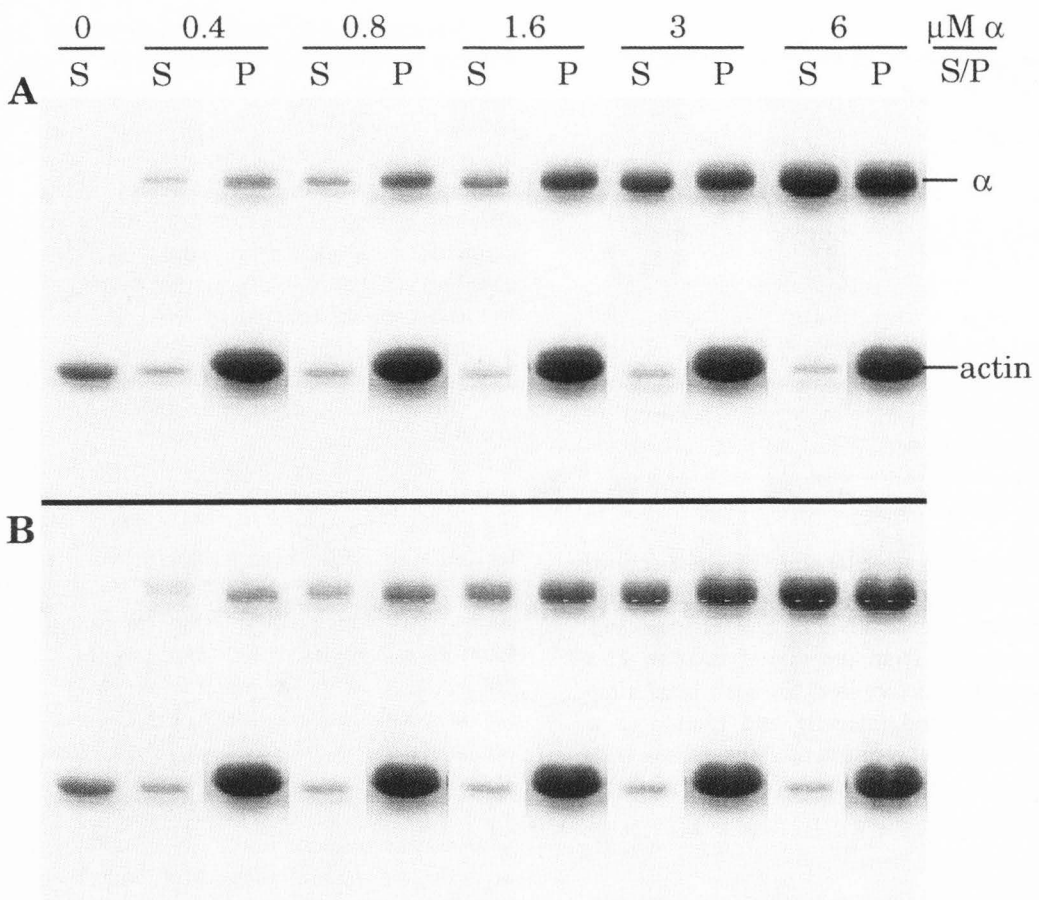

Figure 4. Functionality assay of F-5M alpha-actinin. Different concentrations of either unlabeled (panel A) or F-5M alpha-actinin (panel B) were mixed with a fixed concentration (18 μM) of G-actin under polymerization conditions and incubated for 1h at 20°C. Samples were centrifuged at 13,000g for 30 min at 20°C and aliquots of the supernatant (S) and pellet (P) were assayed by SDS/PAGE followed by Coomassie staining. Numbers above lanes designate alpha-actinin dimer concentration. Note that the supernatant and pellet samples were run on separate gels and the images were made by digitally montaging the appropriate lanes giving the odd appearance of the actin band in the pellet lanes.

