Fluorescently labelled molecules as probes of the structure and function of living cells

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A new approach to cell biology has been created by combining the techniques of micromanipulation of cells, fluorescence spectroscopy and low level light detection. These methods are now being used to study the spatial and temporal distribution, interaction and activity of specific molecules in living cells.

CELL functions such as locomotion, cell recognition, endocytosis, exocytosis, and cell division involve highly dynamic and transitory molecular interactions. Early cell physiologists studied these functions by probing living cells and developed sophisticated techniques for micromanipulation and microinjection[1-4]. A new approach bridging the gaps between cell physiology, biochemistry and ultrastructure is required to define the molecular mechanisms of complex cell functions. Such an approach would require a combination and coordination of (1) techniques for manipulating living cells, (2) techniques which provide signals from specific molecules with high sensitivity, and (3) techniques which detect weak signals from living cells. Recently, the methods of micromanipulation of cells[1-5], fluorescence spectroscopy[6-8], and low-level light detection[9-13] have been combined to provide such a new approach to cell biology. This new concept involves labelling purified molecules covalently with fluorescent probes, and incorporating the fluorescent conjugates into or onto living cells. Cells with associated fluorescent conjugates are then either viewed with an image intensifier coupled to a microscope or are investigated with a microspectrofluorometer. Therefore, the high sensitivity ($\sim 10^3$–10^6 molecules can be detected[14]), and versatility[6-8] of fluorescence techniques can be fully utilised in living cells to yield information at the molecular level.

This review is limited to applications of this approach in which purified molecules are labelled, administered and then studied at the light microscopical level. The technical aspects will be discussed in detail since this new approach requires careful application and interpretation. In addition, three categories of fluorescent conjugates will be discussed: (1) nonperturbing indicators of physiological processes, (2) biologically active agents and (3) functional cellular components (molecular cytochemistry)[15]. Some of the studies referenced could be included in more than one of these categories.

Experimental methods

The application of fluorescent conjugates to studies in single living cells demands the use of highly fluorescent fluorophores which absorb in the visible spectrum, so that adequate signals are obtained while minimising radiation damage and interference from autofluorescence[16]. Furthermore, the conjugates must be associated by stable covalent bonds and be devoid of non-covalently bound fluorophores. Recent advances in the preparation of fluorescent reagents have provided several compounds suitable for applications *in vivo*. Fluorochromes such as fluorescein[15-17], eosin[18], 7-chloro-4-nitrobenzo-2-oxadiazole (NBD)[19], and a series of long-wavelength rhodamine dyes[15,20-21] have been used successfully with living cells. In addition, various reactive derivatives of these fluorophores such as iodoacetamide[15], isothiocyanate[20] and sulphonyl chloride[13] can be obtained commercially. Many of the long-wavelength fluorophores used primarily by neurophysiologists should be useful when reactive derivatives become available[22].

Classical microinjection techniques are still the most direct methods for incorporating exogenous components into living cells[1,23]. Several different microinjection systems, using both hydraulic pressure[4,5,17] and compressed air[13,24,25], have been described in detail. Cells ranging in size from giant protozoans (~600 μm) to human fibroblasts (~15 μm) have been successfully microinjected. These techniques are time consuming and the numbers of cells which can be studied are limited. However, they have the advantages that only a very small volume of material is required for each experiment; components as large as

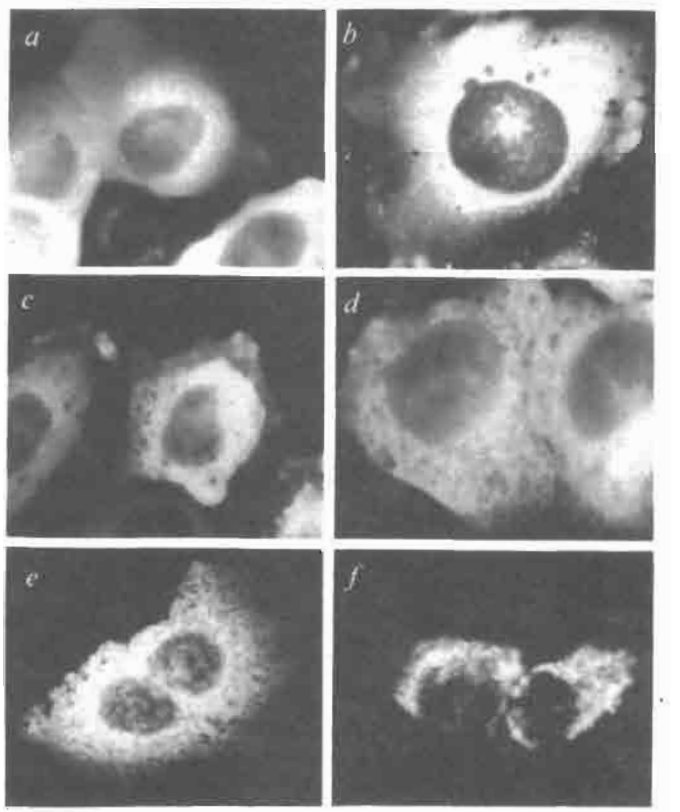

Fig. 1 Autophagy studied by injecting highly concentrated tetramethylrhodamine isothiocyanate labelled bovine serum albumin into HeLa cells. These fluorescence micrographs were taken of injected cells *a*, immediately; *b*, 30 min; *c*, 1 h; *d*, 90 min; *e*, 6 h thereafter. The labelled proteins were taken up into vesicles which ultimately fused with lysosomes (*f*). (From Stacey and Allfrey[23].)

organelles can be microinjected[4], mild solution conditions can be used to deliver the fluorescent conjugates[13,15,17,20], and injection volumes can be controlled.

