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Fluorescence microscopic analysis of cytoskeletal organization and dynamics

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1. Introduction

Fluorescence microscopy represents a simple, efficient approach for examining the distribution of specific molecules in cells. Such spatial information is particularly important for the study of the cytoskeleton, since the molecular components involved often undergo dramatic reorganizations in response to internal and external stimuli. During the past two decades fluorescence microscopy has served as a bridge between biochemical characterization and cellular functions, and has provided important clues to the mechanisms of various motile activities and the functional roles of many cytoskeletal proteins.

Most applications of fluorescence microscopy to date involve fixing cells and staining with either specific antibodies (immunofluorescence) or specific fluorescent ligands such as phalloidin. The application of multiple fluorophores has allowed up to five separate components to be mapped and correlated within a given cell. The main limitation of this approach is that all observations are made with fixed cells, thus it can be difficult to reconstruct dynamic events based on still images of different cells. The second approach, fluorescent analogue cytochemistry, is based on the observation of functional, fluorescently-labelled proteins microinjected into living cells. It allows not only the direct observation of specific components during dynamic processes, but also the analysis of protein interactions and molecular mobilities.

Laboratory procedures for both fluorescent staining and fluorescent analogue cytochemistry have been discussed in numerous articles (1–7). The purpose of the present article is to describe our current procedures for the fluorescence microscopy of cultured animal cells, and to provide additional details that are not already available.

2. Fluorescent staining of fixed, permeabilized cells

Fluorescent staining, including troubleshooting and potential artefacts, has been discussed in the article by K. Wang *et al.* (1). Thus, this section describes only briefly our current methods for indirect immunofluorescence and for staining with fluorescent phalloidin.

The fixation procedure should be chosen on the basis of the specific purpose of the experiment. For example, the glutaraldehyde–Triton protocol developed by Small (1, 8) results in a superb preservation of lamellipodia. However, glutaraldehyde induces fluorogenic side reactions; the resulting background fluorescence can be reduced but not totally removed by treatment with sodium borohydride. The high degree of cross-linking by glutaraldehyde may also destroy some antigenic sites for immunostaining. Fixation–extraction with −20 °C methanol containing 5–50 mM EGTA is also commonly used for microtubule staining (2). However, this method does not preserve lamellipodia and also interferes with the staining of actin filaments by fluorescent phalloidin. On the other hand, formaldehyde usually offers satisfactory results for microfilament structures but is known to disrupt microtubules (2).

The fixation method that we typically use for studying the microfilament system is described in *Protocol 1*. A variation of the method is to include 0.1–0.5 per cent Triton-X 100 in the fixation solution, and delete the acetone extraction step. The cover slip is rinsed three times with PBS after fixation for 5–10 min. This method results in a faster fixation due to the permeabilization by Triton, and a better preservation of the lamellipodia. However, the use of Triton also results in an extensive extraction of soluble contents, including microinjected soluble molecules, from the cell. Cold acetone on the other hand removes primarily lipids while precipitating and retaining most proteins. Other permeabilizing agents, such as saponin, may also be used.

Protocol 1. Fixation and extraction of cells

Materials

- Phosphate buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4.
- Formaldehyde, 4 per cent, in the PHEM buffer (9): 60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 3 mM $MgCl_2$ at pH 6.1. The quality of formaldehyde is critical. We use the 16 per cent 'Electron miscroscope' grade stock solution from Electron Microscopy Sciences, supplied as aliquots in ampoules. Consistent results can also be obtained with paraformaldehyde, prepared as described by Wang *et al.* (1).

- Acetone (analytical grade), chilled to −20 °C: Acetone can be chilled either in an explosion-proof freezer, or in a dry ice–acetone bath and then allowed to sit at room temperature for about 5 min. Precise temperature control of acetone is not necessary but a temperature below −20 °C should be avoided as it leads to formation of ice on the surface of the cover slip and resulting distortion of cell morphology.

Method

1. Grow culture cells on substrates that are free of autofluorescence (most plastic substrates are not suitable), usually glass cover slips.
2. Rinse the culture 2–3 times with warm (37 °C) PBS to remove serum proteins and other components in the medium that may interfere with fixation.
3. Immediately drop the cover slip into warm, freshly prepared formaldehyde solution. Fix for 10 min.
4. Rinse the cover slip twice with PBS at room temp.
5. Dip the cover slip into −20 °C acetone for 5 min.
6. Allow the cover slip to air dry at room temp.

A typical method for staining cells with fluorescent phalloidin or antibodies is described in *Protocol 2*. The most important consideration for antibody staining is the quality of the antibodies and the proper dilution of the antibody solution. The primary antibody should be tested carefully by Western blot analysis of cell lysates. Reliable fluorescent secondary antibodies can usually be obtained from commercial sources (e.g. Sigma Chemical, Organon Teknika-Cappel, or TAGO Inc.), and fluorescent conjugation in the lab is generally unnecessary. The dilution factor should be determined empirically by staining multiple samples with different concentrations. The lowest concentration that gives detectable signals should be used.

