Nile Red: A Selective Fluorescent Stain for Intracellular Lipid Droplets

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ABSTRACT   We report that the dye nile red, 9-diethylamino-5H-benzo[a]phenoxazine-5-one, is an excellent vital stain for the detection of intracellular lipid droplets by fluorescence microscopy and flow cytofluorometry. The specificity of the dye for lipid droplets was assessed on cultured aortic smooth muscle cells and on cultured peritoneal macrophages that were incubated with acetylated low density lipoprotein to induce cytoplasmic lipid overloading. Better selectivity for cytoplasmic lipid droplets was obtained when the cells were viewed for yellow-gold fluorescence (excitation, 450–500 nm; emission, > 528 nm) rather than red fluorescence (excitation, 515–560 nm; emission, > 590 nm). Nile red–stained, lipid droplet–filled macrophages exhibited greater fluorescence intensity than did nile red–stained control macrophages, and the two cell populations could be differentiated and analyzed by flow cytofluorometry. Such analyses could be performed with either yellow-gold or red fluorescence, but when few lipid droplets per cell were present, the yellow-gold fluorescence was more discriminating.

Nile red exhibits properties of a near-ideal lysochrome. It is strongly fluorescent, but only in the presence of a hydrophobic environment. The dye is very soluble in the lipids it is intended to show, and it does not interact with any tissue constituent except by solution. Nile red can be applied to cells in an aqueous medium, and it does not dissolve the lipids it is supposed to reveal.

The formation of cytoplasmic lipid droplets is a normal cellular process. The droplets are neutral lipids, usually triacylglycerols or cholesteryl esters. The former serve as fatty acid energy reserves and the latter function as storage depots for excess cellular cholesterol (1–3). Abnormal accumulation of the cytoplasmic lipid droplets occurs in a variety of pathological conditions. Flow cytofluorometric analysis and fluorescence microscopy are powerful techniques for the analysis of a number of cellular processes. But fluorescent dyes have been lacking that would permit the application of these techniques for the study of lipid droplet formation in cells under normal physiological conditions and of lipid overloading of cells in pathological states (4).

We examined a variety of coloring agents for this purpose and found that nile red (Fig. 1) is an excellent stain. The dye is present as a minor component of commercial preparations of the nonfluorescent lipid stain nile blue (5). Nile red is intensely fluorescent and, if proper spectral conditions are chosen, it can serve as a sensitive vital stain for the detection

of cytoplasmic lipid droplets.

Smith introduced the use of nile blue in 1907 for the histochemical detection of tissue lipids (6, 7). He found that nile blue and similar dyes of the phenoxazine series had the remarkable property of simultaneously staining acid lipids blue and neutral lipids red. In the same year, Thorpe (8) examined the composition of nile blue and other blue phenoxazine dyes and found that they all contained various proportions of oxidation products called phenoxazones. The latter are nonionic and are bright red or yellow. Thorpe demonstrated that phenoxazines could be converted to phenoxazones by boiling in dilute sulfuric acid. Based on Thorpe's observations, Smith correctly deduced that the unusual metachromatic properties of nile blue were the result of two processes: (a) binding of the basic nile blue to fatty acids to form a soap and (b) dissolution of the phenoxazone nile red into neutral lipid droplets (7, 9). The specificity of nile blue staining of lipids has since been studied in detail by Kaufman and Lehmann (10), Lison (11, 12), Cain (13), Lillie (14), and

Dunnigan (15). Both Thorpe (8) and Möhlau and Uhlmann (16), who first synthesized the phenoxazine dyes and their phenoxazone products in 1896, noted the intense fluorescence of the phenoxazones. However, the usefulness of nile red or other phenoxazones as fluorescent lipid stains has received little attention.

We record here our investigations on the suitability of nile red as a fluorescent lysochrome for coloring cytoplasmic lipid droplets. We further demonstrate the use of this dye in flow cytofluorometric analysis of lipid-laden cells. A preliminary account of this work has been presented (17).

MATERIALS AND METHODS

Chemicals

We purchased nile blue chloride (CI 51180, dye content ~95%) and bovine serum albumin (essentially fatty acid free) from Sigma Chemical Co. (St. Louis, MO). The serum albumin was further purified by charcoal treatment to remove fatty acids (18). Filipin was a gift from the Upjohn Co. (Kalamazoo, MI). Other chemicals were analytical reagent grade and were obtained either from Fisher Scientific Co. (Fair Lawn, NJ) or Sigma Chemical Co.

Preparation of Nile Red

We prepared nile red from nile blue by dissolving the latter in 0.5% H_2SO_4 and boiling it under reflux for 2 h (8). (Because of the heterocyclic nature of nile red, we handled it as a carcinogen, though no evidence exists that such is the case.) After cooling, nile red was separated by repeated extraction into xylene. The organic solvent was then removed by flash evaporation. Nile red was identified on the basis of ultraviolet and infrared spectra (19, 20). The preparation appeared to be > 98% pure based on high-pressure liquid chromatography in three solvents.

Isolation of Lipoproteins

We isolated lipoproteins from human plasma by sequential ultracentrifugation in KBr (21). The lipoproteins were exhaustively dialyzed against saline containing 0.01% EDTA, pH 7.4. The purity of the preparation was confirmed by cellulose acetate gel electrophoresis. Low density lipoprotein was acetylated with repeated additions of acetic anhydride as described by Goldstein et al. (22) and dialyzed against 150 mM NaCl-0.01% EDTA, pH 7.4, at 4°C for 24 h. The acetylated low density lipoprotein migrated to a more cathodic position than native low density lipoprotein when electrophoresed in cellulose acetate gels. Oligolamellar lipid vesicles were kindly provided by Dr. Carl T. Bauguess (College of Pharmacy, University of South Carolina, Columbia, SC). They were prepared as described by D'Silva and Notari (23), and most of the vesicles ranged in size from 0.1 to 1 μm.

Preparation of Microsomal Membranes

A microsomal (P) fraction was prepared from a rat liver homogenate as described by deDuve et al. (24). Ribosomes and peripheral proteins were removed by carbonate treatment (25).

Cell Cultures

SMOOTH MUSCLE CELLS: Subcultures of smooth muscle cells were generated from cultures generously provided by Dr. Richard St. Clair (Bowman Gray School of Medicine, Winston-Salem, NC). The cells were derived initially from tissue explants of an adult Rhesus (*Macaca mulatta*) monkey aorta (26). The subcultures were grown in modified Eagle's medium containing antibiotics and 10% (vol/vol) calf serum (27). Cells of passages 15 to 18, grown to confluence, were used for the staining studies. The cultures in our laboratory exhibited the typical growth behavior and morphologic appearance of smooth muscle cells.