During the past few years, new delivery methods using fusion techniques have been developed. The molecules are trapped inside carriers such as red blood cell ghosts[26] and liposomes[27,28] which are subsequently fused with target cells. While these methods allow the 'ultramicroinjection' of large populations of very small cells there are also several limitations: (1) relatively large volumes of fluorescent conjugates are required during the loading process, (2) the conjugates could become exposed to detergents, elevated temperatures, or organic solvents, (3) the proper entrapment and fusion steps must be carefully verified, and (4) the biological effects of the carriers must be controlled. Recently, fibroblasts have been successfully loaded with fluorescently labelled antibodies by red blood cell fusion techniques[29]. Continued improvements in fusion technology should make these approaches more generally useful in the future.

Cell perfusion techniques have been used to deliver small ions and molecules into plant cells[30]. A membrane permeation technique has now been reported which permits the incorporation of small molecules into living animal tissue culture cells[31]. The cells are permeabilised by short treatments with lysolecithin which make the cells leaky to exogenous molecules in the medium for a short period of time. This technique has advantages similar to those of fusion methods, yet does not require the use of carriers. Unfortunately, the application is limited to only very small molecules (molecular weight below 10,000) and to cells grown in monolayer culture. In addition, the cells remain viable for only a short period.

Recent developments in image intensification techniques have provided a more sensitive way of recording fluorescent images than classical photographic methods[10]. The use of standard photographic procedures requires long exposure times and intense illumination. Therefore, fluorescence photobleaching could be extensive, cell damage is possible, and dynamic processes are difficult or impossible to record. In contrast, TV image intensifiers coupled to fluorescence microscopes and video tape recorders allow the continuous recording of weakly fluorescent images in real time or time lapse without significant losses in resolution[10]. Some TV cameras also provide digital output for quantitative image analyses. When these intensifiers are coupled to optical prisms and multichannel analysers, they can also provide rapid microspectrofluorometric measurements[10,32].

Microspectrofluorometers have been developed for quantitative measurements of many fluorescence parameters with very low light intensities[33,34]. In particular, photomultipliers

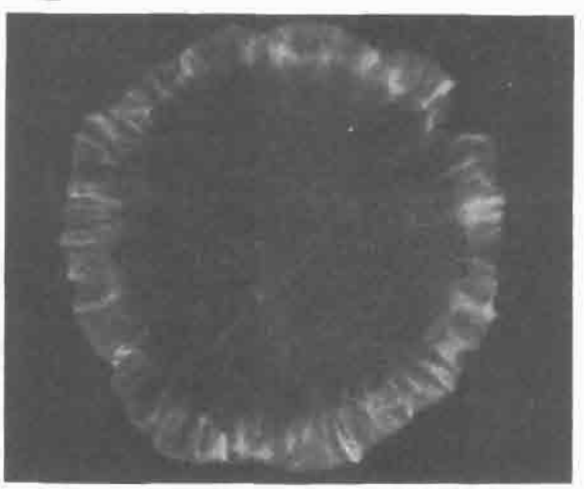

Fig. 2 Fluorescence photograph of a live chick embryo fibroblast that is lysolecithin permeabilised and labelled with NBD-phallicidin[19]. Most of the labelled actin in this spreading cell is located peripherally in the area of membrane ruffling. Some longitudinal stress fibres are also evident. (Micrograph courtesy of L. Barak.)

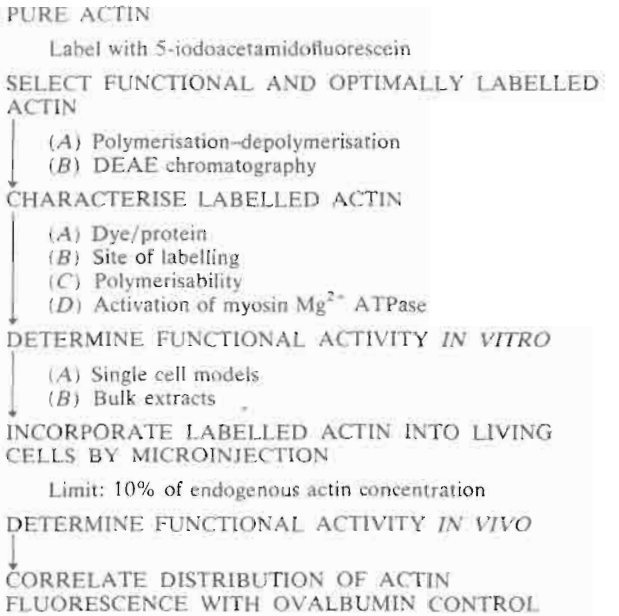

Fig. 3 Flow diagram of the steps involved in molecular cytochemistry. Actin is described as an example.

interfaced with single photon counting devices have provided instruments of high sensitivity[35]. These systems in combination with computer-controlled spectral scanning and data processing form the core of several sophisticated microspectrofluorometers which are capable of performing corrected spectral measurements on single living cells[35–38]. The use of lasers for excitation has also increased the versatility of microspectrofluorometers, because the state of polarisation, spot size, duration and intensity of the exciting light pulse can be controlled over large ranges.