Protocol 2. Staining cells with fluorescent antibodies or phalloidin

Materials

- PBS
- PBS with 1 per cent bovine serum albumin, filtered through filter paper. Sodium azide (0.02 per cent) may be included to inhibit microbial growth.
- Fluorescent phalloidin, 50–100 nM in PBS: Fluorescent phalloidin is supplied by Molecular Probes as a stock solution in methanol, and is usually used after drying in a test-tube and redissolving in PBS with thorough vortexing. The stock solution can also be directly diluted into

Protocol 2. *Continued*

PBS; however, it is important to make sure that the final concentration of methanol is below 5 per cent.

- Primary and secondary antibodies, diluted in PBS–BSA and clarified in a table-top centrifuge for 10–20 min.

Methods

1. Incubate the cover slip from *Protocol 1* with primary antibodies. The required volume of antibody solution can be reduced by cutting a piece of parafilm approximately the area to be stained, and placing a drop (50–100 μl) of the antibody solution on the parafilm. The cover slip is then inverted on to the solution, which spreads uniformly over the surface. It is important to keep the cover slip from drying out after the application of the primary antibody.
2. Place the cover slip face down in a moist chamber (e.g. a closed Petri dish containing a piece of wet paper tissue) and incubate for 45–120 min at 37 °C. Alternatively, the staining can be allowed to proceed overnight at 4 °C.
3. Invert the cover slip (face up) and place in a dish containing PBS/BSA at room temp. The parafilm usually floats off automatically.
4. Agitate the container gently on a shaker table for 5 min.
5. Repeat the washing procedure twice more.
6. Apply the fluorescent secondary antibody as for the primary antibody.
7. Staining with fluorescent phalloidin can be performed, after antibody staining, by incubating the cover slip with 50–100 nM fluorescent phalloidin in PBS for 20 min at room temp., followed by 2–3 rinses with PBS before observation.

The cover slips can be observed after mounting in PBS. However, special 'anti-fading' mounting solutions are commonly used to reduce the photobleaching during observation (2, 10). One solution suitable for long-term preservation of the sample is prepared by dissolving 100 mg/ml 1,4-diazabicyclo [2,2,2] octane in Mowiol mounting medium, as described by Osborn and Weber (2). A solution without Mowiol works in the short term, and is much easier to prepare. In this case the cover slip can be sealed with nail polish around the edge and stored at 4 °C for several days.

Detailed discussions of the potential problems of immunofluorescence and important controls for staining can be found in Wang *et al.* (1). A problem that can particularly affect staining with monoclonal antibodies is the availability of antigenic sites, which can be affected by fixation, extraction, protein conformation, or protein–protein interactions. Thus, either the

entire cell or a part of it may not show staining even though the antibody is functionally active. A survey of different fixation and extraction methods as well as different antibody preparations should be performed if this is in question. In addition, treatment of fixed cells with trypsin or SDS may aid in the exposure of shielded antigenic sites. Although the differential reactivities of monoclonal antibodies generally represent a problem in immunofluorescence, such properties may sometimes be used as a powerful tool for probing the conformational states of molecules in the cell (11).

Another potential problem with fluorescent staining is the redistribution of proteins during fixation. One scenario involves proteins that interact weakly with cytoskeletal structures. Fixation may cause such interactions to become irreversible and result in a significant increase in the association with cytoskeleton (12). Although different fixation methods may be tested, it can be difficult to decide which one to trust if different results are obtained. Thus while immunofluorescence is useful for determining the presence of specific proteins on cytoskeletal structures, the degree of association should be interpreted with caution. The use of fluorescent analogue cytochemistry, as discussed below, represents an independent approach for the localization of specific proteins.

3. Fluorescent analogue cytochemistry

Fluorescent analogue cytochemistry involves the purification, fluorescent labelling, and microinjection of specific proteins into living cells. The approach is based on the assumption that microinjected analogues behave similarly to the endogenous counterpart, and can thus provide information about the physiological behaviour of cellular components based on the fluorescence signal. The major advantage of fluorescent analogue cytochemistry over immunofluorescence is its ability to yield direct information on dynamic changes and even molecular interactions within living cells. The reader is referred to a recent review article for a detailed discussion of previous applications and future potentials of fluorescent analogue cytochemistry (13).

3.1 Preparation of fluorescent analogues

Several detailed protocols for the fluorescent labelling of cytoskeletal components have been published previously (*Table 1*), so the discussion here emphasizes general principles. Proteins to be conjugated should be prepared in at least milligram quantities. Although only a small amount of protein (< 50 μg) is required for microinjection, a minimum of about 0.5 mg is usually necessary for fluorescent labelling. Other important considerations for the choice of proteins have been discussed in Wang (7). For most

morphological studies, members of the rhodamine family, such as tetramethylrhodamine, provide relatively stable and strong signals with minimal interference from autofluorescence. The choice of fluorophores is more critical for fluorescent analogue cytochemistry than for immunofluorescence (4), particularly since anti-bleaching agents cannot be applied to living cells, and strong excitation light can easily cause adverse cellular reactions. Many fluorophores are now available in forms reactive toward either amino or sulphydryl groups. The reagent should be reactive under mild buffer conditions, and form stable, covalent bonds with minimal effects on the function of the protein. Although it is difficult to predict an optimal reactive group, often a number of fluorescent dyes can be used with equally satisfactory results. It is strongly recommended that a 'Handbook' be obtained from Molecular Probes, since it contains valuable information about the physical and chemical properties of a large number of fluorescent reagents. In addition, the book by Means and Feeney on *Chemical modifications of proteins* (14) should be consulted for the characteristics of many common labelling reactions.

