

In Vivo Staining of Cytoskeletal Actin by Autointernalization of Nontoxic Concentrations of Nitrobenzoxadiazole-Phalloidin

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ABSTRACT The blue light-excited fluorescent phalloxin derivative nitrobenzoxadiazole phalloidin (NBD-Ph) was used to stain entire tissue culture monolayers of live L6 mouse cells and other mammalian cell lines without the aid of permeabilization treatment. Although cells tend to exclude the fluorescent toxin, reducing the internal concentration by $\sim 1,000$ times, some of it enters the cells, probably by pinocytosis, and stains actin structures at low intracellular NBD-Ph concentrations (~ 5 – 15 nM), where cell toxicity was negligible or at least not detectable by phase-contrast microscopy. Prolonged treatments with NBD-Ph did induce pharmacological responses similar to those of phalloidin. The dissociation constant for NBD-Ph with F-actin in fixed and extracted L6 cells was determined, from staining intensity measurements at various NBD-Ph concentrations, to be 1.5 – 2.5×10^{-8} M.

A convenient specific fluorescent stain for F-actin and G-actin oligomers in plant and animal cells is obtained by attaching the fluorophore nitrobenzoxadiazole (NBD) to the mushroom phalloxin phalloidin (Ph) (1–3). By permeabilization with lysolecithin, we had incorporated NBD-Ph into entire populations of live fibroblasts in tissue culture to stain the cytoskeletal actin (1). We report here the staining of actin structures in living tissue-culture cells without the help of permeabilizing agents or injection (4–8). Cytoskeletal staining can be obtained under conditions where pharmacological effects of the toxin are mild and reversible. Staining persists after washing away excess toxin. Observations of the processes of internalization suggest mechanisms by which phalloxins penetrate cells and by which the cell responds to and protects itself from the toxin.

The phalloxins are small bicyclic peptides that strongly bind F-actin and G-actin oligomers ($K_d \sim 2 \times 10^{-8}$ M) and stabilize them against depolymerization by agents such as cytochalasin B, DNase I, KI, and heat (9, 10). We have exposed several types of vertebrate cells in tissue culture to nonlethal concentrations of the fluorescence-labeled phalloxin NBD-Ph for various periods of time and observed the resultant fluorescent staining and cell morphology. The particular procedures and results for L6 myoblasts are described here.

MATERIALS AND METHODS

Cell Treatment

L6 myoblasts were grown on 22- \times 22-mm glass coverslips in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum in a humidified

5% CO₂ atmosphere. All media were obtained from Grand Island Biological Co., Grand Island, N.Y. (GIBCO). NBD-Ph was added to supplemented growth medium whenever needed by drying an appropriate amount of NBD-Ph solution in methanol onto the inside of a sterile test tube with a stream of dry nitrogen, and then redissolving it in 125 μ l of medium. At 30–50% confluence of cells, coverslips with L6 cells to be removed for staining were placed cell-side-up on pedestals consisting of neoprene O-rings. The cells were then covered by 125 μ l of supplemented growth medium containing a selected concentration of NBD-Ph by pipetting the solution onto the coverslips. About 2 ml of supplemented medium was placed in the dishes around the pedestals to maintain humidity, and the petri dishes were covered and returned to the 37°C incubator. For the 4°C incubations, the growth medium was supplemented with 15 mM of HEPES at pH 7.3. Upon completion of the incubation period, the cells were washed three times in GIBCO medium 199, and then the cells were immersed face-down in medium 199 with the coverslip edges supported by narrow tape spacers placed on the microscope slide. After each examination, those coverslips that were to be further incubated were carefully lifted and inverted, immersion oil was removed from the back with methanol, and the coverslip was returned to growth medium for the allotted time. Bacterial growth was not a problem, despite the nonsterile handling conditions during examination.

Cell Staining and Fixation

Fluorescein diacetate (FDA) was obtained from Sigma Chemical Co., St. Louis, Mo. It was kept at -20°C at a concentration of 0.1 mg/ml in acetone. Before use, it was diluted 1:200 to 200 with medium 199. Cells were labeled by carefully injecting 25 μ l of the diluted solution under the edges of the raised coverslip. Under illumination at 450–480 nm, live cells started to fluoresce a bright green as the FDA reached them by diffusion.

