ABSTRACT The distribution of actin in proteose peptone-elicited murine peritoneal macrophages is examined with fluorescent analog cytochemistry (FAC), immunofluorescence, and electron microscopy (EM). Living adherent macrophages, microinjected with 5-iodoacetamido-fluorescein-labeled actin, show a rather uniform distribution of actin with punctate and linear fluorescence in the thin peripheral areas of the cell. Apparent incorporation of a portion of the microinjected actin into the cell's actin cytoskeleton is also demonstrated when microinjected cells are subsequently examined for fluorescein fluorescence after fixation and extraction. However, a substantial perinuclear pool of actin, observed with FAC, is lost when microinjected cells are prepared for immunofluorescence using standard fixation methods. These results suggest that part of the cellular actin, possibly nonfilamentous or oligomeric, can be extracted during the normal preparative steps for immunofluorescence. When the dynamic distribution of actin structures is examined in living cells, extension of the cell's periphery is associated with the formation of punctate structures. The distribution of the most stable, nonextractable actin structures in fixed cells at different stages of spreading is quantified using rhodamine-labeled phalloidin and antiactin indirect immunofluorescence. At early stages, the rounded cells show cortical bands of fluorescence surrounding the nuclear region with punctate structures directly above the plane of the attached plasma membrane. At later time periods, fully spread cells contain both punctate and linear fluorescent structures. Adherent macrophage membranes, a preparation in which the attached membrane and membrane-cortex are isolated by shearing away the unattached plasma membrane and underlying cytoplasm, show punctate and linear fluorescence when stained with rhodamine-labeled phalloidin. When the same cell remnant is negatively stained and examined with EM, the fluorescent punctate structures coincide with electron-dense foci and associated radiating thin filaments. We suggest that the optimal approach for elucidating the distribution of cytoskeletal and contractile proteins involved in motile processes is a combined approach using all three techniques. Although each technique is subject to potential artifacts and limitations, the use of FAC can permit the visualization of both the soluble and stabilized components of the cytoskeleton in living, functional cells. A qualitative method for determining differences in local concentrations of proteins is also presented.
extraction steps involved in these techniques could remove at least part of the soluble pools of the proteins. To study the dynamic distribution of actin during spreading, we have compared the results obtained by immunofluorescence and electron microscopy (EM) to those obtained by fluorescent analog cytochemistry (FAC), a technique which allows the study of specifically labeled proteins in living cells (28, 29, 34). FAC involves the incorporation of fluorescently labeled actin into living cells by microinjection, thereby permitting the localization and quantitation of both soluble and stabilized forms of this protein (35). The combination of all three approaches provides a more complete picture of the distribution of actin.

In this study, labeling fixed cells with antiactin antibodies or with rhodamine-phalloidin reveals that fully spread macrophages contain both punctate and linear actin structures associated with the substrate-attached plasma membrane. Fluorescent analog cytochemistry on living cells further shows a rather uniform distribution of actin with punctate and linear structures seen in the peripheral regions of the cell. A substantial perinuclear pool of actin is eliminated when the same cells are fixed and permeabilized, as for immunofluorescence. Therefore, the preparative steps of immunofluorescence may produce misleading conclusions concerning the local concentration of proteins which exist in both soluble and stabilized forms. A qualitative method using both FAC and immunofluorescence for determining differences in local concentrations of proteins is presented. Theories of cytoskeletal/contractile protein distribution and function during spreading as well as potential artifacts of these techniques will be discussed.

Materials and Methods

Preparation of Cells: Peritoneal macrophages are harvested from 20-30 g mice (strain CD-1, Charles River Breeding Laboratories, Inc., Wilmington, MA) previously injected with 1.5 ml of a 10% protease peptone solution (Difco Laboratories, Detroit, MI) 3-4 d before harvest. For microinjection experiments, peritoneal cells are collected in Hank's Balanced Salt Solution (HBSS) (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) with heparin, washed once in HBSS, and plated (approximately 1 x 10^6 cells/well) on four chamber LabTek tissue culture chamber/slides (VWR Scientific Co., Boston, MA) in 1 ml of Roswell Park Memorial Institute 16/40 tissue culture media (RPMI) (Grand Island Biological Co.) with 5% fetal calf serum. After incubation, in a humidified atmosphere of 95% air-5% CO_2 at 37°C for 1 h, nonadherent cells are removed by washing, leaving a population of stimulated macrophages. For rhodamine-labeled phalloidin and indirect immunofluorescence experiments, cells are plated in 2 ml of RPMI (~2 x 10^6 cells/well on double well chamber/slides), allowed to spread at 37°C in humidified 95% air-5% CO_2 atmosphere for various time periods and prepared for staining. For adherent membrane experiments, cells are plated on carbon- and Formvar-coated London finder electron microscope grids (Ernst F. Fullam, Inc., Schenectady, NY) for 30 min. The grids are rinsed and transferred to plastic petri dishes containing RPMI and incubated at 37°C with 95% air-5% CO_2 for 2.5 h.

