

Cellular Dimensions Affecting the Nucleocytoplasmic Volume Ratio

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Abstract. Although it has long been appreciated that larger eukaryotic cells have larger nuclei, little is known about how this size relationship is maintained. Here we describe a method for measuring the aqueous volume ratio of nucleus to cytoplasm, two compartments which are interconnected via the pores in the nuclear envelope. We then use that method to identify proportional cellular dimensions in variously treated cells and in different cell types. Cells were scrape loaded with a mixture of fluorescent dextrans: Texas red dextran, average mol wt = 10,000 (TRDx10), and fluorescein isothiocyanate dextran, average mol wt = 70,000 (FDx70). After introduction into the cytoplasmic space, the TRDx10 distributed into both the nucleus and cytoplasm, whereas the FDx70 was restricted to cytoplasm, due to size exclusion by the nuclear pores. The aqueous nucleocytoplasmic volume ratio ($R_{N/C}$) was determined by measuring, from fluorescence images of spread cells, total cellular fluorescence of each of the two probes and the fluorescence ratio of those probes in the cytoplasm. $R_{N/C}$ was

unaffected by the measurement procedure or by varying temperatures between 23° and 37°C. Loading excess unlabeled dextrans had little effect on $R_{N/C}$, with the single exception that high concentrations of large dextrans could lower $R_{N/C}$ in endothelial cells. Expanding intracellular membranous compartments of macrophages by phagocytosis of latex beads decreased $R_{N/C}$. Expanding the same compartment by pinocytosis of sucrose, which nearly doubled total cell volume, had little effect on $R_{N/C}$, indicating that nuclear volume was more closely linked to the cytoplasmic volume, exclusive of vesicular organelles, than to total cell volume. $R_{N/C}$ was the same in mononucleate and binucleate endothelial cells. Finally, measurements of $R_{N/C}$ in murine bone marrow-derived macrophages, bovine aortic endothelial cells, Swiss 3T3 fibroblasts, PtK₂ cells, and CV-1 cells revealed that nuclear volume scaled allometrically with cell volume. The allometric relationship indicated that cell volume was proportional to nuclear surface area.

A long-appreciated but unexplained observation is that nuclear size increases with cell size. Early cytologists named this relationship the karyoplasmic ratio or nucleocytoplasmic ratio (1, 18). For growing cells and some differentiated cells, there is evidence that cell volume is proportional to the amount of DNA in the nucleus (3, 5). Diploid cells are twice the size of haploid cells (18), and species of amphibians with different C-values (half the quantity of DNA in the haploid cell) contain proportionally large or small erythrocytes (5). However, because an individual organism contains cells of many sizes, but contains for the most part no more than a fourfold variation of cellular DNA content, there should be other factors affecting the nucleocytoplasmic ratio besides the amount of DNA in the nucleus.

An experiment by Prescott (12) indicated that nuclear size is subject to cellular regulation. By surgically removing a portion of cytoplasm from a giant amoeba, thereby reducing cell size, he prevented cell division. Daily surgical operations prevented cell division in one individual for 20 d, despite continued and measurable cell growth. When the surgery schedule was terminated, the amoeba divided, indicating that when allowed to grow larger than a minimum size, the

cell could divide. Interestingly, Prescott observed that after each surgical reduction of cell size, there was an accompanying decrease in the volume of the nucleus. This indicates that the size of the nucleus can adjust to the size of the cell, and that the regulation of the nucleocytoplasmic ratio may be related to a size-dependent regulation of cell division.