Recently, fluorescence photobleaching recovery techniques have been introduced to measure the mobility of surface associated fluorophores[39–42]. These methods utilise a pulsed high-intensity laser microbeam to bleach a small area or volume of fluorescence, and the recovery of fluorescence is monitored with attenuated laser excitation. These measurements can yield diffusion constants of the mobile fraction, the percentage of fluorescent conjugates that are mobile, and information on the bulk directional flow of the conjugates. However, caution must be exercised in controlling the possible biological effects of photobleaching[43], and consideration must be given to the validity of the assumption that cell surfaces are flat and smooth. In addition, care must be taken to ensure that the membrane or surface markers are not internalised before making measurements. These potential problems have recently been addressed critically by Elson and Yguerabide[40].

Physical and chemical considerations

There are several possible physical and chemical problems which could give rise to experimental artefacts, and investigations using this new approach must be judged in part by the manner in which potential problems are considered. Factors which could affect the fluorescence intensity measured for the microscope include: (1) local accessible volume for the conjugate in the cell, (2) local environment of the conjugate, (3) optical properties of the microscope, and (4) local concentration of the conjugate.

Consideration of the local accessible volume is important for both cytoplasmic and membrane-associated components. For cytoplasmic components, the local accessible volume is controlled not only by the actual thickness of the cell, but also by the distribution of organelles which exclude the fluorescent conjugates. For membrane-associated components, the accessible volume is affected dramatically by the presence of membrane folds and microvillar structures. This problem can be

identified and controlled by using a second fluorescent conjugate which would distribute uniformly within the accessible volume. Labelled bovine serum albumin, ovalbumin, or denatured proteins have been used in controls for cytoplasmic components[13,15,17,20] and fluorescent lipid probes[41] can be used in studies on membrane-associated factors. It is imperative that co-incorporation of the experimental conjugate and the control conjugate labelled with a second probe be accomplished in the same cell when the cell shape is irregular[13]. Adequate comparisons cannot be performed in separate cells when they have irregular geometries. However, separate cells can be used when the cell geometry is reasonably constant and simple[17].

The local environment around the fluorescent conjugate could also affect the fluorescence intensity by altering the quantum yield or fluorescence spectral properties. Local variations in ionic parameters such as *p*H and ionic strength as well as binding of other molecules to the conjugates could change the fluorescence parameters[6,7]. The sensitivities of fluorophores can either be utilised in characterising microenvironments or they must be controlled when other parameters are under investigation. Controls for the ionic environments can be performed by comparing the fluorescence of the experimental conjugates with the fluorescence of separate cells containing nonspecific molecules (such as ovalbumin) labelled with the same fluorophore[17], while controls for the effect of specific molecular interactions require the extrapolation of data from solution spectroscopic studies[16].

Knowledge of the optical properties of the microscope is important for interpreting fluorescence images and quantitating local fluorescence properties. The characterisation of the fluorescence image *in vivo* depends on the depth of focus of the microscope. A large depth of focus in relation to cell thickness optimises the formation of an in focus image of the whole cell, while a small depth of focus yields an optical section of the cell. The latter condition would require several changes in focus to reconstruct the three-dimensional image of the cell. Quantitative measurements of fluorescence intensity are further affected by the extent of selecting light from specific planes of the specimen. This latter problem has been solved by using a combination of laser illumination and diaphragms placed in the image plane[39].

The local concentration of the fluorescent conjugates also affects the fluorescence intensity measured or visualised in different parts of cells. The distribution of the conjugates can be determined by applying a combination of the controls used for accessible volume and environmental sensitivity.