MACROPHAGES: We harvested peritoneal cells in saline from unstimulated mice as described by Edelson and Cohn (28). Pooled cells from several animals were collected by centrifugation (400 g, 10 min), washed once in Dulbecco's modified Eagle's medium, and then resuspended in the same medium containing antibiotics and 10% (vol/vol) fetal calf serum. Aliquots of the cells were then dispensed in plastic petri dishes and incubated in a humidified CO_2 (5%) incubator at 37°C for 2 h. We removed nonadherent cells by rinsing the cultures in medium without serum, and induced cytoplasmic lipid

droplets by incubating the adherent cells with acetylated low density lipoprotein (50 μg protein/ml) in 10% fetal calf serum for 40 h as described by Goldstein, Brown and colleagues (22, 29). Control macrophage cultures were incubated for a similar period in the presence of 10% fetal calf serum only. In all experiments the cultures were washed first with phosphate-buffered saline (PBS) containing 2 mg/ml albumin and then washed with PBS alone. For flow cytofluorometric analysis the cells were harvested with a rubber policemen and resuspended in PBS.

Nile Red Staining

Stock solutions of nile red (10, 100, or 1,000 μg/ml) in acetone were prepared and stored protected from light. Staining could be carried out on either fixed (1.5% glutaraldehyde, 5 min) or unfixed cells. Isolated cells (1-2 × 10^6/ml) were suspended in PBS; attached cultured cells were covered with PBS. The dye was then added directly to the preparation to effect a 1:100 dilution, and the preparation was incubated for a minimum of 5 min. For flow cytofluorometry, we analyzed the samples immediately, whereas for fluorescence microscopy we removed excess dye by brief rinsing in PBS. Concentrations of dye used for individual experiments are indicated in the figure legends. The suspension medium should not contain serum or albumin since they could act as a sink to draw nile red out of the cells. Dye in the medium does not hinder observation as nile red fluorescence is quenched in water.

Fluorescence Microscopy

We carried out the fluorescence microscopy studies with a Zeiss Universal Photomicroscope (Carl Zeiss, Inc., Thornwood, NY) equipped for epi-illumination using an HBO 200 high pressure mercury light source, and with 25× and 63× plan neofluar objective lenses. We viewed nile red fluorescence at two spectral settings: yellow-gold fluorescence, using a 450–500 nm band pass exciter filter, a 510-nm center wavelength chromatic beam splitter, and a 528-nm-long pass barrier filter (Zeiss filter set 48-77-11); and red fluorescence using a 515–560 nm band pass exciter filter, a 580-nm center wavelength chromatic beam splitter, and a 590-nm-long pass barrier filter (Zeiss filter set 48-77-14). The stained cells were photographed in color on Kodak Ektachrome film (400 ASA) or in black and white on Kodak Tri-X pan film (400 ASA). Exposures were 8–12 s with exposure meter reciprocity set at 10.

Fluorescence Spectroscopy

Excitation and emission fluorescence spectra were determined with a Perkin-Elmer 650-40 fluorescence spectrophotometer (Perkin-Elmer Corp., Norwalk CT). Except as indicated, spectra were recorded at room temperature with both slits set at 5 nm. The spectra presented were partially corrected by use of the ratio quantum counter system (ratio mode). In some instances, the spectra are shown plotted as wavelength versus normalized fluorescence intensity in which the y-axis scale units have been adjusted to place maximum peak heights at 70% of full chart scale. The relative fluorescence intensity of nile red in the presence of the various samples was obtained after subtraction of both the autofluorescence of the samples and the fluorescence intensity of nile red alone in buffer.

Flow Cytofluorometry

Nile red–stained or unstained control cells were analyzed with a Coulter EPICS V flow cytometer (Coulter Electronics, Inc., Hialeah, FL) equipped with a tunable 4W argon-ion laser (model 164-05; Spectra-Physics Inc., Mountain View, CA) and light scatter and fluorescence sensors. The flow cytometer was interfaced with a Coulter MDADS computer system for six-parameter data acquisition and analysis. Nile red fluorescence detection was carried out with the laser tuned at 488 nm and set at 150–180 mW. We analyzed fluorescence emission simultaneously at two spectral settings by employing a 515-nm-long pass barrier filter to block stray laser light, and a 590-nm center wavelength dichroic mirror to split the incoming emitted light, and by placing a 560-nm short pass filter (yellow-gold fluorescence) and a 610-nm-long pass filter (red fluorescence) in front of the respective fluorescence detectors. A 1.3 neutral density filter was placed before the forward light scatter detector. We carried out all analyses at room temperature and just before counting the cell suspension passed it through a 70-μm mesh nylon screen (Tetko Inc., Elmsford, NY) to eliminate occasional cell clumps.

All fluorescence data collected were gated on forward angle light scatter. We used particles of various diameters (5, 10, 14.6, and 22 μm) to calibrate the instrument. The light scatter threshold used for triggering signal acceptance was set at 7.5 μm. 10-μm diam full bright fluorospheres (Coulter Electronics, Inc.) were used to standardize fluorescence intensity for each study. Photomultiplier

gains were adjusted to place the mode fluorescence signal from the particles in a set standard channel. Autofluorescence of unstained cells was subtracted from the fluorescence profile of the nile red–treated cells. We analyzed at least 10,000 cells for each experiment.

The flow cytofluorometric analyses are presented in the form of frequency distribution histograms. The abscissa is given as the log relative fluorescence intensity, indicating that the fluorescence signal was amplified logarithmically before analog-to-digital conversion.

RESULTS

Chemical and Physical Properties of Nile Red

Nile red is a benzophenoxazone dye (Fig. 1). In older chemical and histochemical literature the dye is sometimes referred to as nile blue A-oxazone. Nile red is poorly soluble in water but it does dissolve in a wide variety of organic solvents (partition coefficients of nile red in organic solvents relative to water are ~200) (Greenspan, P., and S. D. Fowler, manuscript submitted for publication). The dye is intensely fluorescent in all organic solvents. Depending upon the relative hydrophobicity of the solvent, the excitation and emission maxima of nile red fluorescence can vary over a range of 60 nm (Fig. 2); the fluorescence colors range from golden yellow to deep red. Measured at the respective fluorescence maxima, the relative fluorescence intensities of the dye are approximately equal in all organic solvents. In contrast, the fluorescence of the dye is quenched in water. Thus, nile red exhibits the properties of a fluorescent hydrophobic probe (30).

