METHODS IN CELL BIOLOGY

VOLUME 25 THE CYTOSKELETON PART B

EDITED BY LESLIE WILSON

METHODS IN CELL BIOLOGY

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Part B. Biological Systems and in Vitro Models

Edited by

LESLIE WILSON

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PREFACE

Knowledge of the organization of the cytoplasm of eukaryotic cells has expanded greatly during the past decade. The early view of the cytoplasm as a structureless "soup" has given way to our present understanding of it as being exquisitely ordered. This order is conferred by a three-dimensional network of fibrous structures, which include microtubules, microfilaments, and intermediate filaments, and the fiber-associated molecules that mediate the interactions and functions of the fibrous elements with each other and with cytoplasmic organelles. The three-dimensional network of fibrous structures has come to be known as the *cytoskeleton*, though the dynamic nature of the network is not captured by the term.

Early research on the cytoskeleton focused on identification of the major constituents of the cytoskeleton and their characterization, in terms of both their organization in cells and tissues and their biochemical properties. More recently, our attention has been turning strongly toward understanding the functions of cytoskeletal elements in living cells, and has focused on investigation of "less visible" cytoskeletal components (which are considered to interact functionally with the surfaces of the major types of filaments) and on the development of methods and model systems for investigating the functional interactions of cytoskeletal components with one another and with other cell components.

This volume consists of 18 chapters that describe cell systems and *in vitro* model systems presently or potentially valuable for the elucidation of the functions of the cytoskeleton or its components in living cells. The methods involved have been described in considerable detail and are often accompanied by a section of overview perspectives that should aid investigators new to this research area. The previous volume of this publication (Volume 24, The Cytoskeleton, Part A) consists of 24 chapters concerned with the isolation and characterization of cytoskeletal components, and with the development of research tools to enable the study of cytoskeletal components in living cells and *in vitro* models.

I wish to thank all of the authors who have so generously contributed chapters to this volume, and I apologize to readers who are searching for a technique that has not been included. I also wish to thank Susan Overton for her substantial help in preparing the Index.

LESLIE WILSON

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Chapter 1

Fluorescent Analog Cytochemistry of Contractile Proteins

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I. Introduction

A new approach to investigate the distribution and interaction of cytoskeletal and contractile proteins was introduced in 1978 (Taylor and Wang, 1978) by combining the techniques of microinjection, fluorescence spectroscopy, and image intensification. The general approach, which we now call fluorescent analog cytochemistry (FAC; previously referred to as molecular cytochemistry), has been defined as the incorporation of functional fluorescent analogs of cellular components into or onto living cells.

Fluorescent analog cytochemistry is specifically designed to follow the distribution and interaction of molecular components in single living cells. However, as with any technique, errors in experimental design, execution, or interpretation can effectively limit this potentially powerful approach. We have discussed some of the advantages and practical considerations of fluorescent analog cytochemis-

try in our early papers. This chapter is a set of guidelines and procedures for the optimal utilization of the technique. We will emphasize the most critical factors and point out common pitfalls.

II. Procedures

The technique involves four major steps: (1) purification and fluorescent labeling of cellular components; (2) biochemical and spectroscopic characterization in vitro; (3) incorporation into or onto living cells; and (4) image recording and interpretation. The following discussion will be limited to proteinaceous, cytoplasmic components using our results with 5-iodoacetamidofluorescein-labeled actin (5-AF-actin) as illustrations (Taylor and Wang, 1978; Wang and Taylor, 1979, 1980; Taylor et al., 1980). A variety of labeled proteins have been incorporated into various cell types since we first introduced this technique (Feramisco, 1979; Feramisco and Blose, 1980; Kreis et al., 1979; Wehland and Weber, 1980).

A. Purification and Fluorescent Labeling of Cellular Components

Fluorescent labeling of proteins in general has been discussed in several previous reviews (Dandliker and Portmann, 1971; Fairclough and Cantor, 1978; Stryer, 1978). The fluorescent conjugate (hereafter referred to as the "fluorescent analog" of a cellular component) can be prepared either by (a) labeling the purified protein subunits or by (b) labeling a supramolecular complex that contains the target protein, followed by fractionation of the labeled conjugates. This latter approach has the advantage that active sites for structure formation are more likely to be protected from modification.