Proteins are dialysed into an appropriate reaction buffer at a concentration of 1.0–10.0 mg/ml. Components that may interfere with the labelling reaction, including Tris, dithiothreitol (DTT), and ATP, should be removed or reduced in concentration during dialysis. If the cysteine thiol groups are to be labelled, it is recommended that 5–10 mM DTT be added before dialysis to recover any oxidized thiol groups (unless the protein contains structural disulphide bridges). The protein is then dialysed overnight to remove the added DTT.

Fluorescent reagents should be prepared fresh and are usually dissolved in an aqueous buffer before adding to the protein solution. A molar excess of 10 to 20 over the protein represents a reasonable starting point, and can be adjusted up or down based on the resulting stoichiometry of labelling. Since many reactions involve the release or incorporation of protons, it is important

Table 1. Some published protocols for the preparation of fluorescent analogues of cytoskeletal proteins

Protein	Fluorophore	Reference
Actin	Fluorescein	15
Actin	Lissamine rhodamine B	5
Myosin	Tetramethylrhodamine	35
Alpha-actinin	Tetramethylrhodamine	1
Alpha-actinin	Fluorescein	4
Alpha-actinin	Fluorescein and lissamine rhodamine B	5
Vinculin	Fluorescein and lissamine rhodamine B	5
Tubulin	Fluorescein	6

to use a buffer of sufficient strength for maintaining pH. The buffering capacity can be tested by adding the estimated amount of acid or base and measuring the change in pH. We commonly use 50–100 mM borate or Hepes for reactions that require a slightly alkaline pH (8.0–9.0), and phosphate or Pipes for pH values near neutrality.

Of prime importance is the technique for solubilizing the reagent, since variable degrees of solubilization can lead to highly inconsistent labelling. Many reagents are poorly soluble in water and may require hours to dissolve if added directly to an aqueous buffer. The solubilization process is often facilitated by the use of a small amount of organic solvent such as acetone, dimethylsulphoxide (DMSO), dimethylformamide, or ethanol to form a stock solution or fine slurry, which is then added very slowly into the aqueous labelling buffer. Ths solubility of some compounds, such as fluorescein-containing reagents, is also significantly affected by the pH of the buffer.

Incomplete solubilization can be difficult to detect. When in doubt the solution should be clarified in an ultracentrifuge before adding to the protein. The consistency of reaction with such partially soluble reagents can be improved by measuring the absorbance of the supernatant after centrifugation and adjusting the volume used for the reaction. Alternatively, some poorly soluble reagents such as lissamine rhodamine sulphonyl chloride are available pre-absorbed on celite, and can be gradually released after adding directly to the protein solution. Celite particles are then removed by centrifugation at the end of reaction. A simple variation of this method is to dissolve the reagent in a volatile solvent and dry on to the side and bottom of reaction vessels; protein solutions are then added to initiate the reaction.

The optimal reaction time and temperature vary with proteins and reagents and should be determined empirically. A period of 2 h at 4 °C can be used as a starting point. Unless celite particles are used, constant stirring is not recommended after the initial mixing. The reaction should be terminated with quenchers such as lysine, cysteine, or DTT, at molar ratios of 5–10 times over the reagent. The quenching not only makes the reaction more reproducible, but can also facilitate the removal of unreacted non-polar reagents.

Various methods have been used for the removal of unreacted reagents, including dialysis, gel filtration chromatography, adsorption chromatography, and ion exchange chromatography (7). In addition, cycles of precipitation and solubilization can effectively remove a large fraction of unreacted dyes. For proteins with the ability to self-assemble, cycles of polymerization and depolymerization serve both to remove unreacted dyes and to select for assembly-competent molecules.

Purified fluorescent proteins are concentrated to 1–5 mg/ml, usually with an Amicon Centricon concentrator or by vacuum dialysis in Collodion bags (Schleicher and Schuell). The Centricon concentrator is faster, but may become clogged with some proteins. Before microinjection, labelled proteins should be examined by SDS-PAGE to make sure that there is no detectable

residual free dye, which may still be present even after an apparently effective gel filtration step. The fluorophore:protein ratio is determined based on the absorbance of the fluorophore, the estimated extinction coefficient of the fluorophore, and a standard protein assay such as the Lowry or Bradford assay. We avoid the use of absorbance at 280 or 290 nm for the determination of protein concentration, since most fluorophores absorb sufficient UV light to cause considerable errors. The major source of error in measuring the labelling stoichiometry usually comes from the extinction coefficient of the fluorophore, which can vary significantly following the conjugation to proteins. A simple method for the estimation of the extinction coefficient is described in Wang and Taylor (15). An apparent labelling ratio of 1–2 fluorophores per polypeptide is adequate for most experiments. In addition to labelling stoichiometry, possible effects of fluorescent labelling on the function of the protein should be determined based on the known biochemical properties. For experiments involving quantitative fluorimetry, it is also necessary to characterize the spectroscopic properties of the conjugate, such as possible changes in the emission spectrum, as a function of conformation.