When fixation was required, cells were placed in 2.5% formaldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature, extracted with -20°C acetone for 5 min, and stained with 150 nM NBD-Ph for 30 min. Cells were observed on a Nikon Optiphot microscope with a 100-power phase-contrast objective. NBD-Ph fluorescence was excited through standard fluorescein filters at between 450 and 480 nm by an Osram HBO/50 mercury lamp (MacBeth

Sales, Newburgh, N. Y.) and observed through a 515-nm barrier filter to block exciting light. Fluorescence images were recorded directly on Kodak Tri-X film. Film negatives were scanned with a Joyce Mark III densitometer.

Determination of the Binding Equilibrium between NBD-Ph and Cellular Actin

Coverslips of L6 cells plated at the same time and at equal densities were fixed in formaldehyde and extracted with acetone as described above. An individual coverslip of cells was incubated for 1–3 h at room temperature with a particular concentration of NBD-Ph in PBS. Each coverslip was then washed three times in PBS, mounted on a glass slide, and viewed on a Zeiss universal microscope. The fluorescence intensity of NBD-Ph bound to cellular actin was determined by examining sets of coverslips (see Fig. 4, legend) in the following manner. NBD-Ph fluorescence was excited by epifluorescence illumination with the 488-nm line of a Spectra Physics argon-ion laser focused onto a cell through a Zeiss $\times 100$ oil, neofluar objective. Fluorescence collected by the objective lens was separated from the exciting light by a Schott OG530 barrier filter (Schott Optical Glass Inc., Duryea, Pa.) and focused onto the photocathode of an RCA (C31-034) photomultiplier set (RCA Solid State, Somerville, N.J.) behind an adjustable diaphragm. The aperture of the diaphragm was adjusted so that light was collected only from a fixed area on a single cell, and this area remained constant for all measurements. The intensity of the exciting light was attenuated by neutral density filters until bleaching of the NBD was $<10\%$ of the total intensity over the duration of a single measurement. Measurements at various NBD-Ph concentrations were performed under identical conditions.

RESULTS AND DISCUSSION

Various toxin concentrations and periods of exposure were tested to determine the doses of NBD-Ph that would provide visible fluorescence staining of the cytoskeleton in live cells but minimize toxic effects. Best results were obtained by incubating cells for 16–24 h with 15–25 μM of fluorescent toxin in the growth medium. Fig. 1 *a* and *b* show seven live cells in NBD-Ph fluorescence and phase-contrast. In phase-contrast, the morphology of the stained live cells appears normal. In fluorescence, actin stress fibers and some localized but diffusely stained regions are evident. The actin fibers extend to the cell periphery. What we believe are endocytic vesicles seem preferentially aligned along some of the fluorescent fibers. The fluorescence micrograph illustrates the widespread actin labeling that consistently appears in virtually the entire cell population on the coverslip.

FDA staining provides a convenient assay of cell viability (11). Viable cells fluoresce a bright green, indicating that the cell membrane remains intact. More than 95% of cells remaining on a dish show bright green fluorescence after NBD-Ph treatment (not shown). Cell counts and resumption of cell

division after removal of cells from NBD-Ph-containing medium indicate that cell death due to our standard level of toxin for staining (15–25 μM) is negligible. Prolonged incubation with NBD-Ph at higher concentrations inhibits mitosis and eventually causes cell detachment from the glass substrate.

Those cells beginning to exhibit signs of phallotoxin toxicity due to an excessive accumulated dose display features shown in Fig. 2 *a* and *b*. Phase-dense cytoplasm has contracted toward the nucleus, whereas peripheral areas appear flattened. On the nuclear side of this flattened area, a ring of actin which has contracted slightly from the periphery surrounds the cell center. Notice that the cells appear closer to one another in phase-contrast than in fluorescence. This effect is indicated for one pair of cells by the corresponding arrows in Fig. 2 *a* and *b*. These observations are consistent with the pharmacological effects produced by microinjection of phalloidin (4, 5).

The pharmacological effects of the fluorescent toxin seen in phase-contrast are reversible. In phase microscopy, cells removed from the labeling medium are indistinguishable from controls after 8 h. In fluorescence, actin-stress fiberlike structures are still visible at this time and up to 16–24 h after removal of the toxin.