Use of Fluorochromic Properties of Nile Red to Detect Lipids and Hydrophobic Domains

The potential usefulness of nile red as a fluorescent lipid stain is illustrated in Tables I and II. The relative fluorescence
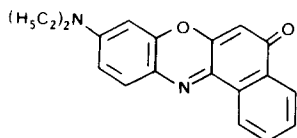
FIGURE 1 Chemical structure of nile red. The dye is hydrophobic and intensely fluorescent.
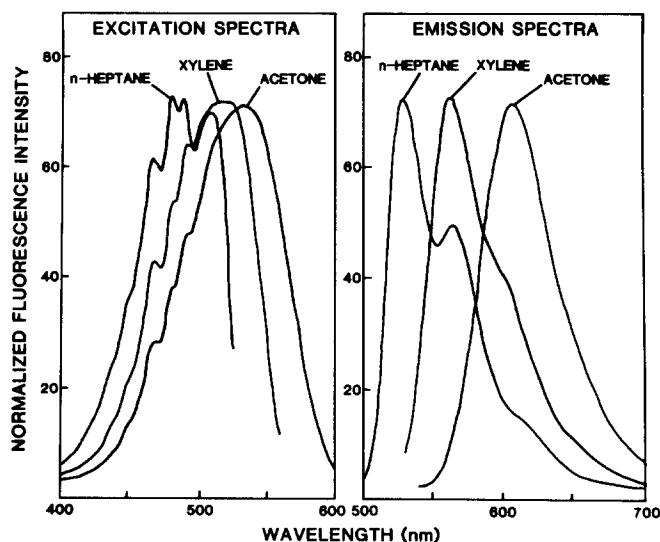
FIGURE 2 Excitation and emission fluorescence spectra of nile red in n-heptane, xylene, and acetone. A 1 μg/ml solution of nile red in each solvent was used. The excitation and emission spectra of nile red were recorded at their corresponding emission or excitation maxima.

TABLE I

Nile Red Fluorescence Intensity in Organic Solvents When Measured at Excitation Wavelengths of 487–489 nm and Emission Wavelengths of 530–550 nm

Solvent	Relative fluorescence intensity (0.1 μg nile red/ml)
n-Heptane	221
Xylene	99
Chloroform	4
Acetone	3
Ethanol	0.1

The excitation wavelength was set at 488 nm with a 2-nm slit width; the emission wavelength was set at 540 nm with a 20-nm slit width. These settings correspond to available filter combinations for fluorescence microscopy and flow cytofluorometry.

TABLE II

Nile Red Fluorescence Intensity in the Presence of Proteins When Measured at Excitation Wavelengths of 487–489 nm and Emission Wavelengths of 530–550 nm

	Relative fluorescence intensity	
Protein*	0.166 μg nile red/ml	1.66 μg nile red/ml
Very low density lipoprotein	44	206
Low density lipoprotein	33	111
High density lipoprotein	12	17
Albumin	0.2	0.5
Hepatic microsomal membranes	4	10

5 μl of an acetone solution of nile red (0.1 or 1 mg/ml) was mixed with 3 ml of the protein solution, and the fluorescence spectra were recorded. The excitation wavelength was set at 488 nm with a 2-nm slit width; the emission wavelength was set at 540 nm with a 20-nm slit width. These settings correspond to available filter combinations for fluorescence microscopy and flow cytofluorometry.
* With the exception of heptic microsomal membranes, proteins were diluted to a concentration of 100 μg/ml with PBS. Hepatic microsomal membranes were diluted to the same concentration with PBS containing 12% metrizamide.

intensity of the dye in different organic solvents, and in the presence of lipoproteins or isolated membranes was measured at fixed excitation and emission wavelengths. The wavelengths chosen (487–489 and 530–550 nm) approximate the excitation and emission maxima of the most nonpolar solvents (Fig. 2). As shown in Table I, the relative fluorescence intensity of nile red in n-heptane is nearly 80-fold greater than that in acetone and ~2,000-fold greater than that in ethanol. We observed similar results for the fluorescence intensity of nile red in the presence of the various proteins (Table II): the fluorescence intensity observed in the presence of very low density lipoprotein is 10- to 20-fold greater than in the presence of hepatic microsomal membranes and is 200- to 400-fold greater than that found with defatted albumin. The latter studies demonstrate that nile red will fluoresce in the confines of a lipid hydrophobic environment. This is observed even in the presence of an aqueous medium.