labeled alpha-actinin. Fig. 6 shows phase-contrast (1P, 2P) and fluorescein-fluorescence (1F, 2F) images of two different rabbit psoas myofibrils at different sarcomere lengths. The F-5M alpha-actinin localized to the Z-line of the rigor myofibrils with some minor binding to regions just adjacent to the Z-line. The Z-lines within a myofibril did not seem to show the same level of incorporation of F-5M alpha-actinin for reasons unknown at present. Pretreatment of myofibrils with unlabeled alpha-actinin does not prevent incorporation of F-5M alpha-actinin into the Z-line suggesting that the mechanism of incorporation is that of exchange of unlabeled for labeled alpha-actinin and this is currently under study. These imaging studies demonstrate the that the labeled protein bound to the predicted in situ location with high specificity.

Discussion

In this paper, I have briefly demonstrated a method to covalently label a protein for use in microscopy. The specific example of covalent labeling of alpha-actinin was presented as well as a functionality test for the labeled protein and application of the label protein in light microscopy. The major steps and factors to be considered in labeling a protein to be used for imaging applications are discussed below.

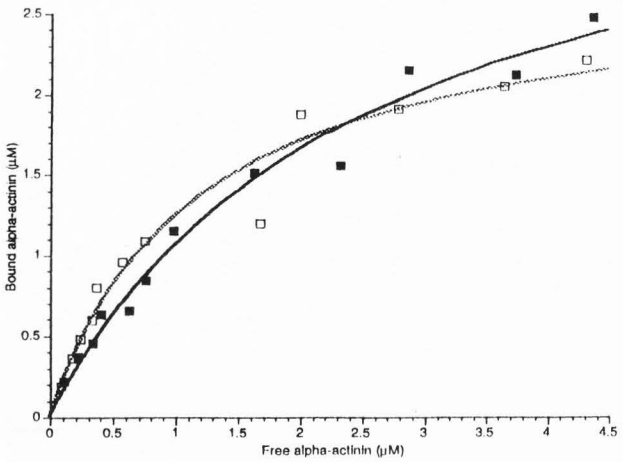

Figure 5. Binding isotherm of unlabeled and F-5M alpha-actinin. The concentration of alpha-actinin in the supernate and pellet were determined by densitometry of Coomassie-stained gels of samples separated by SDS/PAGE. Data were analyzed by non-linear least-square fitting to a hyperbolic function of the form $B = B_{max} * [\text{alpha-actinin}]/(K_d + [\text{alpha-actinin}])$ where B = [bound], B_{max} = [bound] at saturation and K_d = dissociation constant. The B_{max} values were 2.7 and 3.7 μM for unlabeled (open squares) and F-5M alpha-actinin (filled squares) respectively while K_d values were 1.2 and 2.5 μM for unlabeled and F-5M alpha-actinin respectively.

Step 1: Select a reaction chemistry

A good starting point is a reference on chemical modification of proteins which can be found in books such as Means and Feeney (1971) or Hermanson (1996) and a brief review is presented in the handbook by Haugland (1992). When selecting a reaction chemistry, you need to decide whether the dye will be covalently attached to either a primary amine or a cysteine residue. As a general rule, cysteine-specific dyes are the preferred route, because the chemical reactions for coupling to cysteines are usually more efficient under reasonably mild conditions. Thus, the decision is based on whether the protein has reactive cysteines or not and whether cysteine modification alters biological activity. Most all intracellular proteins have available cysteines so this is the chemistry of choice. Some of the cysteine can be buried in the protein structure and not reactive with thiol reagents. In this work, alpha-actinin has a total of 9 cysteines/monomer and of these, 3 are available for reaction, thus, 6 of the thiols are buried and only available under denaturing conditions (Suzuki *et al.*, 1976). Extracellular proteins usually do not have available cysteines as they are oxidized to disulfide crosslinks to aid in protein stability. Thus, for extracellular proteins, reactions involving primary amines are usually employed. In some cases, primary amines may be readily reactive under mild pH conditions but this can be protein specific. Thus, although the reaction conditions are usually more harsh for conjugation at primary amines, the extracellular proteins are usually more stable than intracellular proteins.