The applied NBD-Ph concentrations and exposure times required to stain the cytoskeleton in living cells in the absence of a permeabilizing agent exceed those required to stain fixed cells or living cells in the presence of lysolecithin by several orders of magnitude. This difference suggests that living cells are capable of excluding a substantial fraction of the applied toxin concentrations to maintain a very small ratio of internal to external NBD-Ph concentrations. A minimum value of this ratio of $\sim 1:60$ was estimated by measuring relative fluorescence luminosities of intracellular cells that were briefly exposed to 15–25 μM NBD-Ph in the external growth medium.

To quantify the cytoplasmic NBD-Ph concentration more precisely, we measured the ratio of the intensity of NBD-Ph staining in live L6 cells to the staining intensity of those same cells after fixation, extraction, and restaining with NBD-Ph at the concentrations of NBD-Ph required to saturate nearly all accessible F-actin binding sites.

Fig. 3 shows cells that were fixed and restained at an NBD-Ph concentration of 150 nM after treatment similar to that of Fig. 1. A densitometry scan of the negative film image of this exposure was compared with a scan of the cells pictured in Fig. 1 *a*, after correction for the characteristic response of the film

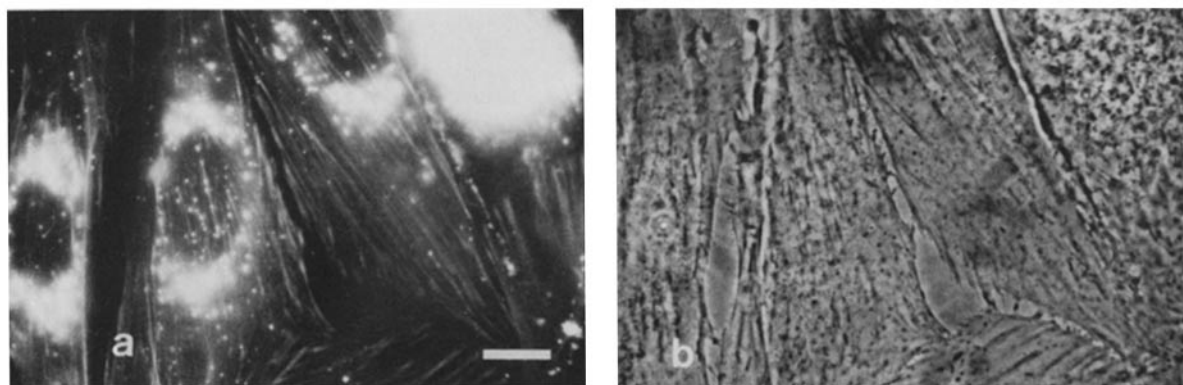


FIGURE 1 L6 cells cultured at 37°C in 5% CO₂ in Dulbecco's Modified Eagle's Medium plus 10% fetal calf serum were grown on glass coverslips to 30–50% confluence. Cells were washed three times and then viewed and photographed in medium 199. Pictured are a typical stained confluent group containing seven live L6 labeled L cells in the presence of 20 μM NBD-Ph for 20 h. The bright area in the corner is a result of out-of-focus vesicles, which can be seen more clearly in the center cell. (*a*) fluorescence and (*b*) phase. There is an admixture of cytoskeletal and vesicular fluorescent staining. Bar, 12 μm . $\times 820$.

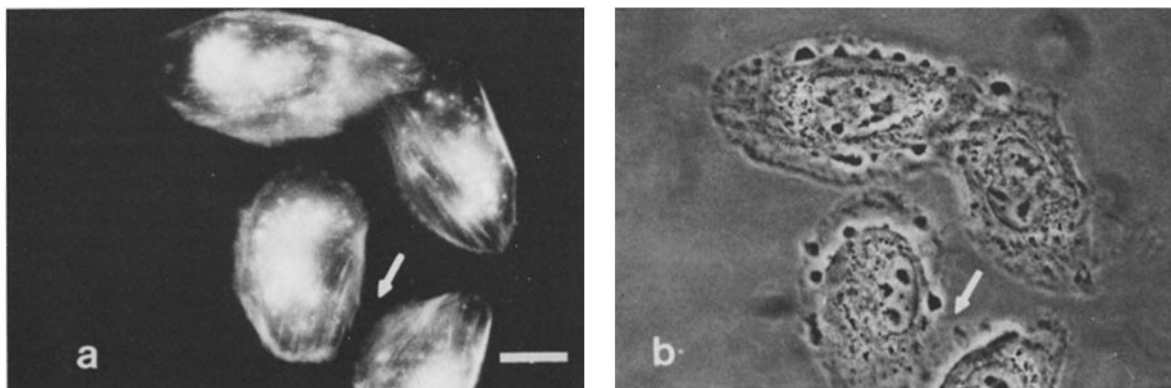


FIGURE 2 L6 cells incubated in 20 μM NBD-Ph until the point where toxicity is first evident. (a) Fluorescent actin fibers have contracted slightly from the cell edges, leaving a stained perimeter that is visible in phase-contrast (b). Note the larger gap between cells in fluorescence than in the phase micrographs (below arrow). Bar, 12 μm . $\times 820$.