Fluorescence Microscopy of Nile Red–stained Cells

Brief treatment of cultured cells with a dilute aqueous solution of nile red produces an intense fluorescent staining of cytoplasmic components (Fig. 3). The staining can be carried out on either fixed or unfixed cells with no apparent
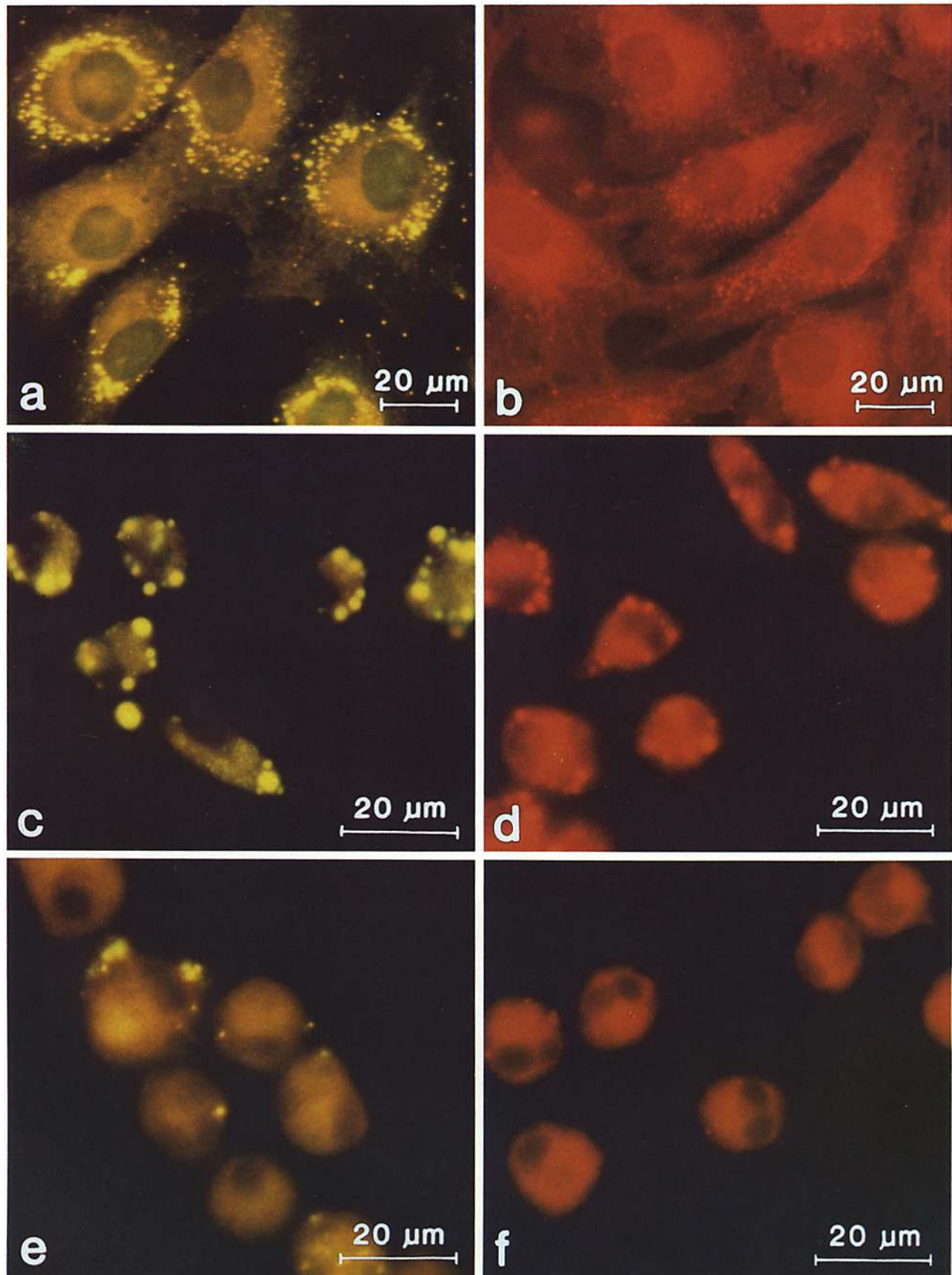
FIGURE 3   Nile red fluorescence of cultured monkey aortic smooth muscle cells and mouse peritoneal macrophages. (a and b) Fluorescence of aortic smooth muscle cells treated with nile red. (c and d) Fluorescence of nile red–stained peritoneal macrophages previously incubated 40 h with acetylated low density lipoprotein. (e and f) Fluorescence of control peritoneal macrophages treated with nile red. Before they were stained, the cells were fixed with 1.5% glutaraldehyde in PBS and then washed with buffered saline. Nile red concentration, 100 ng/ml. (a, c, and e) Excitation wavelength, 450–500 nm; emission wavelength, > 528 nm. (b, d, and f) Excitation wavelength, 515–560 nm; emission wavelength > 590 nm. (a and b) × 660; (c–f) × 1,040.

difference in distribution or intensity of fluorescence. The photographs presented in Fig. 3 show the appearance of the stained cells viewed for yellow-gold fluorescence (excitation wavelength, 450–500 nm; emission wavelength, > 528 nm) and for red fluorescence (excitation wavelength, 515–560 nm; emission wavelength > 590 nm). Viewed for yellow-gold fluorescence (Fig. 3a), the nile red–stained cultured smooth muscle cells exhibit numerous small discrete bodies distributed throughout the cytoplasm, although most are clustered around the dark nonfluorescent nucleus. Viewed for red fluorescence (Fig. 3b), the cultured smooth muscle cells reveal the same intensely stained structures, but the individual bodies are not as readily resolved. In addition, a diffuse general staining of the cytoplasm becomes more apparent. The latter is distributed as an extensive reticulum which may represent intracellular membranes and organelles.

Fig. 3, c–f illustrates the nile red staining obtained with cultured macrophages in which intracellular lipid droplets could be induced by incubation of the cells with acetylated low density lipoprotein (22, 29). After staining with nile red, the cholesteryl ester and triacylglycerol rich cells (Fig. 3, c and d) are seen to have large (up to 5 μm) fluorescent, spherical cytoplasmic structures. Virtually all of the cells in the culture have these structures, although in various numbers per cell. The control cells not incubated with acetylated low density lipoprotein (Fig. 3, e and f), in contrast, have few of these spherical fluorescent bodies and they are always relatively smaller. In fact, many of the control cells show none of them at all. When the nile red–stained cultured macrophages are viewed for red fluorescence (Fig. 3, d and f), the lipid droplets are less apparent. This is due in part to the much more intense reticular staining of the cytoplasm. No significant autofluorescence was observed when unstained cultured macrophages or smooth muscle cells were viewed for yellow-gold or red fluorescence. As further evidence for the preferential detection of lipid droplets by nile red when viewed by yellow-gold fluorescence, lipid-loaded, nile red–stained macrophages were viewed by both phase-contrast and fluorescence microscopy. As can be seen in Fig. 4, a and b, the fluorescent bodies directly correspond to the refractile lipid droplets seen by phase-contrast microscopy. Furthermore, extraction of the macrophages previously incubated with acetylated low density lipoprotein with hexane/isopropanol (3:2, vol/vol) to remove intracellular lipid resulted in the disappearance of the fluorescent bodies (Fig. 4c). This solvent mixture does not extract proteins (31). That a dull diffuse staining of the cells is still present after the cellular extraction of lipids, is presumably a reflection of the remaining hydrophobic domains of cellular proteins.

Application of Nile Red Staining to Flow Cytofluorometry

The lipid droplet–loaded macrophage cultures employed for the fluorescence microscopy studies above were also used to assess the suitability of nile red as a reagent for flow cytofluorometric identification of lipid droplets within cells. Fig. 5 shows the nile red emission spectra, with 488-nm excitation light, obtained for control macrophages and for macrophages incubated with acetylated low density lipoprotein. At 570 nm, we found a nearly fourfold increase in nile red fluorescence intensity in acetylated low density lipoprotein treated cells as compared with control cells. We examined both yellow-gold and red fluorescence intensity distributions in the flow cytofluorometric studies. The spectral window settings used, 515–560 or > 610 nm, determined by available filter sets for the flow cytometer, are represented by the shaded areas of the emission spectra shown in Fig. 5.