Preparation of Labeled Proteins: Actin is purified from rabbit back and leg muscles according to Spudich and Watt (24). F-actin is labeled with 5-iodoacetamidofluorescein (Molecular Probes, Plano, TX); ovalbumin (Worthington Biochemical Corp., Freehold, NJ) is labeled with fluorescein isothiocya- nate as described previously (28, 36). The details of the labeling procedure are presented elsewhere (37). 5-Iodoacetamidofluorescein-labeled actin (AF-actin) is used within 4 d of labeling. Actin and ovalbumin are dialed in the injection buffer (2 mM PIPES, pH 6.2, 0.1 mM MgCl_2, 0.1 mM dithiothreitol, pH 6.9-7.0) before use. Labeled actin is clarified at 100,000 g and microinjected in soluble form at concentrations ranging from 5-10 mg/ml and with dye-to-protein ratios of approximately 0.7-0.9. Labeled ovalbumin is microinjected at similar concentrations and dye-to-protein ratios.

Microinjection of Macrophages: Microneedles with a tip diameter of ~10 μm are prepared from either microstar capillaries (Radnoti Glass Technology, Inc., Monrovia, CA) or omega dot capillaries (Frederick Haer and Co., Brunswick, ME) on a Kopf vertical pipette puller. Needles are loaded by placing a small volume of protein solution on the back end of the needle. The solution is then drawn to the tip by capillary action. Microinjection is carried out by applying back-pressure to needles secured in a Leitz electrode holder. Macrophages are microinjected in RPMI without Phenol red at 37°C on a temperature-regulated microscope stage (Cambion Cambridge Thermionic Corp., Cambridge, MA). In all cases the volume of injected solution is the minimum volume needed to produce detectable fluorescence using an image intensifier system (21, 26) and is measured to be approximately 5-10% of the total cell volume. It is calculated that the amount of actin injected is ~1.3 x 10^7 molecules and only ~4% of the total endogenous actin. Cell viability after microinjection is assessed by examining the morphology of injected cells with Nomarski optics (i.e., damaged cells display a loss of cytoplasmic structure and assume an inflated appearance). Viable cells also retain pre-injected morphology and exhibit phagocytosis of opsinized erythrocytes (Amato, P. A., and D. L. Taylor, manuscript in preparation).

For experiments in which microinjected cells are subsequently prepared for immunofluorescence, the position of the microinjected cell is noted with the stage vernier, as well as other characteristics such as cell shape, location of the nucleus, and lamellipodia. Immediately after recording the distribution of injected proteins in the living cell, the slide is rinsed and immered in fixative. After staining, the injected cell is relocated and the fluorescent pattern is recorded with the video system and on 35mm film.

Fixation and Staining of Whole Cells: Cells to be stained with rhodamine-labeled phalloidin, a cyclic peptide that specifically binds to F-actin (3, 41), or with antiactin are washed twice in phosphate-buffered saline (PBS) and then fixed in 2-3.7% formaldehyde at 37°C for 30 min. After washing twice with PBS, cells are extracted with ~20°C acetone for 3.5 min and air-dried. Rhodamine-labeled phalloidin (a gift of Dr. Thomas Wietand, Max-Planck Institute for Medical Research) is stored in methanol at 0.2 mg/ml. For staining, 100 μl of 0.16 μg rhodamine-phalloidin/ml PBS is applied to the slides and incubated for 20 min at room temperature. Slides are rinsed twice with PBS and immediately viewed and photographed. NBD-phallacidin (a gift of Drs. Larry Barak and Walt Webb, Cornell University) is also used for some experiments. For indirect immunofluorescence, fixed and extracted cells are incubated with 70 μl of purified rabbit antiactin solution (0.05 mg/ml PBS; a gift of Dr. Keiji Fujiwara, Harvard Medical School) at 37°C for 45 min in a chamber of high humidity. After washing three times with PBS, cells are incubated with 70 μl of rhodamine-labeled goat anti-rabbit IgG (0.05 mg/ml PBS; N. L. Cappel Laboratories, Inc., Cochranville, PA) at 37°C for 30 min. After washing three times with PBS, cells are immediately viewed and photographed.

