Chapter 1

Fluorescent Analog Cytochemistry: Tracing Functional Protein Components in Living Cells

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I. Introduction

The central task in modern cell biology is understanding how molecules interact in a cell to perform various functions. Unfortunately, few methods allow us to study directly the function of specific components inside living cells.

There is no reason why many techniques employed by biochemists to study molecules in test tubes or cuvettes cannot be adapted to the living cell. As long as the method has a high enough sensitivity and yields specific signals within a complicated environment, it should be equally useful within living cells. Fluorescence techniques readily satisfy these criteria (see,

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for example, Taylor *et al.*, 1986; Weber, 1986). Fluorescent probes, covalently linked to specific molecules, have been used extensively in biochemistry to report molecular activities and interactions. By incorporating the fluorescently labeled molecules into a living cell, one should gain equally valuable information about the behavior of specific molecules inside living cells. This rationale led to the approach of fluorescent analog cytochemistry (Taylor and Wang, 1978, 1980).

Fluorescent analog cytochemistry involves preparation of fluorescently labeled cellular components (fluorescent analogs), followed by the introduction of the analogs into living cells. When properly prepared, the conjugates can maintain all or most of the original properties and functions. In addition, after entering the cell, many conjugates have been observed to associate with normal physiological structures.

So far, fluorescent analog cytochemistry has been applied primarily to cytoskeletal and surface components. In the simplest application, one can study the ability of the fluorescent analog to incorporate into native cellular structures. One example is the comparison of the incorporation of analogs prepared from different actin isoforms into stress fibers and myofibrils (McKenna *et al.*, 1985). By looking at very early time points after delivery, the site of incorporation can also be identified (Amato *et al.*, 1986). The study can also be combined with immunoelectron microscopy, using antibodies which recognize specifically microinjected components (Amato *et al.*, 1986; Gorbsky and Borisy, Chapter 11, this volume), to reveal the distribution of the analogs at a high resolution.

A second major application is to study the distribution of cellular components during certain processes, such as mitosis, or following treatment with agents which induce specific changes. For example, we have analyzed the distribution of α-actinin in developing muscle cells to determine how myofibrils reach their high degree of organization (McKenna *et al.*, 1986). Finally, fluorescent analog cytochemistry has been combined with fluorescence photobleaching techniques to study the mobility of molecules associated with the cell surface and with the cytoskeletal structures (e.g., Wang *et al.*, 1982c; Wang, 1985). Both bulk translocation and random movement of molecules may be analyzed.

This chapter will focus on the practical methods involved in the application of fluorescent analog cytochemistry to intracellular protein components (see related chapters in this volume for other classes of fluorescent analogs). Readers are referred to several previous articles for general discussions of fluorescent analog cytochemistry (Taylor and Wang, 1980; Wang *et al.*, 1982a, b; Kreis and Birchmeier, 1982; Taylor *et al.*, 1984, 1986; Jockusch *et al.*, 1985; Simon and Taylor, 1986).

II. Equipping the Laboratory for Fluorescent Analog Cytochemistry

The first step of fluorescent analog cytochemistry involves purification and fluorescent labeling of cellular components. Requirements for the methods involved, such as gel electrophoresis and column chromatography, are similar to those for a typical biochemistry laboratory. However, because of the sensitivity of many fluorophores to light, fluorescent labeling should be performed under reduced light; for example, in a room with dimmer control of the overhead illumination.

The cell culture facility is critical. Since cells will shuttle between the CO_2 incubator and the microscope during an experiment, the facility should be located close to the laboratory for fluorescence microscopy, especially if temperature-sensitive mutants are involved. Stocks of cells are maintained regularly. However, it is important to ensure that cells are in a highly healthy state. Poorly maintained cells often spread incompletely on the substrate, respond inconsistently to experimental manipulations, and sometimes emit a high level of autofluorescence. If necessary, culture conditions, such as the medium used or the level of glutamine, should be adjusted. We also routinely use antibiotics, such as penicillin and streptomycin, to prevent contamination during microinjection.

Special vessels are often required for plating cells during the fluorescence observation. Consideration should be given to the requirements for microinjection, cell culture, and fluorescence microscopy. For example, if cells will be injected directly with a needle, it is important to ensure that they are easily accessible for the needle and can be observed clearly at a relatively high (e.g., $\times 400$) magnification. Plastic Petri dishes, although quite accessible on an inverted microscope and ideal for cell culture, offer a very poor quality for both transmitted light and fluorescence optics. To maintain living mammalian cells on the microscope, it is also necessary to ensure proper controls of temperature, humidity, and pH. For extended observations, special devices, such as microscope stage incubators and/or perfusion chambers, should be used (McKenna and Wang, Chapter 12, this volume).