The results of flow cytofluorometric analysis of nile red–stained macrophages are presented in Fig. 6. The lipid droplet formation induced by incubation of the cultures with acetylated low density lipoprotein varies significantly among preparations (29, 32). The experiments presented represent the acetylated low density lipoprotein treated cultures exhibiting, respectively, the least and the most extensive loading of lipid droplets in our series of studies, as judged by fluorescence microscopy. The histograms show that the lipid-loaded cells could be differentiated by their increased fluorescence intensity relative to control cells whether analyzed for yellow-gold or red fluorescence. Some overlap of the fluorescence intensity
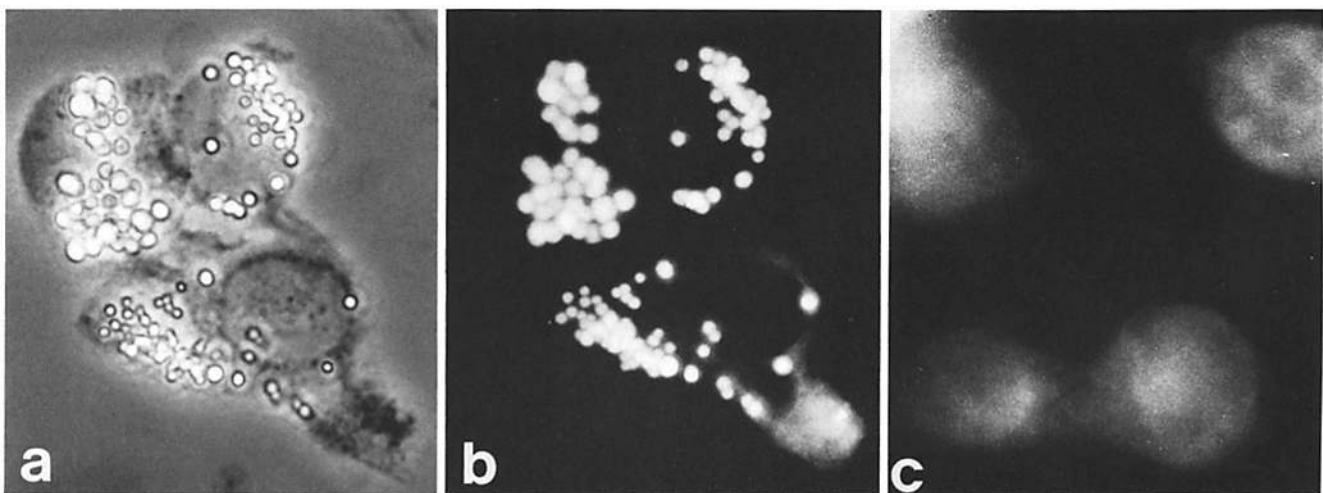

Figure 4 Phase-contrast microscopy and nile red fluorescence of lipid-loaded macrophages. (a and b) Appearance of nile red-stained lipid-loaded macrophages viewed by phase-contrast or fluorescence microscopy. (c) Fluorescence of lipid-loaded macrophages previously treated with hexane/isopropanol (3:2, vol/vol) to extract cellular lipids. The cells were then stained with nile red (100 ng/ml). (b and c) Excitation wavelength, 450–500 nm; emission wavelength, > 528 nm. c was overexposed to permit better cell definition. × 1,040.

distributions of the two cell populations is seen and should be expected since a few cells in the control cultures exhibit lipid droplets in numbers comparable to those found in the least lipid laden cells of acetylated low density lipoprotein treated cultures (see Fig 3). For cells with moderate lipid loading (experiment one), the overlap of the frequency profile with that of control cells is greater for red fluorescence than for yellow-gold fluorescence.

We also examined the influence of nile red concentration
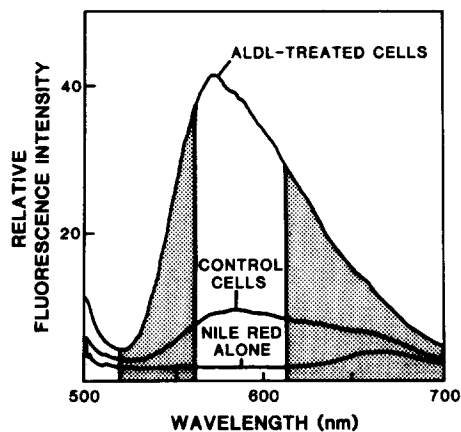
FIGURE 5   Emission fluorescence spectra of nile red-stained mouse peritoneal macrophages using an excitation wavelength of 488 nm. Cultured peritoneal macrophages were incubated for 40 h without (control cells) or with 50 µg protein/ml acetylated low density lipoprotein (ALDL-treated cells). The cells were washed, harvested, and stained with nile red as described in Materials and Methods and then suspended in PBS containing 12% metrizamide (to slow sedimentation of the cells). 5 µl of an acetone solution of nile red (100 µg/ml) was mixed with 3 ml of the cell suspension. The emission spectra were recorded at an excitation wavelength of 488 nm with slit widths at 5 nm. The samples were at equal protein concentrations. The shaded areas represent the two spectral window settings employed for the flow cytofluorometric studies.

on the fluorescence intensity distribution profiles of the cells. As Fig. 7 shows, a 10-fold increase in nile red concentration does not affect the yellow-gold fluorescence intensity of the acetylated low density lipoprotein–treated macrophages but it does increase the intensity per cell of red fluorescence. Apparently, under the conditions employed, the intracellular lipid droplets are saturated with nile red, but other cellular sites remain available for additional binding and interaction with the dye. A similar nile red saturation is observed with high density lipoprotein (Table II).

Since the red fluorescence of nile red is so intense, one must be cognizant of possible red spillover into the yellow-gold fluorescence measurements. (The spillover is a consequence of leaking of stray red light through the filter barriers.) To assess this, we examined nile red–stained oligolamellar lipid vesicles by flow cytofluorometry. (With 488-nm excitation light, the nile red fluorescence of these vesicles exhibits an emission maximum of 624 nm; the fluorescence intensity in the 515–560 nm range is no more than 14% of the intensity at the emission maximum.) Red spillover into the yellow-gold counter did occur with the photomultiplier tube settings used for the cell analyses above. The mode log relative fluorescence intensity occurred at channel 12, as compared with a mode channel of 60 for the same sample in log red fluorescence. If necessary, this spillover could be corrected by use of the flow cytometer's pulse subtraction mode for the yellow-gold counter and a blank of nile red–stained lipid vesicles, whose red fluorescence intensity is comparable to that of the cells being analyzed. In the study shown in Fig. 7, spillover does not appear to be significant, as indicated by a lack of change in yellow-gold fluorescence intensity, although the red fluorescence intensity shifted significantly.