There are some alternatives for labeling of extracellular proteins which do not directly utilize primary amine reactions. For example, intramolecular disulfides can be mildly reduced to give available cysteines followed by labeling with thiol-specific dyes. Alternatively, primary amines can be modified with 2-iminothiolane under mild conditions resulting in an thiolated protein (Jue *et al.*, 1978). This protein can then be modified with a thiol-specific dye. We have utilized this approach to label antibodies with thiol-specific dyes giving good results (Swartz, 1989). Although these alternative methods require some additional steps, they can be utilized to great advantage if one needs a particular labeled protein which is not commercially available.

Once you have decided whether you are going to use a amine-specific or thiol specific dye, your next decision is which reagent to utilize and what reaction conditions to try. For amine-reactive dyes, the general choices are isothiocyanate, sulfonylchloride, and succinimidyl ester derivatives. The general order of preference is succinimidyl ester > isothiocyanates > sulfonylchlorides in terms of reactivity under the same conditions. The pH for reactions with amine-reactive dyes should be in the 8.5-9.0 region such that the amine is unprotonated because this is the reactive species. The buffer for the reaction should be a non-amine containing buffer. Buffers of choice include borate, phosphate, or possibly high pKa Good's buffers. It must be realized that hydroxyl ions compete with the unprotonated amines for the reactive dye. Thus, at higher pH's, more dye will react with hydroxyl ions. As a general rule, the reaction can be done with 5-10 mole dye/mole protein at room temperature for 1-2 h to yield conjugated protein. Poor conjugation levels can be improved by increasing the reaction time and/or increasing the dye level.

With thiol-reactive dyes, the choices are somewhat limited to either maleimide or iodoacetamide derivatives. Maleimides are preferred over iodoacetamides due to their highly efficient and specific reaction with thiols (Gregory, 1955). As shown in Figs. 2 and 3, the reaction of alpha-actinin with F-5M was much faster and more efficient than 5-IAF under the same reaction conditions. A pH of 8.0 was used so that one could compare the reactivity of the two compounds. The maleimide reaction readily occurs at more neutral pH values. Some crossreactivity of maleimide with amines can occur at pH 8.0 (Brewer and Riehm, 1966) but this is

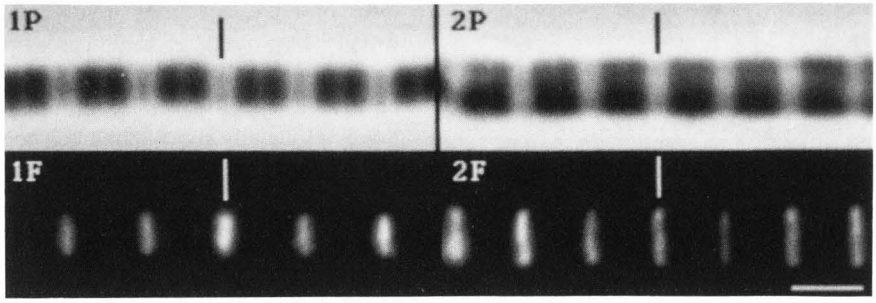

Figure 6. Labeling of rabbit skeletal myofibrils with F-5M alpha-actinin. Myofibrils were incubated with 0.1 μM alpha-actinin for 1h followed by sedimentation and resuspension in buffer without F-5M alpha-actinin, fixation and mounting on slides. Both phase-contrast (P) and fluorescein-fluorescence (F) images are shown. Myofibrils at rest (1) and short (2) sarcomere length are shown. Vertical line denotes Z-line and scale bar is 2 μm.