At least one high-quality fluorescence microscope is required (Taylor and Salmon, Chapter 13, this volume). We have chosen an inverted microscope for the convenience of microinjection and for the ease of the construction of the microscope cell culture system. For needle microinjection, it is important to ensure that the microscope has a sturdy base and is located on a table free of vibration. If necessary, 10-in. inner tube tires may be inserted under the legs of the table as an effective, economical means for the isolation of

vibration. Additional equipment for direct microinjection are listed in the article by McNeil (Chapter 10, this volume). Fluorescence microscopy should be performed in a dark laboratory, to allow observations with dark-adapted eyes and low light level detectors. Air cleaning devices, such as HEPA filters, are also important for maintaining both the performance of the optical equipment and the sterility of the cell culture during microinjection.

In most laboratories, fluorescence microscopes are equipped with mercury arc lamps as the light source. However, unless ultraviolet light is required for excitation, a quartz–halogen lamp is a better and less expensive alternative. For observing living cells, the intensity of excitation light should be attenuated, either by adjusting the input voltage for the quartz–halogen lamp (see, however, Taylor and Salmon, Chapter 13, this volume, for precautions), or by inserting a diaphragm or a set of polarizers in the excitation light path (Wampler and Kutz, Chapter 14, this volume).

The signal level from injected cells is limited both by the very small number of fluorophores in the cell and by the use of very low levels of excitation light in order to minimize radiation damage to living cells (Spring and Lowy, Chapter 15, this volume). In order to collect multiple images from single microinjected cells, low light level detectors are required (Spring and Lowy, Chapter 15, this volume; Aikens *et al.*, Chapter 16, this volume). We have used the Intensified Silicon Intensified Target (ISIT) camera for our experiments. The less-sensitive Silicon Intensified Target (SIT) camera does not offer enough sensitivity for this purpose. Unfortunately, detectors for very low light levels, such as the ISIT camera, are usually quite noisy, and digital image processing is required to obtain acceptable images (Inoue, 1986).

Most investigators using image processing have the difficult experience of choosing an adequate system. For fluorescent analog cytochemistry, the two most crucial functions are frame averaging and background subtraction. They will be used repeatedly during an experiment and should be performed at video speed. A useful feature is the ability to store images on a hard disk. Because of the large number of values for storage, it requires not only a large capacity but also a high speed for the storage and retrieval of data (storing images on video tapes, although more convenient, generally results in a significant loss of resolution. In our experience, except for preliminary studies of short processes or for analyzing sequences stored in a computer, a VCR is not as useful as it might appear). The system should also contain several frame buffers to allow rapid comparison of images and to allow arithmetic manipulations of images. In addition, since images are often very low in contrast, the ability to stretch the contrast is useful. Pseudocolor, on the other hand, has been of little use to us so far. Finally,

one should also consider the ability to perform quantitative analysis on fluorescence images. Even simple measurements of intensity and dimension can often provide invaluable insights. Issues related to quantitative measurement, such as corrections of shading and nonlinearity, are discussed by Aikens *et al.* (Chapter 16, this volume; see also Volume 30, this series).

Even if a computer disk storage is available, photographic equipment, such as a 35-mm camera body, macrolens, and tripod, is required for obtaining high-quality hard copies. It may also be useful to acquire a Polaroid camera for the CRT screen (such as model DS-34) or a thermoprinter for video images (such as Mitsubishi P70-U), to obtain rapid study prints. However, the quality of prints from these devices are not high enough for publication, and electronic or computer contrast enhancement may be required to obtain usable images with the thermoprinter.

III. Preparation of Fluorescent Analogs

Not all proteins are suitable for the application of fluorescent analog cytochemistry. First, considerations should be given to the abundance of the protein inside cells (Wang *et al.*, 1982b). For minor components, it may be impossible to obtain detectable signals without inducing serious disruptions to the cell. Second, the application is limited to proteins which are soluble under conditions compatible with living cells. The presence of detergents, denaturing agents, and extremes of pH and osmolarity will result in cellular damage during delivery. Third, the destination of the analog should be considered. While proteins can be delivered readily into the cytoplasm and nucleus (McNeil, Chapter 10, this volume), incorporation of integral membrane proteins or intraorganelle components may be more difficult or impossible. Fourth, some criteria are required to determine whether the analogs have been utilized properly by the cell. This may be very difficult for proteins not involved in macromolecular assemblies. Finally, analogs in the order of milligrams are often required for each preparation to attain proper final concentration or volume for loading. Therefore it would be more efficient to choose proteins which can be isolated with high yields and stored conveniently.