DISCUSSION

This study demonstrates the utility of nile red as a stain to detect intracellular lipid droplets by fluorescence microscopy
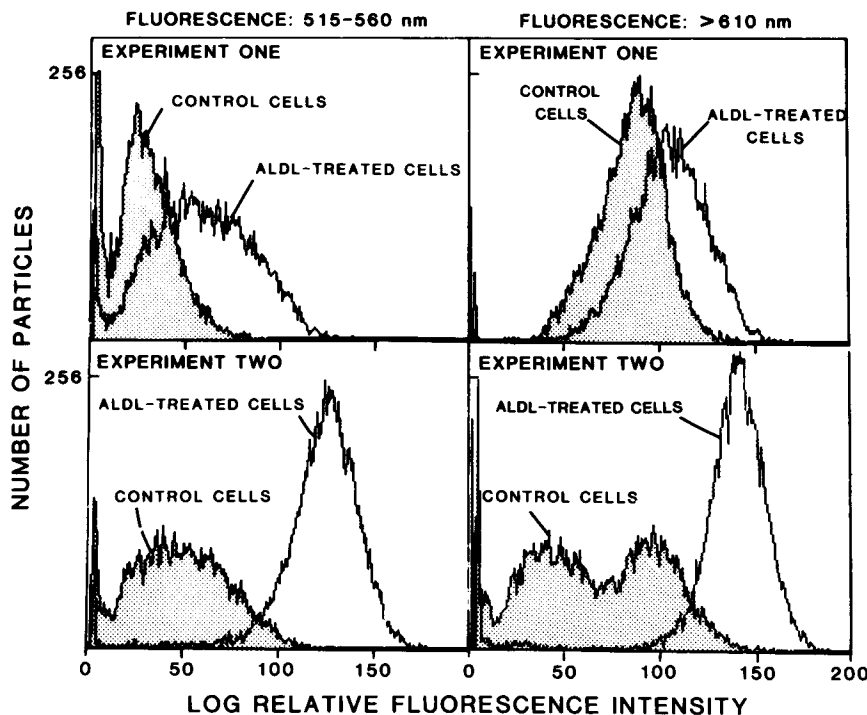

FIGURE 6   Flow cytofluorometric histograms of mouse peritoneal macrophages stained with nile red. Cultured peritoneal macrophages were incubated for 40 h without (control cells) or with 50 µg protein/ml acetylated low density lipoprotein (ALDL-treated cells). The cells were washed, harvested, and stained with 100 ng/ml nile red as described in Materials and Methods. Experiments one and two were performed with ALDL-treated cells exhibiting, respectively, moderate or extensive intracellular lipid droplets, as judged by fluorescence microscopy. Cell fluorescence was measured at a laser excitation wavelength of 488 nm and emission wavelengths of either 515–560 nm (left) or > 610 nm (right).

and flow cytofluorometry. By examination of the fluorescence of nile red–stained cells at wavelengths of ≤570 nm, the fluorescence of nile red interacting with extremely hydrophobic environments (i.e., neutral lipid droplets) is detected preferentially whereas the fluorescence of nile red interacting with cellular membranes is minimized. Under these spectral conditions, the fluorescence of nile red in the presence of microsomal membranes is approximately one-tenth of that observed for lipoproteins bearing large hydrophobic cores of lipid (Table II). It is, nonetheless, the interaction of nile red with cellular membranes that probably gives rise to the observed low intensity background orange fluorescence observed in control macrophages and smooth muscle cells. Nile red staining loses its selectivity for lipid droplets when viewed at fluorescence emission wavelengths >590 nm. Under these spectral conditions, nile red can be thought to be a general stain for lipids since we found that the dye can interact and fluoresce in the presence of phospholipid, cholesterol, cholesteryl esters, and triacylglycerols (Greenspan, P., and S. D. Fowler, manuscript submitted for publication). However,

some caution must be used in interpretation of staining patterns, since nile red also fluoresces in the presence of fatty acid-free albumin, which indicates that proteins containing hydrophobic domains, in addition to lipids, can induce nile red fluorescence.

The dye nile blue has been commonly used as a lipid stain (33). The traces of nile red in commercial preparations of nile blue provide the metachromatic coloring of neutral lipids for which the procedure of Smith and Mair (9) is known. The synthesis of nile red itself and its direct application as a fluorescent probe for lipids appears not to have been done previously, although there are reports on the fluorescence of nile blue–stained tissues (34–36). These reports attribute this fluorescence to be possibly due to the presence of nile red in the commercial nile blue dye. Recently, nile red has been used as a fluorescent laser dye because of its high photochemical stability (37).

In our search for a fluorescent reagent to detect cytoplasmic lipid droplets, we investigated a variety of possible stains (Table III). Nile red proved to be superior to all of the dyes
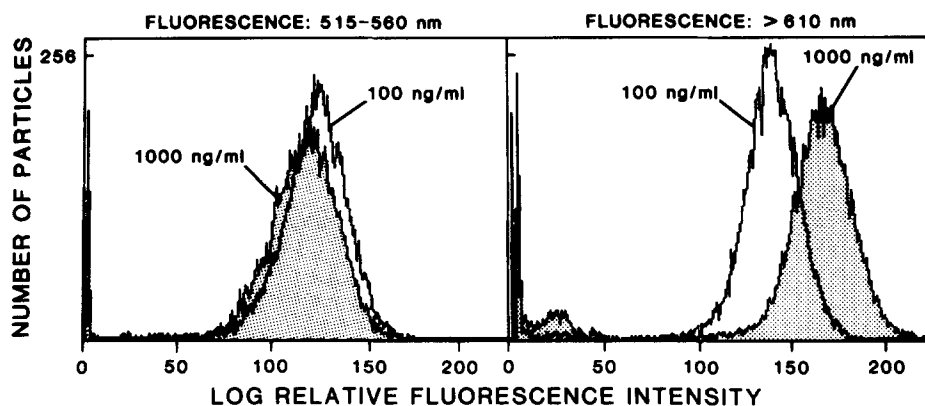

FIGURE 7 Influence of nile red concentration on fluorescence of mouse peritoneal macrophages previously incubated for 40 h with acetylated low density lipoprotein. The cultured cells were washed and harvested as described in Materials and Methods and stained with 100 or 1,000 ng/ml nile red. The flow cytofluorometric histograms show the relative fluorescence intensities of cells when measured at a laser excitation of 488 nm and emission wavelengths of either 515–560 or > 610 nm.