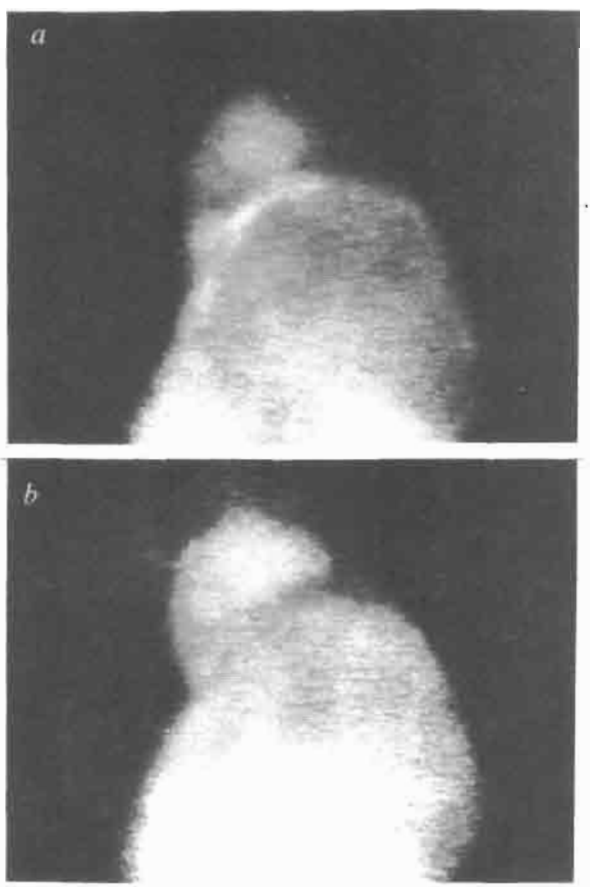

Fig. 4 Fluorescence of 5-iodoacetamidofluorescein-labelled actin (*a*) and lissamine rhodamine B sulphonyl chloride labelled ovalbumin (*b*) in the same specimen of *C. carolinensis*. Actin specific fibrils can be detected in the plasmagel sheets at the tips of advancing pseudopods (*a*). (From Taylor *et al.*[13].)

Nonperturbing indicators of physiological processes

Nonperturbing fluorescent conjugates can be used as indicators of mobility on, within, or between cells. Fluorescence indicators of normal cell-surface mobility have been studied for many years. In an early study Jeon and Bell[44] used fluorescently labelled antibodies to the cell surface of *Amoeba proteus* and a basic protein derived from a papain preparation to label the cell surface. Results based on a double labelling technique suggested that part of the cell surface could move independently of the lipid portion of the membrane.

In an elegant study, rhodamine-labelled peptides of different sizes have been microinjected into living cells coupled by gap junctions to probe the permeability and exclusion limit of the gap junction channels[45,46]. Molecules of molecular weight up to 1,200 pass through the channels of *Chironomus* salivary gland cells which indicates that the channels are ~1.0–1.4 nm in diameter. The selectivities of the various channels have been further characterised by varying the total charge of the labelled peptides[45]. For example, several mammalian cell channels can discriminate between 1–3 negative charges on the peptide backbones. The larger electronegativity inhibits passage. In addition, the permeability of multiple components has been directly compared by using mixtures of conjugates prepared with different probes[45,46]. A control for peptide degradation has also been reported.

Stacey and Allfrey used a similar technique and microinjected a wide variety of rhodamine conjugated proteins into living HeLa cells[47] to study the process of autophagy (Fig. 1). The segregation of microinjected proteins into autophagic vacuoles exhibited a high degree of selectivity with higher molecular weight proteins turning over faster than low molecular weight proteins. One protein (haemoglobin) never became autophagocytosed. The results with fluorescently conjugated proteins were verified with immunofluorescent and autoradiographic techniques.

More sophisticated applications of nonperturbing fluorescent conjugates involve the use of environmentally sensitive fluorophores and spectral analyses to probe intracellular environments. Fluorescein-labelled ovalbumin has been used to measure the cytoplasmic *p*H of single cells[48] based on the observation that the excitation spectra of fluorescein is highly *p*H dependent[49]. The *p*H is measured by determining the ratio of fluorescence emission intensity when the cells are excited at two different wavelengths. The effects of local pathlength are normalised since the ratio of intensities is determined. This fluorescence technique of measuring *p*H is less perturbative and permits better spatial resolution than standard microelectrode methods.

When applying fluorescent conjugates as indicators, the biological effects of the conjugates and the experimental procedures should be carefully examined to make sure that they are actually nonperturbing. The possible degradation of the conjugates inside the cells should also be considered, especially when the conjugates are used as size indicators[46,47]. This latter problem could be checked more critically in the future by isolating the labelled proteins from the cells after incorporation

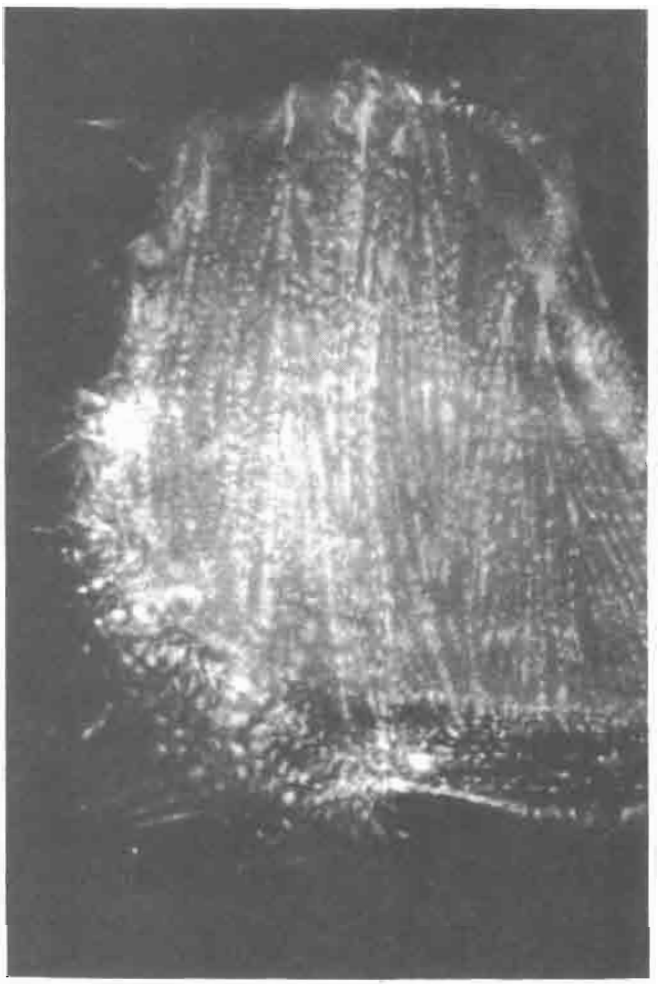

Fig. 5 Tetramethylrhodamine isothiocyanate labelled α-actinin incorporated into a living gerbil fibroma cell. (From Feramisco[21].)