Fixation and Staining of Adherent Membranes: Macrophage adherent membranes are prepared essentially as described by Boyles and Bainton (5) and Clarke et al. (6). Macrophages are allowed to adhere and spread on London finder electron microscope grids and sheared in a stream of buffer (50 mM KCl, 2 mM MgCl_2, 2 mM EGTA, 10 mM PIPES, pH 6.7) from a syringe. The grids are immediately placed in a solution of 2% glutaraldehyde in the same buffer for 30 min. After rinsing, the grids are stained with rhodamine-labeled phalloidin in buffer (dilutions and concentrations the same as for whole cell staining) and incubated for 20 min. Grids are placed under a coverslip on a glass slide and photographed on the light microscope with a 63 x Planapo oil immersion objective (N.A. = 1.4). They are then rinsed with distilled water, negatively stained with 1% uranyl acetate for 30 s at room temperature, blotted dry, and examined on the electron microscope.

Microscopy: A Zeiss photomicroscope equipped with epi-illumination fluorescence and Nomarski optics is used to view the cells. Microinjection is carried out under a 40 x Achromat water immersion objective (N.A. = 0.75) with a working distance of 1.6 mm. Examination of both microinjected cell fluorescence and fixed whole cell fluorescence is carried out with a 63 x Plan-Neofluar water immersion objective (N.A. = 1.2). Fluorescent images of microinjected cells are recorded with a RCA TC 1030/H TV Silicon Intensified Target Camera coupled to an NEC ¾ inch cassette video tape recorder. An HBO 200-W mercury lamp (epi) or a 60-W tungsten lamp (substage) is the light source. Images on the video tape are photographed from the TV monitor (30) with a Nikkormat camera fitted with a 50 mm/1 Biotar rectilinear (10) and Ilford HPS film which is developed with Dassine developer. Cells treated with both rhodamine-labeled phalloidin and antiactin are photographed directly with the photomicroscope. We found that the quality of the images from antiactin and rhodamine-phalloidin-labeled cells is best using the water immersion objective. Mounting the cells in glycerol and covering them with coverslips decreases the image quality.

Negatively stained samples of macrophage adherent membranes are examined on a Philips 300 electron microscope at an accelerating voltage of 60 kV.

Results

Microinjection of Labeled Proteins

Spread macrophages are microinjected with AF-actin, allowed to recover at 37°C, and examined with the fluorescence
microscope. At 30 min postinjection, punctate and linear fluorescence is seen at thinner peripheral regions of the cell (Figs. 1 b and d, and 2 b; photographed from the TV monitor), suggesting rapid incorporation of labeled actin into the cytoskeleton. A large pool of injected actin is seen in the perinuclear region. The nucleus is delineated by a dark area of apparent exclusion of the labeled protein, indicating minimal nuclear-cytoplasmic exchange of labeled actin (Figs. 1 d, 2 b, and 3 a). There is no increase in nuclear fluorescence of labeled actin for as long as 24 h postinjection (results not shown).

Fully spread cells are microinjected with labeled actin, allowed to recover for 30 min, and subsequently prepared for either rhodamine-phalloidin or antiactin staining to compare the results of these distinct methods. Fig. 2 b, photographed from the TV monitor, shows a fully spread cell injected with labeled actin. The nucleus excludes labeled actin and the thin areas of cytoplasm show numerous punctate foci (Fig. 2 b). Most importantly, after fixation, extraction, and staining with rhodamine-phalloidin, the same cell shows a large decrease of fluorescence in the perinuclear region, indicating a significant loss of labeled actin, presumably due to the extraction step required for immunofluorescent staining (Fig. 2 d; photographed directly on the microscope with the automatic camera). In addition, punctate structures within the cell are more clearly observed. The differences in fluorescent images can be explained since Fig. 2 b represents only the distribution of fluorescently labeled injected actin while Fig. 2 d shows staining of total cellular actin (endogenous plus injected) remaining after fixation and extraction.