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not likely at the low levels of maleimide used for labeling. An approach that we have used is to employ the same chemistry to couple different reporter groups to the same protein. For example, we have used maleimide derivatives of fluorescein and Texas Red to label myosin subfragment 1 (S1) (Swartz *et al.*, 1990). The coupling efficiencies are usually very similar between the different reporter groups so that the dye/protein ratios are similar. This is highly advantageous because it affords the researcher many different approaches for double labeling of biological specimens. For example, under appropriate conditions, S1 will bind to either the overlap or I-band region of myofibrils depending on the calcium level. We have used a Texas Red and fluorescein S1 to demonstrate this property in the same myofibril by sequential treatment with one color of S1 followed by the other under different conditions (Swartz *et al.*, 1990). One can also prepare biotin conjugates using the same chemistry. The advantage of having a biotin conjugate is that this can be used with avidin/streptavidin gold conjugates for electron microscopy. Also, the biotin conjugate can be detected with enzyme or fluorophore conjugates of avidin/streptavidin allowing a great latitude in the detection system. We have used the enzyme conjugates to advantage to develop biological function assays described below. One difficulty with the multilabel/same chemistry approach is that a maleimide Texas Red is no longer commercially available. Thus, we have had to modify our procedures to use iodoacetamide derivatives of Texas Red, fluorescein and biotin.

The type of reaction employed can be very protein specific. For example, actin can be specifically labeled with an amine-reactive dye at lys-61 and a thiol-reactive dye at cys-374 (Miki and dos Remedios, 1988). The specific labeling occurs because the residues are likely rendered highly reactive by their micro-environment within the protein. An extensive reference list of protein-specific labeling can be found in Haugland (1992). Another difficulty is that the most reactive residue(s) may be important in biological function. An example of this is S1 which contains two highly reactive thiols which are indirectly involved in ATPase activity. This problem can be dealt with by reversible blocking of the reactive thiols followed by reaction at other, less reactive, thiols and subsequent unblocking (Swartz *et al.*, 1990). Thus, one may have to be creative in the method employed to label the protein and the protocol may be highly protein specific.

Step 2: Pilot studies

The availability of the protein dictates to some extent the amount of development of the conjugation protocol. A simple approach for investigating reaction conditions is the use of SDS-PAGE/UV illumination to follow the time course and efficacy of the reaction as it does not require milligram quantities of protein. A few hundred micrograms of protein may suffice for following the time course of the reaction since one only needs to load 0.2-1 μg protein per lane to follow the reaction. Variables that influence the conjugation reaction include: pH, temperature, reactive dye concentration, protein concentration and time.

pH: The pH conditions are somewhat limited by the stability of the protein at high pH. The preference is for neutral to slightly alkaline conditions. Thiol-reactive dyes are generally used in the pH 7-8.5 range with maleimides in the pH 7 range and iodoacetimides in the 8-8.5 range. For example, to increase the conjugation efficiency of alpha-actinin with 5-IAF, the pH could be raised to 8.5 with the same incubation time as described above. Amine-reactive dyes are used in the pH 8-9.5 range with the succinimidyl ester being used in the pH

8-9.0 range. The buffers used should not be reactive with the dye as discussed above. For the thiol-reactive dyes, the Good's buffers: 3-N(morpholino)propanesulfonic acid (MOPS), piperazine-N,N'-bis(ethanesulfonic acid) (PIPES), N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), etc.) are preferred with phosphate as another alternative.

Temperature: The reaction will be more favored the higher the temperature but one is limited by the stability of the protein, and the possibility of proteolysis of the protein during the reaction due to minor protease contamination. As a general rule, 0-25°C is the functional range of temperature one can use. Lower temperatures are preferred but the reaction will run slower under these conditions. This can be overcome by increasing other parameters which favor the reaction. One must be aware that temperature can influence the exposure of the amino acid side chains for reaction due to temperature-induced changes in protein conformation.

Time: Longer reaction times will favor higher conjugation efficiencies in most cases. For the amine-reactive dyes, the protein amino acid side chains will be competing with hydroxyl ions for the dye. A general time frame is 1-24 h. An easy approach is to start the reaction in the afternoon and let it proceed overnight.