by fusion techniques. Furthermore, when spectral characteristics are used as indicators of local environments such as *p*H, the possible effects of other environmental factors must be ruled out. In all cases, calibration must be performed *in situ*[48].

Biologically active agents

Biologically active agents which can stimulate, alter or block cell functions include lectins, antibodies, drugs and toxins. These agents are used in probing cell structures and functions. Many of these agents can be fluorescently labelled, permitting detailed investigations on the distribution and mobility of binding sites. The relationship between distribution and biological effects of the labelled agents can also be studied.

The plant lectin, concanavalin A (Con A), has been used as a model ligand in investigations on receptor–ligand complexes[51–57]. Fluorescently labelled Con A has facilitated studies on the mobility of Con A receptors, the ultimate fate of the ligand and the relationship between receptor mobility and Con A stimulated cellular events[51–57]. It must be emphasised that Con A has many complex effects on cells[57] and it is difficult to define the complete molecular sequences of events. Similar but more specific experiments have also been performed with antibodies prepared against various cell-surface receptors[56–58]. These cell-surface associated fluorescent conjugates have been used to measure the mobility of membrane components using several different approaches. The simplest method is the observation of large cells such as muscle fibres[59] in the fluorescence microscope. Small labelled spots on the cells can be detected when spread over relatively large distances. A second approach involves fusing cells containing different surface fluorescent conjugates and following the redistribution of the labelled conjugates in the microscope[60]. Poo and colleagues[61] have also studied the accumulation of labelled surface receptors in a uniform electric field applied across the surfaces of cells. Finally, several investigators have used the photobleaching techniques discussed above.

Some drugs can also be fluorescently labelled while maintaining activity. The purpose of labelling the drugs is to study the correlation between the cellular effects and the localisation within the cell. Examples are the tubulin-binding drug colchicine[62], and the actin-binding cyclic peptides phallicidin[19] and phalloidin[63]. At the proper concentration, colchicine can cause microtubule depolymerisation and phallicidin and phalloidin can cause actin polymerisation and stabilisation (see ref. 64 for references). Fluorescent conjugates of these drugs have been used to localise pools of tubulin[62] and F-actin[19,63] respectively in fixed or extracted cells (Fig. 2). However, it is not yet clear whether these drugs have simply identified pools of tubulin and F-actin or have also altered the normal organisation of the target proteins.

Functional cellular components (molecular cytochemistry)

Molecular cytochemistry[13] has been defined as the reincorporation of functional cellular components into or onto living cells following purification, fluorescent labelling and assaying function *in vitro*. The experimental protocol is shown in Fig. 3 using actin as a model. The feasibility of molecular cytochemistry has been initially demonstrated using actin labelled with a non-destructive probe, 5-iodoacetamidofluorescein (IAF). The labelled actin has been proven to be functional in the purified form, in cell-free extracts, and in single-cell models[13,15–17]. Microinjection of this fluorescent

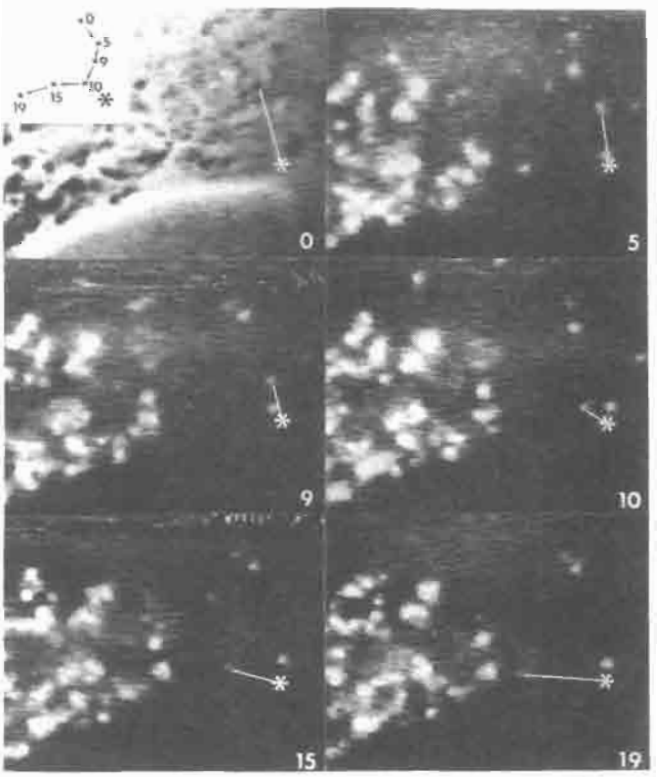

Fig. 6 Saltatory motion of fluorescent vesicles in 3T3 cells 24 h after incubation with rhodamine-labelled α_2-macroglobulin. A time lapse video tape recording at a 9:1 time lapse ratio was made of a cell with vigorous intracytoplasmic vesicle saltatory motion. The numbers in the lower right corner of each single frame image represent the real time in seconds after the beginning of the sequence. A phase image is presented at zero time followed by fluorescence images. The asterisk represents an arbitrary non-moving reference point. The inset summarises the motion of this single fluorescent vesicle. (From Willingham and Pastan[12].)