The fluorescent analogue is then dialysed into a microinjection buffer. We have successfully used as a carrier solution 5–10 mM Tris–acetate at pH 6.95– 7.05, although others have used a variety of buffers for microinjections (e.g. 16). The protein solution should be clarified in an ultracentrifuge (7).

3.2 Cell culture and microinjection

The main concern of cell culture is to maintain the viability of the cell during microinjection and observation, and to provide an optimal optical quality for fluorescence microscopy. We choose to use a stage incubator on an inverted microscope and a relatively simple cover slip chamber/dish (17). Alternatively, some investigators culture cells on cover slips placed inside 60 mm cell culture dishes, and subsequently remove the cover slips and mount in observation chambers. Various approaches have been discussed in McKenna and Wang (17). Depending on the cell type, the cover slip can be used without coating or after coating with polylysine or extracellular matrix proteins such as the ECL matrix (Upstate Biotechnology).

It is critical to maintain the cells under optimal conditions. Inadequately maintained cells may appear normal in phase optics, but recover poorly after microinjection. Different media vary considerably in their level of fluorescence, which can be a problem when signals from microinjected cells are extremely low. The fluorescence of the medium comes mainly from NADH, riboflavins, and phenol red (18) which are present at a relatively high level in Dulbecco's modified Eagle's medium (DME). A method used in this laboratory is to maintain stocks of cells in DME but plate cells in F12K medium for microinjection. For some experiments it may be advantageous to

plate cells in Leibovitz L-15 medium, which uses phosphate buffer and does not require carbon dioxide for the maintenance of pH. However, the medium for maintaining stocks of cell lines should not be changed arbitrarily from that originally used for raising the parent cell line.

Table 2 lists special equipment and supplies used for microinjection. Microneedles are drawn from glass capillaries, using pipette pullers that are available from manufacturers such as David Kopf. Elaborate pulling-control mechanisms are unnecessary for preparing needles for microinjection; however, it is critical to be able to adjust the shape of the needle by changing the current of the heating element and the pulling force. Needles with long tapers tend to trap bubbles, and those with short tapers are usually too fragile at their tips.

Table 2. Special items required for microinjection

Ultracentrifuge and rotors for small volumes
Protein concentrator
Microdialysis system
Vibration-free table
Inverted microscope with phase and epifluorescence optics
Diamond scribe
Microscope stage heater
Microscope stage carbon dioxide outlet
Microcapillaries
Drawn-out Pasteur pipettes
Microneedle puller
Microneedle holder
Air pressure regulator (e.g. syringe) and tubing connections
Micromanipulator

We use capillaries containing inner threads (Frederick Haer) for the preparation of needles. The inner thread allows the needle to be loaded from the back end with a drawn-out pipette. The solution automatically flows into the tip within a few seconds due to capillary action. The glass capillaries from Frederick Haer are used without washing on treatment, except in special cases where the external surface of the capillary is siliconized before pulling to minimize the build-up of materials at the tip of the needle.

Microinjection can be performed conveniently for most cultured animal cells with 25–40× phase objectives and 10× eyepieces. High-quality phase optics are required for detailed observations during microinjections. The microscope and the micromanipulator should be isolated from vibration (7), so that no visible movement of the needle is detectable under the microscope. The micromanipulator should be equipped with coarse controls for tilting and for movement in the x–y direction, and fine controls for movement along the z axis. Other features such as joysticks are useful but not critical. A simple, functional micromanipulator can be assembled with optical positioning devices for under US$1500 (e.g. supplied by the Newport Corp.).

Pressure for microinjection can be simply controlled with an empty 10–50 ml syringe (1, 19). However, some experience is required to maintain the pressure in an optimal range, typically between 0.25 and 0.75 p.s.i. Alternatively, simple electronic or mechanical devices can be designed to allow more precise and reproducible controls of the pressure. The rate of flow during microinjection is affected by the applied pressure, the size of the tip, the viscosity of the material, the amount of materials accumulating at the tip, the presence of any air bubbles in the needle, and the resistance of the cell. Thus the pressure must be adjusted according to the actual response of the cell.

There are also commercial devices for delivering pulses of solution into cells. Although this method reduces to some extent the variability in the volume of microinjection, in our hands short, strong pulses tend to cause more severe shocks to the cell than do gentle, sustained flows. In addition, because of the sensitivity of the rate of injection to the multiple factors mentioned above, it is unrealistic to assume that identical volumes can be microinjected into each cell. In practice, the precision of microinjection experiments is also limited by the variability of cell volume with most cell lines.

Our method of microinjection, described in detail in *Protocol 3*, is derived from that of Graessman *et al.* (19). In this approach, microinjection is achieved by positioning cells under the needle and dipping the needle into the cell, while maintaining the needle at a relatively constant position in the X–Y plane. The flow is maintained constantly at a relatively low level and the amount of microinjection is controlled by the duration that the needle stays inside the cell. Gentle microinjections induce a transient decrease in the phase density of the injected region, and an increase in the contrast of the nucleus against the cytoplasm. In addition, cells may show transient responses to the microinjection, notably the retraction of lamellipodia, but should recover completely within 30 min. Unless the experiment requires an immediate observation, it is best to incubate cells for at least 1 h in a regular incubator following the microinjection to allow complete recovery and incorporation of molecules. For prolonged observations, it is critical to keep the cells in a microscope incubator or perfusion chamber, in order to maintain a proper temperature, osmolarity, and pH (17).