Concentration of dye and protein: The concentration of dye used for the reaction is dictated by the efficiency of the reaction and the final dye/protein desired. A good labeling ratio is 1 mole dye/mole protein (discussed below). With this in mind, a concentration equal to 1-3 fold the protein concentration is recommended for highly reactive dyes such as the maleimides. For less reactive dyes, such as the amine-reactive dyes, 2-10 fold the protein concentration is used. The dyes are generally poorly soluble in water and are dissolved in solvents such as dimethyl sufoxide or dimethyl formamide at final concentrations in the 10-30 mM range. It is not recommended to use dimethyl sulfoxide with sulfonyl chlorides (Boyle, 1966). Once dissolved, the true concentration of the dye can be estimated spectrophotometrically using established, solvent specific, extinction coefficients. The handbook of Haugland (1992) has an extensive list of the extinction coefficients for most dyes. If the dye does not have a good spectral handle (e.g., maleimido-biotin), then you can estimate the concentration from the mass of dye dissolved in solvent. It is a good practice to pre-dilute the dye in the reaction buffer using 5-10% equivalent volume of the protein solution. This is then added to the protein solution rapidly. This approach makes it more likely that you will get uniform labeling of the protein molecules than if you added the dye directly from the stock solution. The protein concentration for the reaction depends on the type of reaction employed. Lower protein concentrations can be used with the thiol-reactive dye than the amine-reactive dyes because there are fewer competing reactions. The protein concentration should be in the 1-5 mg/ml range for reactions. Those using amine-reactive dyes should use the high end of the concentration range to favor conjugation of the protein over hydroxyl ions while the low end of the range work well for thiol-reactive dyes, especially the maleimides.

For the thiol-specific dyes, intracellular proteins should be treated with reducing agents prior to the reaction to ensure that there are no oxidized thiols in the protein. This can be done by incubating the protein with high levels of either DTT (10 mM) or 2-ME (0.1-0.5%) at pH 7-8 and room temperature in a buffer containing EDTA or EGTA and followed by desalting or dialysis to remove the reducing agent. Column desalting is recommended as it is much faster than dialysis. The buffer for the reaction with thiol-specific dyes should be degassed and contain EDTA or EGTA to minimize heavy-metal catalyzed oxidation of thiols.

Step 3: Label protein and subsequent clean-up

Once you have developed conditions which result in good labeling of the protein, you can scale up to label a larger amount to use for functionality tests and for your imaging application. The few days used developing a protocol are well spent if the reaction is successful especially considering the time likely spent isolating the protein. After you have labeled the protein, the unreacted dye can be removed by dialysis, desalting or a combination of the two. If the protein can be precipitated with ammonium sulfate, this will afford some removal of unreacted dye. However, some dyes will salt out with ammonium sulfate (e.g. Texas Red dyes). Some unreacted Texas Red dyes bind quite strongly to Sephadex G-25 so desalting on G-25 is a highly effective method for removing these dyes. A simple assay to confirm removal of unreacted dye is to analyze the protein with SDS/PAGE followed by UV illumination (see Fig. 2). There should be no fluorescence at the dye front if all unconjugated dye has been removed.

Step 4: Determine the labeling ratio

A good labeling ratio is 1 mole dye/mole protein because one has some confidence that subsequent functionality test are truly measuring the labeled protein population and not the unlabeled population. For example, functionality test with a protein labeled at ratios below 0.5 may be difficult to interpret. Higher labeling ratios may be needed to improve sensitivity; however, higher labeling ratios are much more likely to modify biological function. Estimation of the labeling ratio is done by measuring the protein and dye concentration of the same sample after removal of unreacted dye. The protein concentration can be determined using

colorometric protein assays (Lowry, BCA, and Biuret) and if possible, use the unlabeled protein as the standard. It is best to use a sensitive assay read at wavelengths which have minimal overlap with the fluorescence dye. The dye concentration can be estimated spectrophotometrically using published extinction coefficients. The absorbance of most fluorescent dyes is highly solvent specific so it is best to use the solvent for which the extinction coefficient was determined. Protein conformation can also influence the spectral properties of the dye so one may need to denature the protein with urea or guanidine HCl to get an accurate measure of the dye concentration. Once both dye and protein concentration are known, one simply determines the ratio to the two concentrations to determine the labeling ratio.