Fully spread cells microinjected with AF-actin are also examined for fluorescein fluorescence after fixation and extraction. Fig. 3 shows the fluorescence image of an AF-actin-injected cell both before and after fixation and extraction. The living cell (Fig. 3 a; photographed from the TV monitor) shows disperse fluorescence with punctate and linear structures in peripheral regions of the cell. After fixation and extraction (Fig. 3 b; photographed from the TV monitor) the cell retains at least part of the actin in a "ghostlike" image, suggesting that at least a portion of the microinjected actin becomes incorporated into a stable actin cytoskeleton. The same cell, when stained with rhodamine-phalloidin and photographed from the TV monitor (Fig. 3 c), shows intense staining of the stabilized pool of total cellular actin. When photographed directly with the camera in the microscope (Fig. 3 d), distinct linear structures are seen in peripheral regions of the cell indicating a loss of some detail in the video image. The visualization of punctate and linear structures is more clearly observed when fluorescent images are photographed directly on the microscope (with an

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**Figure 1** Fully spread macrophages microinjected with AF-actin, 30 min postinjection. (a and c) Nomarski images. (b and d) Corresponding fluorescence images (from the TV monitor). Note punctate (b, arrowhead) and a suggestion of linear (d, arrowhead) fluorescence. Nucleus (N). (a and b) Bar, 10 μm. × 1,000. (c and d) Bar, 10 μm. × 650.
exposure time of 1-2 min) than when they are photographed from the video monitor (with exposure times of only a fraction of a second). The loss of image quality with the video image is offset by the ability to record living cells with a minimum of damage.

The distribution of actin structures during the spreading process is followed in the same cell (Fig. 4). After recording the initial actin distribution (Fig. 4b), the slides are placed in a petri dish containing complete culture media and the cells are allowed to undergo further spreading at 37°C and 5% CO₂. After 4 h, the cell becomes more flattened as shown in the Nomarski images (Fig. 4a and c); the nucleus is still delineated and remains in the same region of the cell (Fig. 4b and d). In the periphery, newly formed punctate structures are located in an area corresponding to flattened regions of the cell (Fig. 4c and d). There is no observable redistribution or concentration of actin to this leading edge. When fixed, extracted, and stained with rhodamine-labeled phalloidin (Fig. 4f; photographed from the TV monitor), the same cell shows a loss of fluorescence in the perinuclear area.

The distribution of actin is compared to that of ovalbumin, a control protein which is assumed to distribute evenly throughout the cytoplasm. Immediately after microinjection, labeled ovalbumin is apparently excluded from the nucleus (Fig. 5a). After 20 min, however, the nucleus becomes indistinct (Fig. 5b), suggesting that the labeled ovalbumin can diffuse slowly into the nucleus. When the ovalbumin-injected cell is stained with rhodamine-phalloidin (Fig. 5d and e), few punctate structures are seen. Immunofluorescent staining techniques produced a wide range in the number of fluorescent structures for each cell.

In cells microinjected with fluorescent molecules, local differences in fluorescence intensity can be due to differences in local concentration, pathlength of the fluorescent molecules, and/or the accessible volume in that region (29, 34, 36). In Fig. 5b, the thick perinuclear region and the thin cortical region both exhibit a relatively high fluorescence intensity. The high fluorescence intensity in the center of the cell could be due to a large pathlength. In addition, since the thin cortical region of cells usually excludes large organelles, the accessible volume for any small fluorescently labeled molecules should be greater in the cortex relative to other areas of the cell. The accessible volume for a fluorescent molecule in a particular region of a cell can be defined as total cytoplasmic volume minus organelle volume. Hence, local concentrations of labeled proteins cannot be directly inferred from fluorescence intensity alone. Some type of normalization method is required (34).

When compared to staining with rhodamine-labeled phalloidin, the distribution of labeled ovalbumin in the fixed and extracted cell shows a similar high fluorescence intensity in the thin, cortical regions of the cell (Fig. 5c [arrow] and d and e [arrow]). We can therefore conclude that there is no apparent

**Figure 2** Fully spread macrophages microinjected with AF-actin and subsequently fixed, extracted, and stained with rhodamine-labeled phalloidin. (a and b) Nomarski and fluorescein fluorescence image of the living cell (fluorescence image photographed from the TV monitor). (c and d) Nomarski and rhodamine fluorescence image of the fixed, extracted, and labeled cell (photographed directly on the photomicroscope). Note that in the living cell (b), the nucleus (N) is delineated by a dark area of labeled actin exclusion and numerous punctate structures (arrow) are visible in the thin, peripheral areas of the cell. Bar, 10 μm. × 1,300.
elevated concentration of actin in this region relative to the
soluble control protein. In contrast, the high fluorescence inten-
sity of ovalbumin in the perinuclear region (an area with
the longest pathlength of the fluorescent probe) indicates that
the soluble control protein and actin are apparently present in
different concentrations in this area. However, some actin
could have been extracted. Thus, comparing local fluorescent
intensities in ovalbumin-injected cells and in actin injected
cells or actin immunofluorescent images qualitatively normal-
izes for pathlength and accessible volume, thereby, allowing
differences in local concentrations of proteins to be deduced.