Following purification of the protein under study, chemical reactions are used to couple a fluorescent probe to the protein. The optimal probe to use and the labeling condition vary with each protein, and must be determined empirically. In general, fluorophores with relatively long wavelengths of excitation and emission and high quantum yields are preferable (Simon and Taylor, 1986). However, other factors also need to be considered. For

example, phycobiliproteins, although ideal in terms of fluorescence properties (Oi *et al.*, 1982), suffer from their very large sizes, which may affect both the efficiency of conjugation reaction and the properties of conjugates. In addition, some fluorophores (notably, the rhodamine family) tend to associate noncovalently with proteins. In some cases such noncovalently associated probes are very difficult to remove, and a different probe has to be used.

Consideration should also be given to the chemical reaction for the conjugation (Simon and Taylor, 1986). Usually each fluorophore will be available in several different reactive forms, e.g., isothiocyanate and maleimide. The optimal one to use is often difficult to predict and must be determined empirically. Different reactions may have different effects on the functional state of the conjugates, even if the reagents share similar reactive properties. However, the reaction condition should always be mild enough to preserve the native properties of the protein, and the bond should be stable enough to ensure association after introduction into cells. It should be noted that many amine-directed dyes (e.g., isothiocyanates) also react readily with the sulfhydryl group via a relatively labile bond, which may easily break in the cytoplasm. Such conjugates should be treated with dithiothreitol to facilitate removal of reversibly associated probes.

Although many proteins (e.g., α-actinin) can be labeled directly, some may require the protection of active sites. One approach is to label a complex of the protein with ligands or accessory proteins, followed by dissociation and purification of the analog(s). A variation of this approach is to label partially purified preparations while the protein is still associated with native ligands. Besides protecting active sites, these methods may yield several useful analogs simultaneously. Alternatively, proteins may be labeled without protection and subsequently selected for functional conjugates. For proteins that form macromolecular assemblies, such as actin and tubulin, several cycles of polymerization–depolymerization will provide assembly-competent molecules. For other proteins, affinity chromatography with proper ligands may be applied. Such selections will ensure that the conjugates maintain qualitatively the ability to bind, but quantitative characteristics, such as the rate of association, may still be altered.

The most important task after the labeling reaction is to remove unconjugated fluorophores (see also Chapter 2 by Maxfield and Chapter 6 by Edidin, this volume). In our experience, dialysis is rarely adequate for this purpose. Even if the dialysate appears nonfluorescent, the protein may still be heavily contaminated with noncovalently associated fluorophores. The most common method of separation, gel filtration in a column of Sephadex G-25 or G-50, can also be seriously misleading. For example, even after an apparent "clean" separation of the void volume (containing the conjugate)

from the included volume (containing the free dye), the conjugate may still contain a significant amount of noncovalently associated fluorophore. Conversely, commercial tetramethylrhodamine dyes often contain small aggregates which move in the void volume. An alternative, often more effective method for removing unconjugated fluorophores, is the Bio-Bead SM2 (Bio-Rad Laboratories, Richmond, California). These beads remove free fluorophores based on hydrophobic interactions and can be used with fluorescein or tetramethylrhodamine, but not lissamine rhodamine B. Ion-exchange chromatography may also be applied to separate adsorbed probes. In some cases, it has the additional advantage of fractionating conjugates according to the degree of labeling (Dandliker and Portmann, 1971). Finally, it should be noted that the effective method of separation varies not only with fluorophores, but also with reactive groups and proteins. The optimal method should be determined during the preparation of each new conjugate.

IV. Assays of Fluorescent Analogs and Preparations for Microinjection

Serious effort should be made to ensure the absence of noncovalently associated fluorophores. For example, after electrophoresis in a SDS–polyacrylamide gel, noncovalently associated fluorophores will dissociate from the polypeptide and usually move in front of the Bromophenol blue tracking dye. Alternatively, a G-25 desalting column equilibrated in 1–2% SDS will also yield reliable information. Free fluorophores will appear in the included volume. After all fluorophores are proved to be covalently associated, measurements should be performed to determine the molar ratio of labeling (Wang *et al.*, 1982b; Simon and Taylor, 1986). The value, although usually representing no more than an estimate, serves as a useful reference for adjusting the reaction condition and for ensuring consistency among experiments.