Step 5: Biological activity tests

The fidelity of the conclusions one can draw from studies with labeled protein is directly related to the rigors of the biological activity tests employed. The assays used are going to be highly protein specific and dictated by the literature available on the function of the protein. In some cases, protocols can be developed which isolate functional labeled protein. For example, labeled tubulin or actin can be functionally isolated by repeated cycles of polymerization/depolymerization. From this one can conclude that the labeled proteins polymerize reversibly but you cannot conclude that they interact with other proteins in the same way as the unlabeled protein unless you assay for this. We have used reversible actin binding of labeled myosin subfragment 1 (S1) as a method to isolate functional labeled S1. The protein isolated in this fashion had ATPase activities very similar to the unlabeled protein (Swartz *et al.*, 1990). The F-5M alpha-actinin prepared in the current study appears to bundle actin as well as unlabeled actin and locate to the Z-line suggesting that it functions just as unlabeled alpha-actinin. However, we cannot conclude that it binds to proteins other than actin (if there are any) the same as unlabeled alpha-actinin. The more proteins that your labeled protein interacts with, the more complicated the activity assays become.

One approach that we have employed is to use a solid phase competition assay to monitor the binary interaction of a labeled protein with its binding partner. Specifically, we have employed this approach with a labeled subunit of the troponin complex (Swartz *et al.*, 1994). Fluorescein, rhodamine and biotin conjugates of troponin C (TnC) were prepared and assayed for their ability to bind to troponin I coated microtitre plates under high and low calcium conditions. Different levels of the fluorescently-labeled proteins or unlabeled protein were incubated in troponin-I coated wells with a fixed, saturating level, of biotin TnC. The wells were washed and biotin-TnC level was determined by color development with streptavidin-HRP. The assays showed that both unlabeled and fluorescently-labeled TnC displaced biotin-TnC but that the unlabeled TnC competed better than labeled TnC especially at low calcium. This approach has some advantages in that it can be very sensitive and one need not know the properties of the biotin conjugate. What you are interested in is how similar the unlabeled and fluorescently-labeled proteins displace the biotin conjugate.

Step 6: Use the labeled protein

After you have demonstrated that the protein has the biological activity that you are interested in, you can use it to investigate the problem of interest. In the example presented in this study, labeled alpha-actinin was incorporated into skeletal myofibril Z-lines. We have used this in conjunction with labeled S1 to understand the structure of highly shortened myofibrils which are not readily amendable to interpretation from phase-contrast images (Swartz *et al.*, 1993). This was a structural application of the probes instead of a structure/function application. We have also used the labeled S1 as a functional probe to investigate how calcium regulates the binding of myosin to the thin filament (Swartz *et al.*, 1990). We are currently using labeled alpha-actinin as a functional probe to study the dynamics of alpha-actinin exchange at the Z-line.

Another issue along with use of the protein is how to store the labeled protein. The labeled proteins are stored in the dark with reducing agents to prevent bleaching of the fluorophores. Other conditions of storage are protein dependent. The labeled alpha-actinin was stored as ammonium sulfate pellets in the dark at 4°C. Other preparations of these same protein have been stored for over a year under these conditions and still work well for imaging applications. Labeled proteins like actin and tubulin can be stored in liquid nitrogen but activity is lost over storage. Thus, one will have to experiment to determine the best conditions for storage of each specific protein.

Conclusions

In the current work, skeletal muscle alpha-actinin was used to show some of the differences in the thiol-reactive dyes in terms of their reaction efficiency and speed. The ability of the F-5M conjugate of alpha-actinin to bundle actin filaments was also used for functional characterization.

This conjugate was subsequently employed in an imaging application to determine where it was bound in skeletal muscle myofibrils.