TABLE III

Utility of Various Fluorescent Dyes as a Lipid Stain

Dye	Fluorescence of dyes in the presence of low density lipoprotein* (relative fluorescence intensity)	Staining of lipid-loaded macrophages[+]
Nile red	242	Intense staining with excellent definition of lipid droplets
Benz(a)pyrene[§]	10	Weak blue fluorescence but good definition of lipid droplets
Filipin[¶]	0	Weak uniform fluorescence; good definition with fixed cells (39). Reaction specific only for cholesterol
Dil[‖]	14	None
NBD-aminododecanoic acid**	25	Moderate cell staining, but poor definition of lipid droplets
NBD-MANC[‡‡]	7	None
Rhodamine B and 6G	20, 0 (high fluorescence of dye in absence of lipoprotein)	Cells stained intensely red but lipid droplets not seen

Except as indicated, all dyes were prepared as stock solutions in acetone (1 mg/ml).
* Low density lipoprotein (100 μg protein/ml) was mixed with dye (1 μg/ml) and incubated for 10 min. The fluorescence spectra were recorded and the relative fluorescence intensity was measured at excitation and emission maxima, and the contribution of dye and protein fluorescence controls was subtracted.
[+] Lipid-loaded cells were prepared by incubation of mouse peritoneal macrophages with acetylated low density lipoprotein (10 μg protein/ml) for 3 d. Dyes (1 μg/ml in PBS) were added and the cells were incubated 5–10 min at room temperature. Staining was then assessed by fluorescence microscopy with the appropriate filter package.
[§] Stock solution: 20 μg/ml saturated caffeine solution (38).
[¶] Stock solution: 1 mg/ml dimethylformamide.
[‖] 1,1′-dioctadecyl-3,3,3′,3′-tetramethylendocarboxyamine.
** 12-(N-methyl-N-(7-nitro-benz-2-oxa-1,3-diazol-4-yl)) aminododecanoic acid.
[‡‡] 25-(N-methyl-N-(7-nitro-benz-2-oxa-1,3-diazol-4-yl))-27-norcholesterol.

examined. Most fluorescent hydrophobic dyes, such as the rhodamines and some nitrobenzoxadiazol derivatives, uniformly stained cells without distinguishing cytoplasmic lipid droplets. Benz(a)pyrene prepared in caffeine as described by Berg (38) stained cytoplasmic droplets clearly but required excitation in the ultraviolet (not convenient for standard flow cytofluorometry), and the blue fluorescence was weak. Further, benz(a)pyrene is highly carcinogenic. The fluorescent antibiotic filipin is used as a fluorescent stain for cholesterol (39–41) and its use in flow cytometry has just been reported (42). However, filipin stains cholesterol, not triacylglycerols, and the use of fixed cells is required. Lipoproteins have been successfully labeled with 1,1'-dioctadecyl-3,3,3',3'-tetra-methylendocarboxyamine (DiI) (43), but we found that this dye did not produce detectable staining of living cells.

Baker defined lysochromes as dyes used to color tissues by dissolving in them (44). He described the ideal lysochrome as being (a) strongly colored; (b) very soluble in substances it is intended to show; (c) unable to interact with any tissue constituent except by solution; and (d) able to be presented to the tissues in a solvent with the following characteristics: the solvent will not dissolve the substances the lysochrome is intended to show, and the lysochrome is much less soluble in the solvent than in these substances. Nile red is a stain that meets these conditions remarkably well. In particular, the dye can be applied to cells in an aqueous solvent and it dissolves preferentially in lipid. In addition, nile red has two other useful properties: its fluorescence is quenched in an aqueous environment so it is seen only in the substances it is intended to stain, and its fluorescence color changes in the presence of very hydrophobic lipids. In our experience, nile red provides resolution of cytoplasmic lipid droplets in tissues equal to, if not better than, that obtained with the nonfluorescent dye oil red O (Fowler, S. D., and P. Greenspan, manuscript submitted for publication).

Flow cytofluorometric analysis offers a unique approach to the examination of the accumulation of lipid droplets within individual cells. As seen in the fluorescence micrographs shown in Fig. 3, the number of lipid inclusions in control macrophages is not uniform; certain cells may have none whereas others may be filled with numerous lipid droplets. Similarly, when macrophages are incubated with acetylated low density lipoprotein, there is a vast diversity in the number of lipid droplets found in each cell. The use of flow cytofluorometry extends these visual observations (Fig. 6), permitting the counting of thousands of cells per minute. The results from the flow cytofluorometric studies presented also indicate that certain control macrophages treated with nile red exhibit a fluorescence intensity that approximates that of acetylated low density lipoprotein treated macrophages. Through the use of nile red and flow cytofluorometry, it is now possible to select and collect those cells containing the greatest number of lipid inclusions and to study the biochemical basis for their excessive cellular lipid loading.

Note Added in Proof: We have been informed that Kodak Laboratory & Specialty Chemicals (Rochester, NY), Molecular Probes, Inc. (Junction City, OR), and Lambda Physik GmbH (Gottingen, Federal Republic of Germany) now have or will soon market nile red for investigational use.