Protocol 3. Microinjection

1. Plate the cells (17) and allow them to recover for 12–48 h.
2. Scan the dish at a low magnification to choose a good area for microinjection. The cells should be well attached at this time, otherwise they may dissociate from the dish during microinjection. For most experiments, the cell density should be somewhat below confluency to

provide an adequate space for cells to spread and for manoeuvring the microinjection needles.

3. Gently mark areas to be microinjected with a diamond-tipped scribe on the exterior surface of the cover slip.
4. Locate regions marked for microinjection and bring cells into focus. Place a nearby cell-free area in the centre of the field; use 250–400 ×.
5. Draw microneedles with a pipette puller. Needles may be prepared beforehand and stored in a dust-free container. However, prolonged storage may affect sterility of the needle and is not recommended.
6. Load a microneedle. When capillaries containing inner threads are used, the solution is loaded from the back end of the needle near the tapered region using a drawn-out Pasteur pipette.
7. Mount the microneedle on the needle holder and the micromanipulator.
8. Carefully introduce the needle into the medium, but keep the needle at a level well above cells. Apply pressure to prevent backflow of the medium into the needle. Bring the needle near the centre of the field. The needle should be out of focus in the microscope, appearing only as a shadow which can be discerned most easily by moving the needle back and forth with the coarse X–Y control of the micromanipulator.
9. Bring the needle close to focus but still above the level of the cells. Position a cell to be microinjected directly under the needle. For well spread cells, microinjections have to be performed in the thicker, perinuclear region.
10. Carefully bring the needle to focus, i.e., lower the needle down into the cell. Microinjection sometimes starts as soon as the needle is brought into focus. Otherwise a gentle tap on the micromanipulator is used to puncture the cell membrane.
11. Terminate the microinjection by raising the needle. Adjust the injection pressure if necessary. Move the next cell under the needle, and repeat the microinjection.
12. Replace the medium after microinjection, to correct for any evaporation that may have occurred during microinjection and to remove materials expelled from the needle into the medium.
13. Incubate cells for at least 1 h in a regular incubator.

The volume of microinjection can be calculated from the integrated intensity of fluorescent molecules injected into the cell. A method derived from Lee (20) is described in detail in *Protocol 4*. Typically the pressure microinjection technique allows the volume of delivery to vary between 0.5 and 10 per cent of the cell volume. However, in a particular experiment, the

volume can be maintained within a factor of 2–3 by consistent performance of the injection technique.

Protocol 4. Measurement of microinjection volume

Materials

- Lucifer Yellow–dextran (10 kDa, Molecular Probes); 2 mg/ml and 10 mg/ml in 5 mM Tris–acetate, pH 7.0.

Methods

1. Microinject cells with 10 mg/ml Lucifer Yellow–dextran.
2. Measure the total fluorescence intensity from each injected cell.
3. Measure the background intensity and subtract from the signal obtained in the previous step. Background image can be obtained after defocusing the microscope. Alternatively, background intensity can be determined by measurement at several spots in the vicinity of the injected cell and multiplying the average by the number of pixels covered by the cell.
4. Perform shading correction if high accuracy is required.
5. Prepare an observation vessel containing type FF immersion oil (Cargille Laboratories), 2–4 mm in depth.
6. Load 10–20 μl of 2 mg/ml Lucifer Yellow–dextran into an atomizer, which can be constructed by attaching tubing to a dusting spray can.
7. Spray the solution of Lucifer Yellow–dextran into the immersion oil.
8. Observe the droplets of dextran solution under the microscope. Obtain the integrated fluorescence intensity from droplets of different sizes. Also measure the diameter of each droplet.
9. Plot the droplet fluorescence intensity against volume.
10. Find the droplet volume that yields the same fluorescence intensity as that measured from the cell. Divide this volume by 5 to obtain the volume of microinjection.
11. Measure the average cell volume by inducing cell rounding (e.g. by gently trypsinizing the culture) and measuring the average diameter. This is used for the calculation of injection volume relative to the cell volume.

3.3 Limitations and potential problems

As with other methods for living cells, microinjections of fluorescent analogues may be complicated by the unpredictable nature of living cells and perturbations caused by the physical manipulation. In addition, the success of

the approach may be affected by the possible modification of the injected analogues in the cell and potential problems of incorporation into physiological structures. The problem of cellular disruption is usually analysed by examining cellular activities such as locomotion and division, and cellular structures based on immunofluorescence staining or ultrastructural analyses. The issue of incorporation is usually addressed by performing immunofluorescence microscopy to compare the distribution of the injected analogue with that of the endogenous counterpart. The two should appear similar if not identical to each other throughout the course of any process under study. However, as immunofluorescence may suffer from its own artefacts, a discrepency between the two does not necessarily indicate a problem with the incorporation of analogues.

4. Image detection and processing

The use of low-light-level detectors and image-processing systems has revolutionized the analysis of fluorescence signals from single cells. These powerful devices not only play a critical role in the detection of extremely weak signals of microinjected fluorescent analogues, but also greatly facilitate the analysis of fluorescence staining of fixed cells (*Table 3* lists equipment required). For a comprehensive discussion of the technical aspects of video microscopy, the book by Inoue (21) is an excellent reference and contains many important details that are not covered here. The following discussion will emphasize the detection of signals in fluorescent analogue cytochemistry.