actin analogue into living cells has provided a direct way of visualising actin-containing structures and following the changes in actin distribution during cellular processes such as fertilisation of eggs[17], cytokinesis[17], amoeboid movement[13,15], pinocytosis[13], and Con A capping[13]. Extensive controls *in vitro* and *in vivo* have made the interpretation of the fluorescent images possible[13,15–17] (Fig. 4). This approach has recently been applied to other cells[20,21] and other contractile proteins[20] (Fig. 5).

The use of fluorescent conjugates specific for receptors in the cell surface has permitted the characterisation of the kinetics of the distribution and the ultimate fate of many ligands. These investigations have included studies on α_2-macroglobulin[12,65,66], epidermal growth factor[66–68], insulin[66–69], a chemotactic peptide[70], low-density lipoproteins[71], and acetylcholine receptors[71–74]. All of these fluorescent ligands initially label the cell surface uniformly, but at least some of the ligand–receptor complexes aggregate into patches within a few minutes. Most of the fluorescent conjugates eventually become internalised[69,75]. The role of internalisation has not yet been defined in detail. The redistribution of some of these ligand–receptor complexes in the plane of the membrane has been quantitated using the fluorescence photobleaching techniques.

Proper use of molecular cytochemistry demands the application of various biological controls in addition to the analysis of physical considerations discussed above. The functional activity of the conjugates must be carefully demonstrated. It requires that: (1) the labelling reaction does not abolish the normal biochemical activity of the substrate, (2) the conjugates have access to the intracellular domains where the function is performed, and (3) the conjugates are not rapidly degraded *in vivo*. The biochemical activities can be readily tested using *in vitro* assays, cell-free extracts or lysed cell models[13,16]. The use of site-specific probes would provide functionally homogeneous conjugates and would yield unequivocal results with *in vitro* assays[16]. The studies with labelled actin have yielded the most definitive biological controls[13,15–17] to date.

The function inside living cells is much more difficult to assay. For IAF-labelled actin mentioned above, well characterised cellular responses such as cortical wound healing, can be used to test the functionality *in situ*[13]. Furthermore, a comparison of the fluorescent images of biologically active conjugates with non-functional conjugate controls would also indicate the formation of structures related to biological functions[13,15,17]. Note that the absence of fluorescence from a cellular domain, such as the nucleus[13,17], does not necessarily imply that the endogenous component is not present. Negative results could be due to physical exclusion of the conjugate, slow turnover time for the incorporation into specific structures, as well as the absence of a cellular component from a particular region. Therefore, negative results must also be interpreted with caution.

Future prospects

This new approach to cell biology is likely to develop extensively both technically and in its range of biological applications. At the technical level, improvements in the hardware and software applied to intensified image recording and spectral analysis should permit sophisticated manipulations of the experimental data. Measurements of fluorescence polarisation[14,76,77], resonance energy transfer[60,78–80] and fluorescence lifetime could be performed and factors such as accessible volume could be corrected automatically. Detailed molecular information including microviscosity, local polarity, rotational freedom and formation of supramolecular structures could be determined.

The application of this new technique is expected to extend to many different areas of cell biology, through the use of fluorescent conjugates of nonperturbing indicators, biologically active agents and functional cellular components. Components binding specific ions or ligands could be labelled with environmentally sensitive probes and used as indicators of intracellular environments. One such possibility would be the calcium binding protein calmodulin[81]. Some drug studies previously relying on radioactive derivatives could be performed with fluorescent conjugates which would allow the direct observation of uptake, distribution and turnover. Furthermore, the new techniques of blocking cell functions with specific antibodies[82,83] or modified cellular components[84] can be combined with the present approaches yielding a more powerful technique. The most dramatic advances will probably be seen in the area of molecular cytochemistry using functional conjugates of proteins, lipids, nucleic acids, carbohydrates and even whole organelles. Careful use of molecular cytochemistry is expected to bring new insights into areas as diverse as cell motility, virus–cell interactions, nuclear–cytoplasmic interactins, cell–cell interactions in tissues, axonal transport, gene expression, and assembly of organelles at both morphological and molecular levels. A direct connection between cell physiology, biochemistry and ultrastructure would then become a reality.