REFERENCES

1. Renold, A. E., and G. F. Cahill, Jr., editors. 1965. Adipose tissue. *In* Handbook of Physiology. American Physiological Society, Washington, DC. 824 pp.
2. Goldstein, J. L., and M. S. Brown. 1977. The low-density lipoprotein pathway and its relation to atherosclerosis. *Annu. Rev. Biochem.* 46:897–930.
3. Brown, M. S., and J. L. Goldstein. 1983. Lipoprotein metabolism in the macrophage: implications for cholesterol deposition in atherosclerosis. *Annu. Rev. Biochem.* 52:223–261.
4. Kruth, H. S. 1982. Flow cytometry: rapid biochemical analysis of single cells. *Anal. Biochem.* 125:225–242.
5. Conn, H. J. 1953. Biological Stains. Sixth ed. Williams & Wilkins, Baltimore. 367 pp.
6. Smith, J. L. 1907. On the simultaneous staining of neutral fat and fatty acids by oxazine dyes. *J. Pathol. Bacteriol.* 12:1–4.
7. Smith, J. L. 1910. The staining of fat by Nile-Blue sulphate. *J. Pathol. Bacteriol.* 15:53–55.
8. Thorpe, J. F. 1907. A reaction of certain colouring matters of the oxazine series. *J. Chem. Soc.* 91:324–336.
9. Smith, J. L., and W. Mair. 1911. Fats and lipoids in relation to methods of staining. *Skand Arch. Physiol.* 25:247–255.
10. Kaufman, C., and E. Lehmann. 1926. Sind die in der histologischen technik gebräuchlichen fettdifferenzierungsmethoden spezifisch? *Virchows Arch.* 261:623–648.
11. Lison, L. 1935. Sur le mécanisme et la signification de la coloration des lipides par le bleu de Nil. *Bull. Histol. Appl.* 12:279–289.
12. Lison, L. 1935. Etudes sur la métachromasie colorants métachromatiques et substances chromotropes. *Arch. Biol.* 46:599–668.
13. Cain, A. J. 1947. The use of nile blue in the examination of lipoids. *Quart. J. Microsc. Sci.* 88:383–392.
14. Lillie, R. D. 1956. The mechanism of nile blue staining of lipofuscins. *J. Histochem. Cytochem.* 4:377–381.
15. Dunnigan, M. G. 1968. The use of nile blue sulphate in the histochemical identification of phospholipids. *Stain Technol.* 43:249–256.
16. Möhlau, R., and K. Uhlmann. 1896. Zur kenntniss der chinazin-und oxazinfarbstoffe. *Liebigs Ann. Chem.* 289:90–130.
17. Greenspan, P., E. P. Mayer, and S. D. Fowler. 1984. Use of nile red in fluorescence microscopy and flow cytometer analysis of intracellular lipid loading. *J. Cell Biol.* 99(4, Pt. 2):58a.
18. Chen, R. F. 1967. Removal of fatty acids from serum albumin by charcoal treatment. *J. Biol. Chem.* 242:173–181.
19. Davis, M. M., and H. B. Hetzer. 1966. Titrimetric and equilibrium studies using indicators related to nile blue A. *Anal. Chem.* 38:451–461.
20. Stužka, V., V. Šimánek, and Z. Stránský. 1967. Infra-red spectroscopy of benzo-(a)phenoxazines. *Spectrochim. Acta. Part A Mol. Spectrosc.* 23:2175–2183.
21. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* 34:1345–1353.
22. Goldstein, J. L., Y. K. Ho, S. K. Basu, and M. S. Brown. 1979. Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. *Proc. Natl. Acad. Sci. USA.* 76:333–337.
23. D'Silva, J. B., and R. E. Notari. 1982. Drug stability in liposomal suspensions: hydrolysis of indomethacin, cyclocytidine, and p-nitrophenyl acetate. *J. Pharm. Sci.* 71:1394–1398.
24. deDuve, C., B. C. Pressman, R. Gianetto, R. Wattiaux, and F. Appelmans. 1955. Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat liver tissue. *Biochem. J.* 60:604–617.
25. Fujiki, Y., A. L. Hubbard, S. Fowler, and P. B. Lazarow. 1982. Isolation of intracellular membranes by means of sodium carbonate treatment: application to endoplasmic reticulum. *J. Cell Biol.* 93:97–102.
26. St. Clair, R. W., B. P. Smith, and L. L. Wood. 1977. Stimulation of cholesterol esterification in rhesus monkey arterial smooth muscle cells. *Circ. Res.* 40:166–173.
27. Fowler, S., H. Shio, and H. Wolinsky. 1977. Subcellular fractionation and morphology of calf aortic smooth muscle cells. Studies on whole aorta, aortic explants, and subcultures grown under different conditions. *J. Cell Biol.* 75:166–184.
28. Edelson, P. J., and Z. A. Cohn. 1976. Purification and cultivation of monocytes and macrophages. *In In Vitro* Methods in Cell-mediated and Tumor Immunity. B. R. Bloom and J. R. David, editors. Academic Press, New York. 333–340.
29. Brown, M. S., J. L. Goldstein, M. Krieger, Y. K. Ho, and R. G. W. Anderson. 1979. Reversible accumulation of cholesteryl esters in macrophages incubated with acetylated lipoproteins. *J. Cell Biol.* 82:597–613.
30. Lakowicz, J. R. 1983. Principles of fluorescence spectroscopy. Plenum Press, New York. 496 pp.
31. Hara, A., and N. S. Radin. 1978. Lipid extraction of tissues with a low-toxicity solvent. *Anal. Biochem.* 90:420–426.
32. Falcone, D. J., N. Mateo, H. Shio, C. R. Minick, and S. D. Fowler. 1984. Lipoprotein-

heparin-fibronectin-denatured collagen complexes enhance cholesteryl ester accumulation in macrophages. *J. Cell Biol.* 99:1266–1274.

33. Adams, C. W. M., and O. B. Bayliss. 1975. Lipid histochemistry. *In* Techniques of Biochemical and Biophysical Morphology, Vol. 2. D. Glick and R.. M. Rosenbaum, editors. Wiley-Interscience, New York. 99–156.

34. Mix, M. 1959. Zum mechanismus der sphärosomenfluoreszenz mit oxazinen und anderen basischen farbstoffen. *Protoplasma.* 50:434–470.

35. Bozzo, L., and B. D.-C. Vidal. 1968. Nile blue sulphate and brilliant cresyl blue in fluorescence methods for the histochemical demonstration of lipids. *Ann. Histochim.* 13:177–183.

36. Canete, M., M. J. Hazen, and J. C. Stockert. 1983. Nile blue sulfate staining for demonstration of lipids in fluorescence microscopy. *Acta Histochem. Cytochem.* 16:286–288.

37. Basting, D., D. Ouw, and F. P. Schäfer. 1976. The phenoxazones: a new class of laser dyes. *Opt. Commun.* 18:260–262.

38. Berg, N. O. 1951. Histological study of masked lipids. *Acta. Pathol. Microbiol. Scand.*
Suppl. 90:1–192.

39. McGookey, D. J., and R. G. W. Anderson. 1983. Morphological characterization of the cholesteryl ester cycle in cultured mouse macrophage foam cells. *J. Cell Biol.* 97:1156–1168.

40. Börning, H., and G. Geyer. 1974. Staining of cholesterol with the fluorescent antibiotic "filipin." *Acta Histochem.* 50:110–115.

41. Kruth, H. S. 1983. Filipin-positive, oil red O-negative particles in atherosclerotic lesions induced by cholesterol feeding. *Lab. Invest.* 50:87–93.

42. Muller, C. P., D. A. Stephany, D. F. Winkler, J. M. Hoeg, S. J. Demosky, Jr., and J. R. Wunderlich. 1984. Filipin as a flow microfluorometry probe for cellular cholesterol. *Cytometry.* 5:42–54.

43. Pitas, R. E., R. L. Innerarity, J. N. Weinstein, and R. W. Mahley. 1981. Acetoacetylated lipoproteins used to distinguish fibroblasts from macrophages *in vitro* by fluorescence microscopy. *Arteriosclerosis.* 1:177–185.

44. Baker, J. R. 1958. Principles of Biological Microtechnique. A Study of Fixation and Dyeing. Methuen & Co. Ltd. London. 357 pp.