As discussed previously (21, 22), fluorescence should be observed with objective lenses of high numerical apertures. The lenses most commonly used in this lab are 25× Plan-Neofluar/NA 0.8, 63× Neofluar/NA 1.25, and 100× Neofluar/NA 1.30 objectives. A great degree of flexibility in magnification is gained by using a coupling system between the microscope and the detector that allows changes of projection lenses (21). The level of excitation light for fluorescence observation should be maintained as low as possible, to avoid both photobleaching and radiation damage to living cells. For the detection of fluorescent analogues, it is also important to minimize the number of images recorded by adjusting the frequency of image recording according to the rate of the event under study. Proper heat filtration in the excitation light path is crucial for minimizing the heating of cells and removing infrared light that can be picked up by many video detectors.

The detection of images represents a special challenge in fluorescent analogue cytochemistry, since the level of signals is limited both by the number of molecules that can be incorporated and by the low excitation light that can be used with live cells. As a result, the required sensitivity of detection is generally higher than that for immunofluroescence or ion imaging. A simple guideline is that the detector should be sensitive enough to pick up images

Table 3. Equipment for low-light-level image acquisition and processing

Items	**Considerations**
Optical coupling between microscope and detectors	Ability to change magnification Light throughput
Detector	Sensitivity Resolution Response time Geometric distortion and linearity Requirement of image-processing systems Communication with computer
Image-processing hardware	Real-time processing of video frames Number of frame buffers Resolution of frame buffers Bit-width of frame buffers Look-up tables Speed of calculation and input/output Speed of communication with the computer system
Computer system	Speed of calculation and input/output Number of communication ports Backup systems (e.g. streaming tape) Maintenance and supervision required
Computer software	Availability of key functions Ease of use Efficiency of programs
Hard-disk drives	Capacity Speed
Pointing devices	Resolution Response time Ability to draw precisely Ease of pointing/defining specific points
Monitors	Resolution Range of contrast and brightness DC restoration circuit
Optional equipment	
Image printers	
Video recorders	

barely visible to the dark-adapted naked eye. This can be achieved with intensified SIT (ISIT) or intensified CCD (ICCD) video cameras, or with a cooled, slow-scan CCD detector commonly used in astronomy. Detailed discussions of the characteristics of various detectors can be found in several articles (23–26).

As the signal level decreases, more experience is needed to bring raw images into focus with ISIT or ICCD cameras. In some cases it is helpful to perform a 'rolling average' with an image processor to improve the image

quality during focusing. However, this also causes a significant delay in response time and makes it necessary to focus very slowly. The problem is much more serious with slow-scan CCD detectors, which may need an accumulation time of 0.5–2 s to yield even low-resolution images for focusing. The only satisfactory solution so far is to place the CCD detector parfocal with an ISIT/ICCD camera at two separate ports, and to use the video camera for focusing.

In order to obtain images of high resolution, it is critical to perform signal integration to improve the signal/noise ratio of low-light-level images. This is typically achieved with an image processor for video cameras and a timing circuit for slow-scan CCD detectors. Since the signal/noise ratio is proportional to the square root of the integration time, the improvement is most noticeable during the first few seconds and levels off subsequently. With ISIT video cameras, an integration of 2–10 s represents a compromise between signal/noise ratio and integration time. An integration time of 5–20 s is typically used with a cooled slow-scan CCD detector (Star I; Photometrics). In our experience little can be gained with a longer integration if the signal is too low to yield a usable image with these integration times. At such low levels of signal, stray light and the fluorescence of the medium often become the limiting factors, raw images may be almost impossible to focus, and focusing may also show a noticeable drift during prolonged integrations.

Background images, which are obtained by switching off the excitation light and integrating for the same period of time, are usually subtracted from integrated fluorescence images. The subtraction can be done either before or after appropriate division (usually by the number of integrated frames from a video camera) before viewing in a frame buffer. When the signal level is very low, it is better to subtract the integrated background first. The results are then divided in a stepwise fashion until no overflowing pixels are observed in the frame buffer. This method minimizes the loss of information associated with division and rounding, and has been used successfully to image structures that are barely above the noise level (27).

The images are best stored as digital files, both for preservation of information and for ease of analysis, as discussed later. Simple programs that automatically generate a series of file names, and imprint the time and date on the image or image files, can greatly improve productivity. The only drawback of digital files is that a lot of computer disk space is required—about 0.25 Mb for a 512 × 480 × 8 bit image. We have alleviated this problem by compressing the image files. Briefly, since most fluorescence images are characterized by a large number of pixels of similar intensities and gradual transitions in intensities, a program can be used to convert the pixel intensities into differences between neighbouring pixels. The differences are then coded into variable bits, such that the most frequent difference (i.e. 0) is stored only as a single bit and infrequent differences stored with up to 12 bits (28). A 2–3 fold compression can be obtained using this approach, typically

within 5 s on a graphics workstation. However, the ideal solution to the problem of image storage is the use of removable, optical read–write disks of gigabyte capacities, which are now commercially available.