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1. Chambers, R. & Chambers, E. L. *Explorations into the Nature of the Living Cell* (Harvard University Press, Cambridge, Massachusetts, 1961).
2. Lorch, I. J. in *The Biology of Amoeba* (ed. Jeon, K. W.) 1–36 (Academic, New York, 1973).
3. Jeon, K. W., Lorch, I. J., & Danielli, J. F. *Science* **167**, 1626–1627 (1976).
4. Hiramoto, Y. *Expl Cell Res.* **27**, 416–426 (1962).
5. Nichols, K. M. & Rikmenspoel, R. *J. Cell Sci.* **29**, 233–247 (1978).
6. Guilbault, G. G. *Practical Fluorescence. Theory, Methods and Techniques* (Dekker, New York, 1973).
7. Fluorescence Spectroscopy (eds Pesce, A. J., Rosen, C. G. & Pasby, T. L.) (Dekker, New York, 1971).
8. Thaer, A. A. & Sernetz, M. (eds) *Fluorescence Techniques in Cell Biology* (Springer, Berlin, 1973).
9. Reynolds, G. T. *Quant. Rev. Biophys.* **5**, 295–347 (1972).
10. Reynolds, G. T. & Taylor, D. L. *BioScience* (in the press).
11. Sedlacek, H. H., Gundlach, H. & Ax, W. *Behring. Inst. Mitt* **59**, 64–70 (1976).
12. Willingham, M. C. & Pastan, I. *Cell* **13**, 501–507 (1978).
13. Taylor, D. L., Wang, Yu-Li & Heiple, J. *J. Cell Biol.* (submitted).
14. Sengbusch, G. V. & Thaer, A. in *Fluorescence Techniques in Cell Biology* (eds Thaer, A. A. & Sernetz, M.) 31–39 (Springer, Berlin, 1973).
15. Taylor, D. L. & Wang, Y.-L. *Proc. natn. Acad. Sci. U.S.A.* **75**, 857–861 (1978).
16. Wang, Yu-Li & Taylor, D. L. *J. Histochem. Cytochem.* (submitted).
17. Wang, Y.-L. & Taylor, D. L. *J. Cell Biol.* **82**, 672–679 (1979).
18. Cherry, R. J., Cognoli, A., Oppliger, M., Schneider, G. & Semenza, G. *Biochemistry* **15**, 3653–3656 (1976).
19. Barak, L. S., Yocum, R. R., Nothnagel, E. A. & Webb, W. W. *Proc. natn. Acad. Sci. U.S.A.* (in the press).
20. Feramisco, J. R. *Proc. natn. Acad. Sci. U.S.A.* **76**, 3967–3971 (1979).
21. Kreis, T. E., Winterhalter, K. H. & Birchmeier, W. *Proc. natn. Acad. Sci. U.S.A.* **76**, 3814–3818 (1979).
22. Waggoner, A. S. *A. Rev. Biophys. Bioengng* **8**, 47–68 (1979).
23. Diacumakos, E. G. *Meth. Cell Biol.* **7**, 288–311 (1978).
24. Blinks, J. R. *et al. Meth. Enzym.* **58**, 292–328 (1978).
25. Rose, B. & Lowenstein, W. R. *J. Membrane Biol.* **28**, 87–199 (1976).
26. Schlegel, R. A. & Richsteiner, M. C. *Meth. Cell Biol.* **20**, 341–354 (1978).
27. Tyrrell, D. A., Heath, T. D., Colley, C. M. & Ryman, B. E. *Biochim. biophys. Acta* **457**, 259–302 (1976).
28. Gregoriadis, G. *New Engl. J. Med.* **295**, 704–770 (1976).
29. Yamaizumi, M., Uchida, T., Mekada, E. & Okada, Y. *Cell* **18**, 1009–1014 (1979).
30. Williamson, R. E. *J. Cell Sci.* **17**, 655–668 (1975).
31. Miller, M. R., Castellot, J. J. Jr & Pardee, A. B. *Expl Cell Res.* **120**, 421–425 (1979).
32. Kohen, E., Kohen, C. & Thorell, B. *Expl Cell Res.* **81**, 477–482 (1973).
33. West, S. S. in *Physical Techniques in Biological Research* Vol. 3C (ed. Pollister, A. W.) 253–321 (Academic, New York, 1969).
34. Ploem, J. S., Starke, J. A., De, Bonnet, J. & Wasmund, H. *J. Histochem. Cytochem.* **22**, 668–677 (1974).
35. Malmstadt, H. V., Franklin, M. L. & Horlick, G. *Analyt. Chem.* **44**, 63A–76A (1972).
36. Wreford, N. G. M. & Schafield, G. G. *J. Microsc.* **103**, 127–130 (1974).
37. Rost, F. W. D. & Pearse, A. G. E. *J. Microsc.* **94**, 93–105 (1971).
38. Cova, S., Prenna, G. & Mazzini, G. *Histochem. J.* 279–299 (1974).
39. Axelrod, D., Koppel, D. E., Schlessinger, J., Elson, E. & Webb, W. W. *Biophys. J.* **16**, 1055–1060 (1976).
40. Elson, H. & Yguerabide, J. *J. supramolec. Struct.* **12** (in the press).
41. Smith, B. A. & McConnell, H. M. *Proc. natn. Acad. Sci. U.S.A.* **75**, 2759–2763 (1978).
42. Koppel, D. E. *Biophys. J.* **28**, 281–292 (1979).
43. Sheetz, M. P. & Koppel, D. E. *Proc. natn. Acad. Sci. U.S.A.* **76**, 3314–3317 (1979).
44. Jeon, K. W. & Bell, L. G. E. *Expl Cell Res.* **33**, 531–539 (1964).
45. Flagg-Newton, J., Simpson, I. & Loewenstein, W. R. *Science* **205**, 404–407 (1979).
46. Simpson, I., Rose, B. & Loewenstein, W. R. *Science* **195**, 294–296 (1977).
47. Stacey, D. W. & Allfrey, V. G. *J. Cell Biol.* **75**, 807–817 (1977).
48. Heiple, J. & Taylor, D. L. *J. Cell Biol.* (submitted).
49. Ohkuma, S. & Poole, B. *Proc. natn. Acad. Sci. U.S.A.* **75**, 3327–3331 (1978).
50. Bittiger, H. & Schnebli, H. P. (eds) *Concanavalin A as a Tool* (Wiley, New York, 1976).
51. Schlessinger, J. *et al. Proc. natn. Acad. Sci. U.S.A.* **73**, 2407–2413 (1976).
52. Yahara, I. & Edelman, G. M. *Expl Cell Res.* **81**, 143–155 (1973).
53. Ryan, G., Borysenko, J. Z. & Karnovsky, M. J. *J. Cell Biol* **62**, 351–370 (1974).
54. Oliver, J. M., Ukena, T. E. & Berlin, R. D. *Proc. natn. Acad. Sci. U.S.A.* **71**, 394–398 (1974).
55. Condeelis, J. R. *J. Cell Biol.* **80**, 751–758 (1979).
56. Taylor, R. B., Duffus, P. H., Ross, M. C. & DePetris, S. *Nature new Biol.* **233**, 225–229 (1971).