As emphasized in previous articles (Wang *et al.*, 1982b; Taylor *et al.*, 1984), functional assays should be performed to determine the effect of labeling on the protein. Changes or partial loss of activities is not uncommon and should not necessarily prevent one from using a particular fluorescent conjugate, as long as the effect is taken into account in the interpretation. Spectroscopic characterizations should also be performed if the experiment involves quantitative fluorometry of analogs in different structures or environments.

Sometimes extracted cell models are used to test the binding of the analog to residual structures (e.g., Sanger *et al.*, 1984). However, while the infor-

mation is valuable, the interpretation as a functional assay may be complicated by the possible disruption or creation of binding sites during the extraction.

For direct microinjection, it is usually much more desirable to introduce a small volume of concentrated solution (≥1 mg/ml), rather than a large volume of dilute solution. The total volume of the stock required for each experiment is very low (in the order of microliters). We regularly use the Amicon Centricon and Centriprep filters for concentrating protein analogs. However, some proteins (e.g., tropomyosin) appear to stick to the membrane. These proteins may be concentrated by vacuum dialysis through a Colloidin bag (Schleicher & Schuell, Keene, New Hampshire). Besides concentration, colloidin bags are also ideal for microdialysis, which is required to bring the analog into the solution for microinjection (Wang *et al.*, 1982b), and to remove any toxic materials which might be introduced during the concentration process. Finally, in order to perform direct microinjection, the solution should be clarified thoroughly. We have used a Beckman-type 42.2Ti rotor in a regular ultracentrifuge (20-minute spin at 25,000 rpm) and an Airfuge (23 psi, 20 minutes) for this purpose. Centrifugation in a microfuge or 0.22-μm filtration usually yields only marginal results. Clarified solutions should be handled very carefully: introduction of air bubbles readily induces aggregates which greatly aggravate the microinjection process.

V. Delivery of the Conjugates and Handling of Living Cells

Although the most common method for delivering the analog into living cells is needle microinjection, there is no reason to reject other, perhaps more convenient, methods (McNeil, Chapter 10, this volume). Some of these methods may actually be better tolerated by cells which are exceptionally sensitive to pressured flow from a microneedle (e.g., *Xenopus* myotomal muscle cells; N. M. McKenna, unpublished observations). However, bulk loading methods generally require a much larger amount of fluorescent analogs, and may not be compatible with some proteins. For example, both actin and myosin polymerize in balanced salt solutions used for bulk loading. Many bulk methods also have a serious limitation regarding the size of molecules to be delivered (McNeil, Chapter 10, this volume).

Fine adjustments are required for microinjecting different proteins. The amount of analogs a living cell can incorporate varies. For example, there is a clear limit for the incorporation of vinculin into adhesion plaques. Microinjecting a large amount of vinculin results in an increase in diffuse cytoplasmic fluorescence and obscures adhesion plaques. The shape of the

needle should also be tailored according to the property of the solution. In general, a more rapid taper and larger tip opening should be used for more viscous solutions. For actin and myosin, it is also helpful to maintain a continuous flow between cells, otherwise the influx of salt in the medium will cause the protein to polymerize at the tip of the needle.

It is often necessary to identify a particular microinjected cell. This can be achieved by marking the surface of the cover slip with a diamond pen before microinjection. The position of a particular cell may then be noted relative to the scratch mark. Alternatively, a diamond marking device, which screws into the nosepiece of the microscope, can be purchased from Leitz, Inc. (Rockleigh, New Jersey). It makes neat, circular marks of different diameters around the center of field. A less expensive version of this approach uses rubber stamps. However, the ink dissolves in microscope immersion oil and is incompatible with oil-immersion objectives.

After microinjection, the medium should be replaced to remove any fluorescent analog located outside the cell. The dish is then returned to the incubator for 1–3 hours before observation. This allows both the recovery of cells from any damage inflicted by microinjection, and the incorporation of analogs into structures. Time required for the latter varies from 10 to 15 minutes for actin and from 2 to 3 hours for myosin.

Microinjected cells should first be examined for possible disruptive effects and intracellular distribution of the analog. Some fluorescent analogs may have toxic effects. For example, we found fluorescent caldesmon to cause rounding and detachment of fibroblasts (S. K. Stickel and Y.-L. Wang, unpublished observations). The analog may also form artifactual structures, and immunofluorescence should be performed to compare the distribution of the analog with that of the endogenous counterpart, although immunofluorescence itself may yield artifacts. Some proteins also exhibit variable behavior in different cells. For example, fluorescently labeled muscle actin incorporates consistently into native structures in chick embryonic fibroblasts and IMR33 gerbil fibroma cells, but forms paracrystalline structures in NRK cells and PtK cells (Wehland and Weber, 1980). In addition, different fluorophores also appear to affect the extent of actin incorporation into stress fibers (McKenna *et al.*, 1985).