FIGURE 3 Coverslip of Fig. 1 fixed in 2.5% formaldehyde for 10 min, extracted with -20°C acetone for 5 min, and restained with 150 nM NBD-Ph for 30 min. The fixed cells produced three to six times more fluorescence compared with the live cells of Fig. 1, given equal exposure and processing conditions, as determined by densitometry tracings of the negatives. Therefore, it was necessary to increase the printing time and aperture to produce comparable prints. Bar, 12 μm . $\times 820$.

(2). The exposures were taken and processed under identical conditions. The fixed restained cells were three to six times more fluorescent than the live cells. The amount of NBD-Ph in solution in the cytoplasm of the live cells could then be estimated by identifying the NBD-Ph concentration for the same ratio on an equilibrium binding curve with F-actin in fixed L6 cells. This procedure assumes equivalence between NBD-Ph accessible F-actin pools in fixed and live cells. Microinjection experiments with phalloidin (12) indicate this equivalence.

Fig. 4 shows the equilibrium binding curve of NBD-Ph to F-actin of fixed and extracted L6 cells. The dissociation constant K_d is equal to the concentration of NBD-Ph at half-maximal binding; thus, K_d equals $1.6\text{--}2.5 \times 10^{-8}$ M in agreement with values for other phalloxins (9, 10). For staining intensity values three to six times below the saturation level, the concentration of NBD-Ph in solution is 5–15 nM. This suggests that the available cytoplasmic concentration of NBD-Ph within the live cells remains near 5–15 nM, even when the external concentration is 15–25 μM .

Thus, the cell membrane presents a substantial barrier to

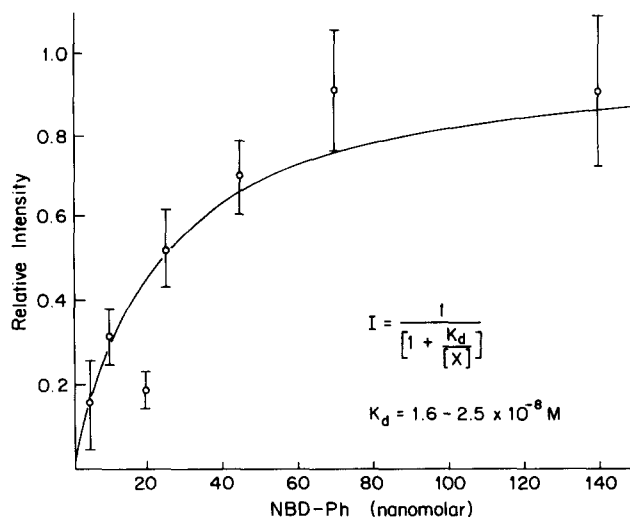


FIGURE 4 Equilibrium binding of NBD-Ph to accessible cellular F-actin of fixed and extracted L6 cells. The fluorescence intensity of NBD-Ph on cellular F-actin was measured after incubation of the cells at each of the concentrations shown on the graph. For NBD-Ph binding to actin described by $[A] + [\text{NBD-Ph}] = [A - (\text{NBD-Ph})]$, the relationship between relative fluorescence intensity and phalloxin concentration is given by the equation $I = 1.0 / (1 + K_d / [X])$ where $[X]$ = concentration of NBD-Ph in the external labeling solution at equilibrium and I equals the relative fluorescence intensity of the stained L6 cells at concentration $[X]$. K_d is the equilibrium dissociation constant for NBD-Ph and F-actin. The average intensity was normalized to produce an intensity of 1.0 at infinite NBD-Ph concentration. Each experimental point on the graph represents the averages of two to seven separate experiments, except those at 20 and 25 nM, which were the results of single experiments. Each experiment represents the average of intensity measurements from 14–16 randomly selected, NBD-Ph-stained cells from a single coverslip.