The fluorescent-labeling reagents should be chosen based on several criteria. The fluorophore should absorb in the visible range, optimally 450-650 nm in wavelength, so that radiation damage to living cells and interference from autofluorescence can be minimized. Furthermore, the fluorophore should be stable under recording conditions. Optimally, fluorophores with high quantum yields and high extinction coefficients should be chosen, in order to maximize the signal from single cells. The reactive group should create a covalent bond that is stable inside the cell, without affecting the biochemical functions of the protein. The optimal reactive group will be determined to a large extent by the properties of the specific protein. For some well-characterized proteins such as actin, it is possible to choose site-specific reagents directed toward an apparently nonessential site (e.g., sulfhydryl reagents react predominantly with cys-373 in F-actin; Elzinga and Collins, 1975; Wang and Taylor, 1980). If nonspecific reagents are

used, it is important either to isolate a functional, labeled fraction after reaction or to protect the active sites furing the reaction.

To date, several fluorescent reagents have been used for fluorescent analog cytochemistry. 5-Iodoacetamidofluorescein, rhodamine isothiocyanate (RITC), and dichlorotriazinyl aminofluorescein (DTAF) (Wang and Taylor, 1980; Feramisco, 1979; Keith *et al.*, 1980). Many other fluorescent reagents such as 7-chloro-4-nitrobenzo-2-oxa diazole (NBD-Cl), eosin isothiocyanate, and cyanine dyes fit into the above criteria and remain to be explored (Ghosh and Whitehouse, 1968; Cherry *et al.*, 1976; Waggoner, 1979). Of particular interest is the "piggyback" labeling techniques using Rhod τ a-lactalbumin introduced by Shechter *et al.* (1978). This technique has great potential for preparing fluorescent analogs of minor cellular components and yielding high fluorescence intensity from a very small number of labeled molecules.

B. Biochemical and Spectroscopic Characterization in Vitro

It is very important to characterize various properties of the fluorescent analog *in vitro* before microinjection into living cells. These assays not only minimize the possibility of artifacts after microinjection, but also provide necessary information for interpreting results from injected cells.

The solution of the fluorescent analog should be free of unbound or noncovalently associated fluorophores; otherwise, a confusing background fluorescence will be observed in the injected cell, and the results will be impossible to interpret. The test can be performed by using either a desalting column preequilibrated with SDS or SDS-gel electrophoresis. A single fluorescent band comigrating with the protein should be detected.

The average number of fluorophores per protein molecule (F/P ratio) must be determined in order to allow proper evaluation of biochemical assays. The concentration of fluorophore can be determined most conveniently by light adsorption (Dandliker and Portmann, 1971). However, it is necessary first to determine the extinction coefficient of the bound fluorophore. A simple method to obtain approximate values has been described by Hartig et al. (1977). The method assumes that the extinction coefficient of the fluorophore bound to unfolded protein (in 7.5 M urea) is equal to that of the original fluorescent reagent in the same solvent. For those fluorescent reagents that undergo significant changes in electron distribution on reaction, model compounds prepared by reaction with small molecules (i.e., amino acids) should be used.

If a low F/P ratio is obtained, it is necessary to isolate the labeled fraction to obtain a F/P ≥ 0.5 , in order to allow unambiguous evaluation of biochemical assays. For reactions that alter the net charge of proteins, the fractionation can be carried out by ion-exchange chromatography (Wang and Taylor, 1980).

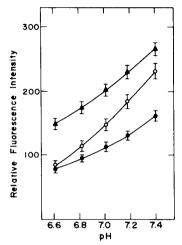


Fig. 1. Peak fluorescence intensities of 5-AF-actin are affected by both pH and the state of actin assembly. The graph indicates that the detection of G-actin (open circles) in the cell will be slightly favored over that of F-actin (solid circles). The binding of HMM (triangles) has a significant effect under saturation conditions, but given the low myosin/actin ratio in nonmuscle cells, the net effect inside cells should be limited. (From Wang and Taylor, 1980.)

Detailed biochemical assays should be carried out to assess the functional activities of the analog. For 5-AF-actin, the assays include viscosity measurement, activation of heavy meromyosin Mg²⁺ ATPase, and formation of Mg²⁺ paracrystals. The results are compared to unlabeled controls and are evaluated in relation to the percentage of protein molecules that are labeled.

Along with assays with purified proteins, additional assays can be performed using crude cell-free extracts. For example, motile extracts of *Dictyostelium discoideum* have been used to test the incorporation of 5-AF-actin into contracting fibrils and pellets (Taylor and Wang, 1978; Wang and Taylor, 1980). The extract system allows assays to be performed at a level between living cells and purified proteins and could cover functions not well characterized with purified proteins.