The covalent labeling of proteins for imaging

applications is dependent upon the specific objectives and the protein to be labeled. It is a straight forward process of deciding the type of protein modification, doing pilot experiments to define conditions for labeling, labeling enough protein to allow for functionality testing, estimation of dye/protein and performing some trial imaging applications. Once you have established a protocol that gives functional protein that has sufficient signal for your imaging application, you can repeat the protein labelling on a larger scale and have plenty of labeled functional protein for more extensive experimentation.

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Discussion with Reviewers

M. Malecki: In studies on incorporation of proteins, not only would you record an increase of fluorescence intensity due to accumulation of protein derivatized with fluorochrome, but also you would experience reduction in that intensity as the result of fading and further exchange with underivatized molecules. Would you find it feasible to quantitate the final number of incorporated molecules based upon fluorescence?
Author: Yes, I would find it feasible to quantify the number of incorporated molecules based upon fluorescence intensity. One does not obtain an absolute value for number of molecules but monitors a relative change in fluorescence intensity. The competition between unlabeled and labeled molecules for incorporation can be a problem but the experiments are usually done with the labeled molecule in great excess over unlabeled such that this in not a problem. You can use the opposite approach of getting maximal incorporation of labeled molecules followed by wash-out of labeled molecule with unlabeled by following the decrease in fluorescence intensity. Photobleaching is an over-riding experimental problem when trying to measure fluorescence intensity. A few ways of dealing with it are minimizing exposure time to excitation light, adding oxygen or free-radical scavengers to the medium for in vitro experiments, and characterizing the bleaching kinetics for your system then correcting intensity values using the kinetic constants obtained from the bleaching time-course. For the systems that I work in, I do not focus using the fluorescence image, rather, I focus using phase contrast then obtain and image in the fluorescence channel only using one exposure time. Thus, bleaching complications are minimized.

M. Malecki: In your experience, what is the most efficient, but least toxic anti-fading agent applicable for studies on living systems?
Author: There is no readily accepted agent to minimize fading problems in living systems since oxygen is required for the cells. Rather, the approach is to optimize the imaging system sensitivity such that minimal exposure to excitation light is used. This is accomplished by minimizing exposure during focus and image acquisition. Currently available sensitive cameras (both video and CCD) allow one to obtain high quality images with short exposure times. Selection of more photo-stable dyes also facilitates imaging in live cells.

M. Malecki: How would you quench formation of free radicals to reduce phototoxicity for living systems?
Author: The cell itself has free-radical scavengers in the form of reduced glutathione and ascorbate but these could be depleted during long exposure to excitation light. Again, the best approach is to use minimal exposure excitation light and photo-stable dyes.

J.R. Lakowicz: Many authors have noticed that the fluorescence intensity of labeled proteins does not increase linearly with the number of covalently attached groups. Have you noticed such effects in your labeling of alpha-actinin? If you have noticed such effects, which probes seem to show the greatest amount of self quenching?
Author: Yes I have observed this most noticeably upon over-conjugation of alpha-actinin with Texas Red. I have not noticed it as readily with fluorescein. Part of this self quenching may be because the probe becomes buried in hydrophobic regions of the protein thus changing its excitation and emission properties and there is the possibility of inner-filter effects when many dye molecules are confined to the small volume of the protein. We try to use dye to protein ratios of 1 for most proteins that we use and use low amounts of dye for the conjugation reaction so that we do not over-label the protein. High labeling ratios are also much more likely to alter biological function.

J.R. Lakowicz: Many probes have been reported to photobleach rapidly in microscopy. What types of fluorophores have you found to photobleach most rapidly in microscopy? What types of probes have you found to be most stable in microscopy?
Author: From my experience, fluorescein and lower excitation wavelength probes are the most susceptible to photobleaching while the longer wavelength dyes, such as Texas Red and the Cye dyes are less susceptible whether antifade agents are added or not.

J.R. Lakowicz: What would you consider to be the characteristics of ideal fluorescent probes?
Author: The ideal fluorescence probe would be readily reactive with cysteines (preferably a maleimide derivative), have excitation above 340 nm so that special optics would not be needed, and be photostable so that bleaching is minimal.