The present work is an effort to identify the cellular dimensions critical to maintenance of the nucleocytoplasmic ratio. We first describe a method for measuring the aqueous nucleocytoplasmic volume ratio, which we define as the ratio of free water distribution between the nucleus and its contiguous cytoplasmic space. The nuclear compartment is separated from the cytoplasm by the nuclear envelope, and that envelope helps to maintain the distinct identities of the compartments. Pores in the envelope are large enough to allow free exchange of water, ions, and macromolecules of diameters <45 Å (10, 11), and they are small enough to prohibit exchange of many larger macromolecules. Our method exploits the size selectivity of the nuclear pores; large, fluorescein-labeled dextran molecules are loaded into the cytoplasmic space and are excluded from the nucleus by the nuclear pores, whereas smaller, Texas red-labeled dextrans loaded

into the cytoplasm distribute into both nucleus and cytoplasm. Analysis of fluorescence images from cells loaded with both probes yields the volume ratio of the two compartments. We report that the size of the nucleus is more closely related to cytoplasmic volume, exclusive of vesicular organelles, than to total cell volume. Moreover, we identify a proportionality between cytoplasmic volume and the surface area of the nucleus.

Materials and Methods

Cells

Bone marrow-derived macrophages were obtained from femurs of C3H.He or ICR mice (Trudeau Institute, Saranac Lake, NY), as described previously (16). Bone marrow exudate was cultured for 5 d in bone marrow growth medium (BMM: DME plus 30% L cell-conditioned medium plus 20% heat-inactivated FBS; 16). For the study of actively growing macrophages, day 5 cultures were resuspended from their culture dishes and replated onto 35-mm Lab-tek culture dishes (Nunc, Roskilde, Denmark) at 10^6 cells per dish. They were then left in BMM overnight before analysis.

Bovine aortic endothelial cells (BAEC, provided by Dr. Paul McNeil, Harvard Medical School; BAED, provided by Dr. Patricia D'Amore, Harvard Medical School), Swiss 3T3 fibroblasts, CV-1 cells (obtained from Dr. David Knipe, Harvard Medical School), and PtK₂ cells (obtained from Dr. Paul McNeil) were cultured in DME plus 10% heat-inactivated FCS (DM10F).

Fluorescent Labeling Procedures

Fluorescent dextrans were introduced into the cytoplasm of fibroblasts by scrape loading (9) or bead loading (8). A mixture of 3.5 mg/ml Texas red-labeled dextran, average mol wt = 10,000 (TRDx10; Molecular Probes Inc., Junction City, OR)¹ plus 3.5 mg/ml FITC-labeled dextran, average mol wt = 70,000 (FDx70; Sigma Chemical Co., St. Louis, MO) was prepared in PBS (137 mM NaCl, 3 mM KCl, 7 mM phosphate buffer, pH 7.4). Macrophages in culture dishes were rinsed twice with PBS at 37°C, and then PBS was removed and 50–60 μ l of dextran solution was added to the dish. Cells were scraped off the dish with a rubber policeman, and then warm medium was added to the dish. These scrape-loaded cells were plated into wells of culture dishes containing No. 1 coverslips (12-mm diameter). After 10 min at 37°C, medium was changed, thereby lowering the extracellular dextran concentration, and cultures were left for another 1–3 h in fresh medium (5% CO₂, 37°C).

Endothelial cells, fibroblasts, PtK₂ cells, and CV-1 cells on coverslips were loaded with dextrans by bead loading (8), in which 150–200- μ m-diameter glass beads are dropped onto cells on coverslips, thereby loading extracellular macromolecules (dextrans) into continually attached cells. After bead loading, coverslips were rinsed in warm PBS to remove the glass beads, and then they were placed into culture medium for 1–2 h before microscopic analysis.

For microscopic analysis, coverslips were rinsed in PBS with added divalent cations (137 mM NaCl, 3 mM KCl, 0.5 mM MgCl₂, 1 mM CaCl₂, 7 mM phosphate buffer, pH 7.4); then they were mounted on glass slides to form chambers of PBS sealed with valap, as described previously (15). The loading methods produced very little labeling of intracellular vesicles, either by endocytosis or sequestration of fluorescent dextrans (data not shown). Occasional preparations which displayed punctate intracellular fluorescence were rejected.

Cell Volume Alteration and Measurement

Macrophage size was increased either by phagocytosis of latex beads or by vacuolation. Macrophages were scrape loaded with fluorescent dextrans and plated onto coverslips. During the next 2 h, several coverslip cultures were incubated in 0.5 ml of medium containing 7- μ m-diameter latex beads (Duke Scientific Corp., Palo Alto, CA) at 8×10^6 beads/ml. Macrophages with varying numbers of phagocytosed beads were then identified in an inverted microscope. Images were collected only from cells in which all phagocytosed beads were in one plane of focus.