Characterization of the Stable Fraction of Actin
during Spreading: A Quantitative Study

Rhodamine-phalloidin and antiactin antibodies are used to
label the stable fraction of actin (fixed and maintained in the
cell) during the spreading process. These methods are used to
characterize the distribution of this pool of actin quantitatively.
Cells are plated and allowed to spread for 15 min, 30 min, 1,
2, and 3 h. After fixation, extraction, and staining with rhoda-
mine-labeled phalloidin, the slides are photographed and
scored for both cell shape and the appearance of fluorescent
structures. After 15 min of incubation, most of the cells (Fig.
6) are round (Fig. 7a) while a small percentage are beginning
to spread. At a high plane of focus, a cortical band of fluores-
cence is seen (Fig. 7b); this is probably due to the distribution
of actin structures surrounding the nucleus of the cell. At a low
plane of focus (Fig. 7c), punctate structures are visible in the
plane of substrate-membrane contact. After 30 min of spread-
ing, a higher percentage of cells are spread with some cells
assuming an elongated shape (Fig. 6); the cortical band of actin
is seen at a high plane of focus (Fig. 8b) along with punctate
structures in a low plane of focus (Fig. 8c). Linear fluorescent
structures (referred to as fluorescent fibers in immunofluores-
cent studies) were only rarely seen after 30 min of spreading.
However, after 1 h of spreading, linear fluorescence (i.e., fibers)
is seen in all four categories of possible cell shapes (Fig. 6).
After 3 h of incubation, most cells are spread with punctate
and linear fluorescence dominating the images (Fig. 9a).

To compare indirect immunofluorescent images with those
using rhodamine-labeled phalloidin, cells are allowed to spread
for 15 min and for 3 h, and are subsequently fixed, extracted,
and stained with either antiactin antibody or rhodamine-la-
beled phalloidin. Using the antibody, some cells exhibit only
diffuse fluorescence at 15 min and no punctate structures are
observed. After 3 h of incubation, many cells are fully spread
and punctate structures can be seen above the attached mem-
brane (Fig. 9b). Although linear fluorescent structures are
observed using the antibody technique (Fig. 9b), they are seen
less frequently and not as distinctly as they are when stained
with rhodamine-labeled phalloidin. However, both techniques
produced images which were nearly indistinguishable. A con-
trol experiment, in which cells were not exposed to the primary
antibody but are incubated with rhodamine-labeled goat anti-
rabbit IgG, shows no specific staining (not shown).
Macrophone Adherent Membranes

Adherent membranes are stained with rhodamine-labeled phalloidin, examined with the fluorescence microscope, then negatively stained and examined with the electron microscope in order to correlate fluorescent structures with ultrastructure in the same cell. Fluorescent images of adherent membranes resemble those of whole cells showing varying numbers of punctate structures as well as linear fluorescence (Fig. 10a and b). In the same cell, fluorescent structures coincide with electron dense areas at the ultrastructural level (Fig. 10c and d, and Fig. 11a and b). At high magnification, the electron dense foci are closely associated with meshworks of thin filaments (Fig. 11b) and may be similar to actin foci described for macrophages and other adherent cells using similar techniques (5, 14, 22, 23, 27, 31).
FIGURE 5 Distribution of FITC-labeled ovalbumin in a fully spread macrophage. a–d are photographed from the TV monitor; e and f on the photomicroscope. a shows the fluorescence image immediately following microinjection; labeled ovalbumin is apparently excluded from the nucleus (N). After 20 min (b), the nucleus becomes indistinct. c shows the fluorescein fluorescence after fixation and extraction; a portion of the labeled ovalbumin is retained in the cell. Note the relative high fluorescence intensity of the thin cell periphery in both living and fixed ovalbumin-injected cell (b, and c, arrow). d and e shows the actin distribution in the same cell as revealed by staining with rhodamine-labeled phalloidin. A local high fluorescence intensity and apparent concentration of actin in the cortical region of the cell (d and e, arrow) corresponds to highly fluorescent cortical region in the labeled ovalbumin images (b and c, arrow). f is the Nomarski image of the fixed, extracted cell. Bar, 10 μm. × 1,100.