VI. Data Collection and Interpretation

If the purpose of the experiment is to study incorporation, then simple photography may be adequate. This often requires several minutes of exposure and results in photobleaching and cellular damage. In order to perform prolonged observations, low light level detectors should be em-

ployed (Spring and Lowy, Chapter 15, this volume; Aikens *et al.*, Chapter 16, this volume). However, even with a highly sensitive detector, cells should not be exposed to continuous illumination. Structural damage (e.g., breakdown of actin filament bundles) in cells injected with rhodamine-labeled actin may be noticed, for example, after 20 minutes of continuous recording with a video tape recorder. Efforts should also be made to minimize the time of visual observation of fluorescence. We usually use visual observation only to search for areas of microinjection, then switch immediately to a low light level video camera. The image detector should be used at the maximal acceptable sensitivity, while the excitation light should be at the minimal acceptable level. These adjustments are dependent on the purpose of the experiment (e.g., qualitative imaging or quantitative photometry) and on the availability of digital image-processing systems. Under ideal conditions, a single cell may be recorded off and on for at least 3 days.

If a digital image processor is unavailable, images may be recorded with a 35-mm camera and a motor winder mounted in front of the TV monitor. A simple electronic controller can be assembled to trigger the shutter for excitation light and the camera at the same time. This reduces unnecessary exposure of the cell to the excitation light. By adjusting the exposure time between 1/4 and 2 seconds, images may also be "integrated" on the film to improve the signal-to-noise ratio.

Ideally, images should be acquired with a digital image-processing system. In my laboratory, we first record an image of the "dark count" with no light entering the low light level video camera. Then the illumination shutter is activated briefly, and 64–128 frames of cellular fluorescence images are averaged. The image of dark count is then subtracted from the cellular image and the result stored in a disk file. This process requires typically 5–20 seconds, depending on the performance of the image-processing system. Although this method may not be suitable for very fast processes, it preserves the data with little distortion.

Many experiments require detailed comparison of fine differences among images. For images stored on computer disks, this may be done theoretically by loading one picture after another into the viewing frame buffer. However, the speed for loading is usually not high enough to allow perception of a continuous motion. If the number of images is small, the comparison may be done by loading different images into multiple frame buffers. A simple program can be written to continuously switch different frame buffers for display, and the differences will be much easier to perceive. If a large number of images are involved, such as in time-lapse studies, a time-lapse video recorder can be used. The images are retrieved one by one from the computer disk while recording at a low speed. Playing back at a high speed will then turn those images into motion pictures.

Most image-processing systems will also perform at least some quantitative analyses. This issue is covered in detail in the companion volume (Volume 30, this series). Moreover, fluorescence photobleaching recovery may be used, using equipment identical to that for membrane-bound probes (Wolf, Volume 30, this series), to study the mobility of incorporated analogs.

An important last step is obtaining hard copies of the images for presentation. High-quality prints should be prepared with a photographic camera aligned properly in front of the monitor screen. It should be noted that the large variation in the quality of published video images is due largely to the adjustment of monitors and the photography, not the quality of the monitors. The most important, often overlooked, adjustment is the vertical hold of the monitor. The method for adjusting vertical hold, as well as a general description of the photography of CRT screens, may be found in the book by Inoue (1986).

VII. Prospectus

The discussion in this chapter represents our experience in using currently available equipment to trace protein molecules in living cells. The application so far has been limited primarily to cytoskeletal components, and the potential to study other classes of protein components as well as large complexes remains to be investigated. Similarly, little has been done to take advantage of various sophisticated analytical methods, as discussed in Volume 30 in this series. The basic application of fluorescent analog cytochemistry is also expected to undergo continuous changes with the advancement of related technologies. For example, the development of new detectors (Spring and Lowy, Chapter 15, this volume; Aikens *et al.*, Chapter 16, this volume), the application of confocal scanning microscopy (Brakenhoff *et al.*, Volume 30, this series), and the improvement of video recording equipment will all affect future applications of this approach. Advances in these areas should further enhance the power of fluorescent analog cytochemistry and facilitate an understanding of how different protein molecules work together in living cells.

Acknowledgments

The author wishes to thank Dr. Nancy McKenna for reading the manuscript. The work was supported by grants from National Institutes of Health (GM-32476), National Science Foundation (DCB-8796359), and the Muscular Dystrophy Association.

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