entry of the toxin. This barrier and the mechanism of entry were investigated as follows. Cytoskeletal fluorescence was monitored as a function of both time and temperature. Fluorescence increased in proportion to the incubation time and concentration of NBD-Ph in the external medium. Approximately 5 h were required for stress fibers to become visible, whereas many tiny fluorescent points were evident within the cells after only 1–2 h (Fig. 5a). Cells labeled at 4°C for 24 h with 15 mM HEPES added to the medium were only slightly more fluorescent than cells incubated in an identical manner

but without NBD-Ph. Binding of NBD-Ph was not inhibited by 2 μM cytochalasin B, an inhibitor of phagocytosis (13).

Four alternative mechanisms may be invoked to explain the toxin internalization: (a) passive diffusion through the lipid bilayer; (b) active transport; (c) absorptive pinocytosis; and (d) fluid phase pinocytosis. Because internalization and staining are inhibited at 4°C, and because phalloidin and phallotoxins in general are not lipid soluble, passive diffusion seems unlikely. Active transport and absorptive pinocytosis cannot be entirely excluded. Cells briefly exposed to up to 20 μM NBD-Ph and then washed show no evidence of NBD-Ph fluorescence above background, suggesting the absence of a high-affinity membrane receptor.

The following observations are suggestive of, but not sufficient to establish, fluid phase pinocytosis as the cellular mechanism of entry for NBD-Ph. The process is time, temperature, and concentration dependent. It is nonsaturable, at least in the range of concentrations used, because the cytoplasmic fluorescence increases for increasing concentrations of NBD-Ph in the external medium between 0.1 and 20 μM . It is not inhibited by cytochalasin, and it is accompanied by the early appearance of small fluorescent points that are presumably pinocytotic vesicles. To account for actin labeling following internalization via pinocytosis, NBD-Ph must escape intact from pinosomes or subsequent to accumulation in lysosomes (1, 2, 14–16). Neither

photolysed forms of NBD-Ph nor NBD-ethanolamine nor NBD-Cl stain in a way similar to NBD-Ph (1). Therefore, it is unlikely that the observed fluorescence distribution is due to release of the fluorophore after degradation of the fluorescent toxin.

Following the recovery of L6 cells after staining and removal of the toxin, we observed that the internal actin filamentary structures remain stained for 16–24 h. Meanwhile, the cell morphology is normal. After 36–48 h, the fluorescence becomes concentrated in spherical vesicles of various sizes (Fig. 5b). Whether these vesicles also contain actin within them is not known. However, chloroquine, a drug whose effects are exerted in lysosomes (14–16), at a concentration of 0.05 mM changes the distribution of fluorescence. Fluorescence appears in large vesicles, and in some cases cytoskeletal labeling by NBD-Ph is abolished (Fig. 6). The NBD-Ph vesicular fluorescence is much stronger than the weak fluorescence observed in cells treated with chloroquine alone. In Fig. 6a, correspondence between phase and fluorescent vesicles (see arrow) is evident. It has been reported that pinosomes either fuse or exchange their contents with chloroquine-induced vacuoles (14). It thus appears that chloroquine inhibits NBD-Ph cytoskeletal labeling by trapping the toxin in the chloroquine-induced vacuoles.

We have observed fluorescent staining of actin structures without permeabilization in living human fibroblasts, rat kan-