DISCUSSION
The Use of FAC to Study Actin Distribution

Electron and immunofluorescence microscopy have revealed that actin plays an important role in both cell adhesion and spreading (2, 9, 11, 13, 22, 23, 39). Reaven and Axline (20) demonstrated the presence of microfilaments on the cytoplasmic surface of the plasma membrane of macrophages. Scanning electron microscopy further revealed that the spreading lamellae and lamellipodia of macrophages are principally composed of dense foci, interconnected by radiating filaments and filament bundles, which may serve as elements linking actin to the substratum (31). Other studies, using actin antibodies to visualize actin-containing structures in macrophages, have similarly demonstrated the presence of fluorescent punctate structures which may serve as actin-substratum attachment sites (4, 16, 17). Although several studies using immunofluorescence have reported the absence of fluorescent actin fibers in these cells (15, 16, 18), Berlin and Oliver (4) have noted oriented fibers in fully spread macrophages. Hence, immunofluorescent and electron microscopic examination of macrophages has provided a good deal of information concerning the more stable structures of the cytoskeleton. However, conventional fixation and extraction procedures can result in the loss of cellular protein and even a reorganization of cellular components. Using various fixatives, Willingham and Yamada (40) report that as little as 50–70% of total cellular protein is retained in fixed cells under some conditions. Therefore, we applied the technique of flu-
the soluble actin pool in a living cell and stabilized actin variety of mechanisms (19, 38). It should be emphasized that injection volumes, allows for its use as a dynamic probe of both injected labeled actin is functionally active and is able to within 30 min (7). The incorporation of labeled actin may result from the exchange of actin subunits in the cell by a variety of mechanisms (19, 38). It should be emphasized that injection of fluorescently labeled actin, especially in small volumes, allows for its use as a dynamic probe of both the soluble actin pool in a living cell and stabilized actin structures. In addition, the visualization of distinct actin structures using FAC should not be expected to be as clearly detectable as with immunofluorescence since we are only looking at a small percentage of total actin. Microinjected macrophages show a rather uniform diffuse distribution of actin and both punctate and linear structures in thin, peripheral regions, suggesting that the labeled actin is accessible to cortical regions of the cell. The diffuse perinuclear fluorescence can effectively mask distinct fluorescent structures and may represent the soluble actin pool and/or overlapping bundles of actin filaments. Fixation and extraction of microinjected cells could remove the less stable pool of actin thereby allowing visualization of distinct actin structures when stained with rhodamine-labeled phalloidin or antiactin antibody. Using immunofluorescence, what may be simply interpreted as a local concentration of a particular protein may in fact be due to an area of substantial organelle exclusion and thus large accessible volume of cytoplasmic proteins. The cortical regions of the macrophage would fall into this category. Therefore, suggestions of high concentrations of cytoplasmic proteins in this area made on the basis of immunofluorescence intensities (4, 25, 32, 33) must be interpreted with caution since this area represents a region of large accessible volume. Microinjection of a soluble control protein, such as labeled ovalbumin, prior to preparation for immunofluorescence staining, can identify these areas in the cell and can be used to normalize the relative fluorescence intensities seen in immunofluorescence images. However, it must be remembered that the mobility of fluorescent molecules in a living cell may be affected by the different structural properties of the cytoplasm in a particular region,
Figure 8 Nomarski (a) and fluorescence images (b and c) of fixed cells stained with rhodamine-labeled phalloidin after 30 min of spreading. A cortical band of fluorescence is seen in a high plane of focus (b). In a low plane (c), punctate structures are visible. Rarely can linear structures (c, arrow) be seen after only 30 min of spreading. Bar, 10 μm. × 1350.

Figure 9 Fluorescence images of fixed cells after 3 h of spreading. (a) Stained with rhodamine-labeled phalloidin. (b) Antiactin indirect immunofluorescence. Both fluorescent punctate and linear structures are visible above the attached membrane. Note that images produced by both techniques are nearly indistinguishable. Bar, 10 μm. × 1700.