Spectroscopic characterization is very important for the interpretation of fluorescence signals from injected cells. It is necessary to determine whether the fluorescence intensity of spectra are affected by solvent parameters, such as pH and free Ca²⁺ ion concentration, which could vary in different regions of the cell or during different physiological states of the cell. In addition, the effect of conformational change or structural transformation on fluorescence properties should be investigated, since a particular conformation or structure could be favored at certain sites of the cell and affect the pattern of fluorescence distribution (Fig. 1).

C. Incorporation into Living Cells

The optimal target cell is determined to a large extent by the questions under investigation. For example, cultured mammalian cells are particularly amenable to whole-cell image analysis using tv-image intensifiers and computers, because of the short pathlength and distinct cytoskeletal structures. On the other hand, large cells, such as sea urchin eggs, giant amoebas, and large cultured cells (e.g., myotubes and newt lung fibroblasts), can be measured with large measuring spot sizes and yield strong fluorescence signals, and thus are more readily applicable to microspectrofluorimetric measurements.

A number of microinjection techniques, including direct-pressure microinjection, red cell ghost fusion, liposome fusion, and cell permeation, have recently been introduced or refined to deliver macromolecules into living cells (for a review, see Taylor and Wang, 1980). Two important precautions should be followed when applying these techniques.

First, the solvent condition should be compatible with both the target cell and the fluorescent analog. The cytoplasm should be kept from exposure to harmful conditions such as high pH (>7.2) or high free Ca²⁺ ion concentration (>10⁻⁵M). In addition, appropriate solvent conditions should be chosen to stabilize the analog. For example, G-actin should be kept in the presence of nucleotides and divalent cations. Conditions that favor actin polymerization, such as high ionic strength (>10 mM) and high Mg²⁺ ion concentration (>0.2 mM), should be avoided.

Another consideration is the amount of the fluorescent analog to microinject. The final concentration of the analog in the cell should be sufficiently high to yield detectable signals, but low enough to minimize disturbance of the cell. For proteins with enzymatic activities, this precaution is especially important. We have set an upper limit at $\sim 10\%$ of the concentration of the corresponding endogenous component. Unfortunately, for most microinjection techniques (except direct-pressure microinjection; see Kiehart, Chapter 2, this volume), reliable ways to control the volume of delivery have yet to be developed.

An important question is whether the analog can actually be utilized by the cell after microinjection into the cytoplasm. The incorporation of analogs into cellular structures could be affected by such factors as the rate of turnover, the accessibility of incorporation sites, and the mechanism of structure formation. Using mass-incorporation methods such as ghost fusion, it should be possible to apply biochemical techniques to study the turnover of the injected analog (Rechsteiner, 1979). The question of incorporation can be studied by using model systems in which the behavior of the component has been characterized in detail. For example, the single-cell model of *Chaos carolinensis* has been used to test the incorporation of 5-AF-actin (Taylor and Wang, 1978; Taylor *et al.*, 1980). A significant fraction of 5-AF-actin is found to remain associated with the ghost

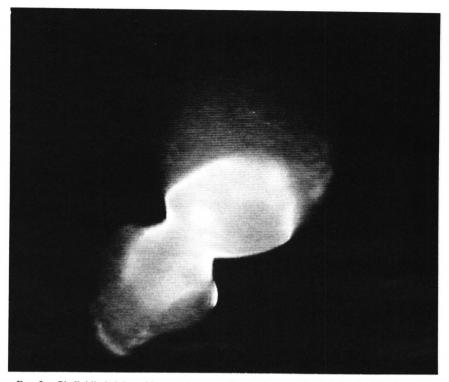


Fig. 2. Phalloidin is injected into a *Chaos carolinensis* that contains 5-AF-actin. The fluorescent fibrils contract irreversibly to one end of the cell and recruit most of the 5-AF-actin. Image recorded using a RCA SIT coupled to a NEC videotape recorder.

and membrane-free cytoplasm on rupturing a preinjected cell in a stabilizing solution. Furthermore, 5-AF-actin becomes incorporated into cytoplasmic fibrils when a cell with 5-AF-actin is postinjected with phalloidin (Fig. 2). These observations are consistent with the known characteristics of endogenous actin in amoebas (Taylor *et al.*, 1976; Stockem *et al.*, 1978). The use of model systems indicates whether the analog can be utilized by the cell under specified conditions, but does not prove that the analog can be incorporated into all possible cell structures or under all conditions. When interpretating results from injected cells, the possibility of "false negatives" should always be considered.

D. Image Recording and Interpretation

The fluorescence image of injected cells can be recorded using photography, and high-quality records can be obtained from nonmotile processes (under condi-