5. Analysis of fluorescence images

Images of fluorescence microscopy are usually analysed in terms of the distribution of fluorescence intensities and/or changes in fluorescence as a function of time. Both qualitative and quantitative analyses can be greatly facilitated by the use of computers. Discussed below are several useful techniques that can be readily implemented into most image-processing systems.

Table 4. Useful computer programs for image processing

Programs	Considerations
1. Basic functions for image acquisition and processing	
Average frames	Handling of overflow
Accumulate frames on frame buffers	Bit-width for the accumulated frame
Take rolling average	
Subtract two frames	Correction of negative values Bit-width
Divide two frames	Speed Handling of scaling factors
Divide images by a constant	Speed when dividing by power of 2 Ability to divide by a decimal number
Store images on files	Speed
View image files	Ability to compress/decompress during storage/retrieval
View images in composite form	Ability to load/store images in specific frame buffers Ability to load a series of images automatically
Stretch contrast	Ease to specify and change the extent of stretching Ease to remove the stretching
Show motion picture	Ability to change speed
Alternate frame buffers for display	Ability to stop, step, and reverse direction Ability to roam images for correcting misregistration
2. Basic functions for image analysis	
Define areas of interest	Ability to define lines, rectangular regions, and regions of irregular shapes Ease of moving or editing defined areas

Measure distances	Ability to convert into actual distances according to the magnification
	Ease of performing statistics
Measure point intensities	Ease of specifying random points
	Ability to perform averaging over local areas
Integrate intensities over an area	Ability to integrate over irregular areas
	Display of number of pixels within the defined area
Display intensity profile	Ability to perform averaging over local areas
3. Miscellaneous functions	
Remove noise	Minimal effect on resolution
Convolute	Limitation on the size of the matrix
	Ability to normalize results when the sum of the matrix elements is not equal to 1
	Speed
	Correction of overflow and underflow
Display pseudo-colour	Ability to change colour interactively
Generate series of file names automatically	
Stamp time-date on images	
Clear frame buffers	
Copy images among frame buffers	
Translocate images in frame buffers	
Compress/decompress image files	
Change image size	
Superimpose two images in different colours	
Display scale bars	

5.1 Qualitative analysis

Fluorescence images, especially those of fluorescent analogues, often show a very low contrast. It is not uncommon for an image to contain only pixel values between 0 and 30 in an eight-bit frame buffer, which allows possible grey values from 0 to 255. The simplest approach for contrast enhancement is to expand linearly the pixel values within a specified range to fill the entire grey scale. Although the enhancement does not provide any new information, it greatly facilitates the visualization of faint structures. Similar methods are already used widely in polarization and DIC optics. The enhancement should be implemented through look-up tables in the image processor, and ideally should allow the user to change the extent of enhancement interactively while viewing the image.

A second highly useful technique facilitates the detection of motion and differences among images. When a small number of images are involved, as in double immunofluorescence, the images are simply loaded into several frame buffers and the display is changed back and forth among the images, either automatically or manually. Most image processors allow three or four images to be compared in this fashion. For the analysis of motion, it generally

requires the successive display of more than five images in a time frame shorter than that required for retrieving images from the computer disk. A simple technique to achieve this is to divide the frame buffers into multiple domains and load different images at a reduced resolution into each domain. The display is zoomed into individual domains, such that only one image is displayed at a time, and is switched rapidly among different images. The division of a 512 × 480 frame buffer into four 256 × 240 domains quadruples the number of images that can be loaded without a serious sacrifice in resolution, and allows motion pictures to be constructed with 12–16 frames in most image processors. Ideally, the computer program should allow repeated displays of a sequence of images in either the forward or backward direction, at a speed that can be altered while viewing the motion picture.

Various techniques have been developed for the sharpening of images or the removal of noise (28, 29). The success of such techniques depends heavily on the nature of the image. For fluorescence images of a limited signal/noise ratio, the main challenge is to distinguish structural details from random noise.

In our experience, removal of noise with simple convolution techniques usually results in a significant loss of image resolution. We have developed a simple approach for noise removal based on the usually gradual transition in the fluorescence intensity around true structures, but sharp changes around the noise. At each pixel, calculations are performed using its intensity value and neighbouring intensity values to find a most likely value, e.g. by least squares fitting into a second-order equation. The fit values are then averaged with the original pixel value to obtain the final value for the pixel. This method results in a significant reduction in random noise with only a slight decrease in the 'sharpness' of the image.

Sharpening of images involves both an increase in the rate of transition of pixel values and the removal of out-of-focus signals. The latter has been done with dramatic success for the imaging of chromosomes, through calculations based on through-focusing image series (30). Since the amount of out-of-focus signals may be limited in well-spread cells, it is uncertain whether equally impressive results can be obtained with this approach for the study of cytoskeletons in cultured cell lines. The increase in the rate of pixel value transition is readily achieved using convolution techniques. The most serious problem with commonly used matrices is the significant increase in noise and the possible creation of artefacts. Limited success is achieved in the author's laboratory with matrices such as

$$\begin{matrix} 0 & -1 & -1 & -1 & 0 \\ -1 & 2 & 2 & 2 & -1 \\ -1 & 2 & 5 & 2 & -1 \\ -1 & 2 & 2 & 2 & -1 \\ 0 & -1 & -1 & -1 & 0 \end{matrix}$$

that suppress noise in local domains but amplify changes over longer distances.