Interpretation of Actin Distribution during Spreading by FAC and Immunofluorescence

Rhodamine-labeled phalloidin and antiactin indirect immunofluorescence of whole fixed, extracted cells also reveals fluorescent punctate and linear structures associated with the substrate-attached plasma membrane. Linear structures seem to be accentuated by staining with rhodamine-labeled phalloidin as compared to antiactin. This may be due in part to the binding of phalloidin to F-actin structures in contrast to antiactin antibodies, which may bind both filamentous and residual nonfilamentous actin. It should be emphasized, however, that linear structures were seen with both techniques and that no dramatic difference between phalloidin labeling and antiactin labeling was detected. Future studies will evaluate other fixation procedures since it has been demonstrated (22, 23) that different fixation procedures can optimize the preservation of cytoskeletal elements, particularly actin filaments in thin, peripheral regions of attached cells.

The appearance of nonextractable fluorescent structures as a function of time is correlated with the degree of spreading in
FIGURE 10  (a and b) Macrophage adherent membranes stained with rhodamine-labeled phalloidin. Varying numbers of punctate and linear structures are visible. Bar, 10 μm. × 1,200. (c and d) After staining with rhodamine-labeled phalloidin, the same adherent membrane is negatively stained and examined with electron microscope. Fluorescent structures coincide with electron dense areas at the ultrastructural level (c and d, arrows). Bar, 10 μm. × 1,800.

FIGURE 11  Macrophage adherent membrane stained with rhodamine-labeled phalloidin and negatively stained and examined with electron microscope. Note that a represents only a portion of the cell which remains after shearing. The boxed area of a corresponds to the negatively stained image shown in b. The four fluorescent punctate structures represent the four electron dense foci shown in b. The foci are closely associated with meshworks of thin actin filaments (arrow). (a) Bar, 10 μm. × 1,600. (b) Bar, 0.5 μm. × 33,800.
actin which was previously associated with either unattached membrane or cytoplasm in the perinuclear region becomes associated with the substrate-attached membrane. The actual extension of the cell's boundary may involve the elongation of membrane-associated actin especially from foci on the cytoplasmic surface of the attached membrane.

FIGURE 12 Schematic drawing summarizing our results on the organization of actin during the spreading process. During initial attachment to the substrate (A), punctate structures (bold dots) are formed. The nucleus (N) is surrounded by a spherical distribution of actin structures (thin lines) which appear as a cortical band of fluorescence in a high plane of focus. As spreading is initiated, the cell begins to flatten, thereby increasing the surface-to-volume ratio. As the cell continues to flatten (B), an increasing percentage of cytoplasmic actin comes into contact with the spreading membrane. Thus, actin which was previously associated with either unattached membrane or cytoplasm in the perinuclear region becomes associated with the substrate-attached membrane. Fluorescent linear structures (thick bars) are formed as spreading continues. The actual extension of the cell's boundary may involve the elongation of membrane-associated actin especially from foci on the cytoplasmic surface of the attached membrane.

fixed cell preparations. Cells are plated and allowed to spread for various times and fluorescent structures are quantified by scoring cells in a number of fields. Punctate structures are formed. The nucleus (N) is surrounded by a spherical distribution of actin structures (thin lines) which appear as a cortical band of fluorescence in a high plane of focus. As spreading is initiated, the cell begins to flatten, thereby increasing the surface-to-volume ratio. As the cell continues to flatten (B), an increasing percentage of cytoplasmic actin comes into contact with the spreading membrane. Thus, actin which was previously associated with either unattached membrane or cytoplasm in the perinuclear region becomes associated with the substrate-attached membrane. Fluorescent linear structures (thick bars) are formed as spreading continues. The actual extension of the cell's boundary may involve the elongation of membrane-associated actin especially from foci on the cytoplasmic surface of the attached membrane.

In summary, the optimal approach for elucidating the distribution of cytoskeletal and contractile proteins involved in motile processes is a combination of three techniques. Immunofluorescence and electron microscopy can yield a great deal of information concerning the structural components of the cytoskeleton while FAC allows us to follow dynamic changes of both the soluble and structural pools of cytoskeletal proteins in living, functional cells (35). Results from one technique must be interpreted with caution due to the potential artifacts and limitations of each technique. The concomitant use of FAC and immunofluorescence can minimize artifacts such as local differences in pathlengths and accessible volume, thereby permitting qualitative determinations of the local concentrations of proteins in different regions of the cell.

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