57. Schreiner, G. F. & Unanue, E. R. *Adv. Immun.* **24,** 38–165 (1976).
58. Woda, B. A., Yguerabide, J. & Feldman, J. D. *J. Immun.* **123,** 2161–2167 (1979).
49. Edidin, M. & Fambrough, D. *J. Cell Biol.* **57,** 27–53 (1973).
60. Keller, P., Person, S. & Snipes, W. *J. Cell Sci.* **28,** 167–177 (1977).
61. Poo, M-m, Poo, W. J. H. & Lam, J. W. *J. Cell Biol.* **76,** 483–501 (1978).
62. Clark, J. & Garland, D. *J. Cell Biol.* **76,** 619–627 (1978).
63. Wulf, E., Deboben, A. Bautz, F. A., Faulstich, H. & Wieland, T. *Proc. natn. Acad. Sci. U.S.A.* **76,** 4498 (1979).
64. Taylor, D. L. & Condeelis, J. S. *Int. Rev. Cytol.* **56,** 57–144 (1979).
65. Pastan, I., Webb, W. W. & Elson, E. L. *Proc. natn. Acad. Sci. U.S.A.* **74,** 2909–2913 (1977).
66. Schlessinger, J., Schechter, Y., Willingham, M. & Pastan, I. *Proc. natn. Acad. Sci. U.S.A.* **75,** 2659–2663 (1978).
67. Scheckter, Y., Hernaez, L., Schlessinger, J. & Cuatrecasas, P. *Nature* **278,** 835–838 (1979).
68. Haigler, H., Ash, J. F., Singer, S. J. & Cohen, S. *Proc. natn. Acad. Sci. U.S.A.* **75,** 3317–3321 (1978).
69. Maxfield, F. R., Schlessinger, J., Schechter, Y., Pastan, I. & Willingham, M. C. *Cell* **14,** 805–810 (1978).
70. Niedel, J. E., Kahane, I. & Cuatrecasas, P. *Science* **205,** 1412–1414 (1979).
71. Krieger, M. *et al. J. supramolec. Struct.* **10,** 467–478 (1979).
72. Axelrod, D. *et al. Proc. natn. Acad. Sci. U.S.A.* **73,** 4594–4598 (1976).
73. Anderson, M. J. & Cohen, M. W. *J. Physiol., Lond.* **237,** 385–400 (1974).
74. Block, R. J. *J. Cell Biol.* **82,** 626–643 (1979).
75. Silverstein, S. C., Steinman, R. M. & Cohn, Z. A. *A. Rev. Biochem.* **46,** 669–722 (1977).
76. Axelrod, D. *Biophys. J.* **26,** 557–574 (1979).
77. Nihei, T., Mendelson, R. A. & Botts, *J. Biophys. J.* **14,** 236–242 (1974).
78. Stryer, L. *A. Rev. Biochem.* **47,** 819–846 (1978).
79. Wu, C. W. & Stryer, L. *Proc. natn. Acad. Sci. U.S.A.* **69,** 1104–1108 (1972).
80. Fernandez, S. M. & Berlin, R. D. *Nature* **264,** 411–415 (1976).
81. Potter, J. D. *et al.* in *Calcium Binding Proteins and Calcium Function* (eds Wasserman, R. H. *et al.*) 239–249 (North-Holland, New York, 1977).
82. Mabuchi, I. & Okuno, M. *J. Cell Biol.* **74,** 251–263 (1977).
83. Rungger, D., Rungger-Brändle, E., Chaponnier, C. & Gabbiani, G. *Nature* **282,** 320–321 (1979).
84. Meeusen, R. L. & Cande, W. Z. *J. Cell Biol.* **82,** 57–65 (1979).

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