5.2 Quantitative analysis

A great deal of information can often be obtained by the simple measurement of fluorescence intensities at specific structures. With images stored as arrays of pixels in a frame buffer, it is relatively easy to obtain intensity values at specific points or as profiles along specific lines. An equally useful program allows the integration of intensities within an enclosed area of any irregular shape. A pointing device, such as a graphics tablet or mouse, is commonly used to facilitate the definition of points and lines in the image. Due to the presence of noise in most fluorescence images, it is usually more useful to calculate an average over a local area, e.g. over 5×5 pixels, rather than using values of single pixels for point measurements and linear profiles.

Common sources of error for intensity measurements are the non-uniform detection of fluorescence across the field and the deviation from linearity of many video cameras. The former is caused primarily by the non-uniform throughput of the optical system and the uneven response of the detector, and can be corrected by dividing the image with the image of a uniformly fluorescent object, e.g. a uniformly dispersed fluorescent solution. The problem of non-linearity is associated with the limited dynamic range of many cameras, and can be alleviated by transforming the intensity values with a look-up table. It should also be noted that many experiments require only relative comparison of intensities, and can be performed simply by using a constant, central region of the field and by staying well within the dynamic range of the detector.

Equally useful is the ability to determine the relative position and dimension of specific structures. This also allows the calculation of the rate of any movement. A simple approach is to define two specific points on the screen with a pointing device, and to ask the computer to calculate the distance based on the number of pixels between the points, the aspect ratio of pixels (typically 5:4), and the magnification. Similar programs can be developed to calculate the surface area of any irregular shapes based on the number of pixels and the magnification.

Two sources of error should be noted in dimension measurements. First, some intensified cameras, notably ISIT cameras, introduce significant distortions, especially near the periphery of the field. The error affects primarily the measurement of dimensions that approach the width of the field. Methods have been developed for the correction of such distortions (31), and have been implemented in some image processors (e.g. Inovision). However, the extent of error may be insignificant for many applications, and it may be easier just to avoid the measurement of objects near the periphery of the field.

The second source of error affects primarily the measurements of small objects approaching the limit of resolution of the microscope, and often shows a strong dependence on the intensity of the object. Specifically, the apparent size is affected not only by the point spread function of the microscope, but also by the frequency response and the blooming of the video camera (21). Thus the measurement of diameters of less than 0.5 μm can become unreliable, even when the density of pixels allows a much better resolution. The limitation is less serious for the measurement of minuscule displacements provided the signal/noise ratio is high, since in this case the position can be determined based on the intensity profile.

Ratio imaging (32), developed primarily for the analysis of intracellular ion concentrations, can also yield valuable information on the distribution of fluorescent staining or analogues. For example, the true distribution of the concentration of fluorescent molecules can be obtained by dividing the fluorescence image with an image of control fluorescent molecules (with a different wavelength) that assume a uniform distribution in the same cell. Otherwise, the apparent fluorescence intensity can be easily affected by the distribution of organelles and the variation in the thickness in different regions of the cell (33). Ratio imaging can also be used to compare the distribution of different structural components or protein isoforms, and to identify sites where specific subpopulations of molecules are localized. One recent example involves the microinjection of living cells with a fluorescent analogue, followed by the extraction of cells after a short period of incubation (34). The preferred sites of incorporation are mapped by dividing the image of injected analogues with the image of staining, which indicates the overall distribution of the corresponding endogenous component. A high ratio thus represents a high incorporation rate on a per unit mass basis. Without the ratio imaging, it would be impossible to determine the true active sites of incorporation, since the apparent intensity of the analogue is determined by the rate of incorporation as well as the distribution of structures, both of which can vary dramatically in different regions of the cell.

Many technical aspects of ratio imaging have been discussed in a recent article by Bright *et al.* (32). An important problem which can seriously affect the performance of ratio imaging is the misregistration of images. This problem is most serious when the two images are recorded with different dichroic mirrors and barrier filters, since slight inconsistencies in the alignment of these components can cause significant shifts in images. Sophisticated algorithms have been developed to align two images (31), but the implementation of such programs in image-processing systems is not a trivial task.

We have developed a simple strategy for aligning images that relies on visual judgment. Briefly, the two images for ratioing are displayed back and forth at a high speed. Misregistration is readily visualized as wobbling of the images, and can be eliminated by moving the images relative to each other

until any wobbling disappears. This method effectively removes misregistrations due to translation, but not those caused by rotation, differential magnification, or distortion. However, these latter problems are usually insignificant over limited domains of the cell.

6. Conclusion and prospects

Although immunofluorescence is a well-developed technique, there is still great potential for the development of new fluorescent ligands or peptides as agents for the detection of specific components (13). In addition, many potential applications of fluorescent analogue cytochemistry remain to be explored. For example, cells microinjected with fluorescent analogues can be readily treated with various chemical agents or probed with other micromanipulation methods for mechanical or electrophysiological properties. The fluorescence signals can be analysed with photobleaching or photoactivation and various spectroscopic techniques, and provide information much beyond the resolution of the light microscope, e.g. on the conformation, association, and mobility of molecules. The method is also easily combined with ion imaging for the analysis of possible roles of second messengers in cytoskeletal dynamics. The use of caged second messengers represents a particularly attractive approach for such studies.

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