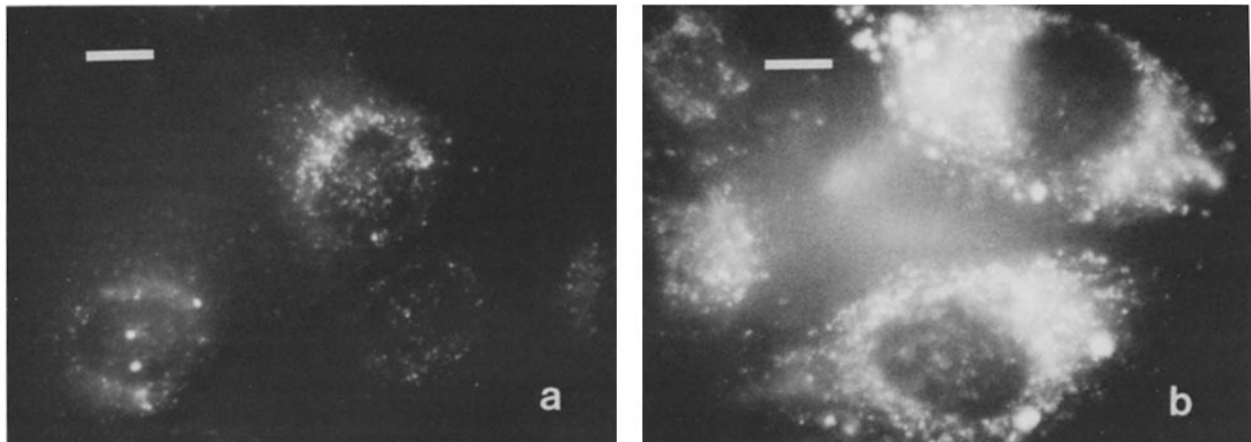


FIGURE 5 (a) Live L6 cells after 2 h of 20 μM NBD-Ph incubation. There is a lack of cytoskeletal staining, but pinpoint fluorescence is now evident. (b) Live L6 cells treated with 20 μM NBD-Ph 36 h after removal from incubation with the toxin. Fluorescence is now concentrated in large vesicles. Bar, 12 μm . $\times 820$.

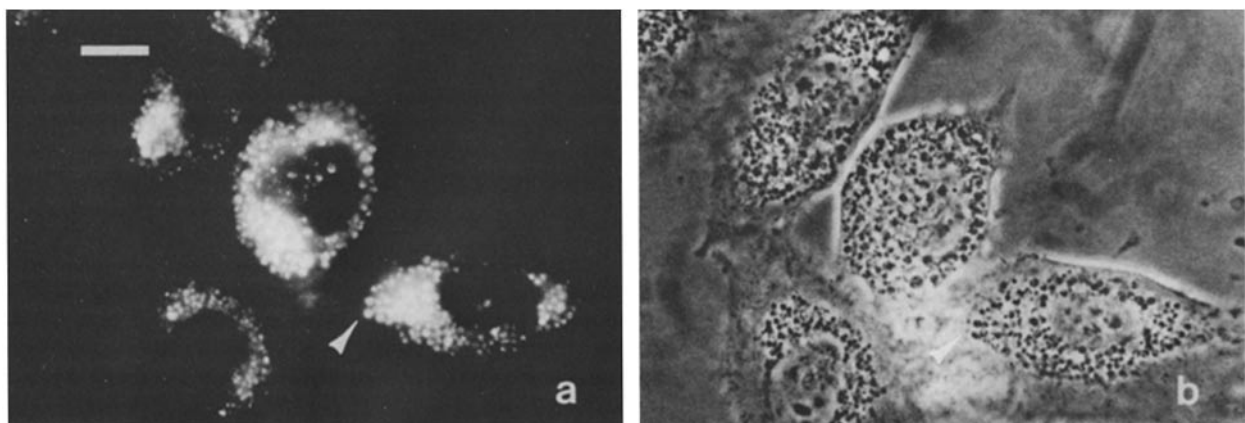


FIGURE 6 Live L6 cells simultaneously treated with 0.05 mM chloroquine and 20 μM NBD-Ph for 24 h. Fluorescence is contained in spherical structures that coincide with phase vacuoles. Bar, 12 μm . $\times 820$.

garoo fibroblasts, and mouse embryo fibroblasts similar to that seen in L6 cells. We also have observed staining in lymphocytes.¹ However, we have not yet investigated hepatocytes, the cells most affected by phalloidin in animal injection experiments (9). We think that phallotoxins probably enter living cells by pinocytosis, escape from pinosomes (14–16) or lysosomes, and then bind actin to exert their toxic effect. The external concentration of NBD-Ph required to stain actin moderately in live cells is 15–20 μ M; this is at least two orders of magnitude greater than the concentration of NBD-Ph (50–150 nM, see Fig. 4) that will intensely stain actin in slightly permeabilized cells (1).

L6 cells remain nearly normal in appearance after treatment at levels of NBD-Ph suitable for staining actin structures. They return to completely normal appearance after the treatment is ended, although they retain the staining of actin structures. Therefore, the fluorescence-conjugated toxin NBD-Ph may be suitable for studies of the *in vivo* association of actin with other molecules that can be marked with appropriate fluorescent ligands.

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¹ E. S. Wu. Personal communication.

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