1. Abbreviations used in this paper: FDx70, fluorescein isothiocyanate dextran, avg. mol wt = 70,000; R_{NC}, aqueous nucleocytoplasmic volume ratio; TRDx10, Texas red dextran, avg. mol wt = 10,000.

To vacuolate cells, macrophage cultures were incubated overnight in medium containing 20 mg/ml sucrose. After vacuolation by pinocytosis of sucrose, macrophages were scrape loaded with fluorescent dextrans, plated onto coverslips, and incubated another 2 h in medium with sucrose. Control populations were treated over the same period with medium lacking beads or sucrose.

Cell volumes were measured in living, resuspended cells using an ocular micrometer on the light microscope. Cells were resuspended from culture dishes either by chilling them in cold PBS (macrophages) or by trypsinization. Diameters of 81 spherical cells were measured, and the total volume of each cell was calculated ($V_T = 4\pi r^3/3$) before determining the mean volume, SD, and SEM.

Image Collection and Analysis

Determination of the nucleocytoplasmic volume ratio first required that we obtain two digitized video images from each scrape-loaded cell. Cells on coverslips were observed using an inverted microscope (model IM-35, Carl Zeiss, Inc., Thornwood, NY) equipped for epillumination of fluorescent specimens. Light from a tungsten lamp passed through either a fluorescein or a rhodamine filter set and a 40 \times water-immersion objective lens (NA 0.75). Fluorescence images were collected using a multichannel plate intensifier coupled to a nuicon video camera (Video Scope International, Ltd., Washington, DC.).

Video signals were collected and processed using a Tracor Northern TN 8500 image analysis system (Noran Instruments Inc., Middleton, WI). Each video image was stored in digitized form as the Kalman average of 50 video frames. Labeled, well-spread cells were identified using the fluorescein filter set. The cell was then viewed through the video monitor using the rhodamine filter set to adjust illumination intensity and cell position on the screen. For each cell, four images were collected: (a) after adjusting the Texas red fluorescence image, the stage was adjusted to a nearby cell-free region of coverslip to collect a background image (BACK_{TR}); (b) the cell was again positioned in the center of the screen and the image of the cellular Texas red fluorescence was collected (CELL_{TR}); (c) without moving the stage, the filters were changed, the intensifier gain was adjusted, and then the cellular fluorescein image was collected (CELL_F); and (d) the stage was again moved to a cell-free region to collect a background image for the fluorescein filter set (BACK_F). Background-subtracted images were then created by digital image processing: the Texas red background-subtracted image (SUBT_{TR}) = CELL_{TR} - BACK_{TR} and the fluorescein background-subtracted image (SUBT_F) = CELL_F - BACK_F. The background-subtracted images were stored for subsequent analysis.

Measurement of the nucleocytoplasmic volume ratio required that we obtain four values from the background-subtracted images of each cell. These were the total Texas red fluorescence in the cell (TR_T), the total fluorescein fluorescence (F_T), the Texas red fluorescence from a region of cytoplasm not overlying the nucleus (TR_{C1}), and the fluorescein fluorescence from that same region of cytoplasm (F_{C1}). As shown in Fig. 1, the method for obtaining these values was to prepare two binary images (B1 and B3) which could be applied to the grey images SUBT_{TR} (I1) and SUBT_F (I2) for selective quantitation of the cellular fluorescence. (step 1) To create a binary mask for measuring total fluorescence (TR_T and F_T), SUBT_{TR} (I1) was used to generate a binary image of the cell (stored in B1) by including all regions of I1 with grey value greater than 10 (scale, 0–255). This binary image was then edited such that the portion of the binary overlying the cell was the only positive area of the binary (i.e., the fluorescence of other cells on the screen was masked out).

Next, we created the binary image used to measure cytoplasmic fluorescence (TR_{C1} and F_{C1}) (Fig. 1, steps 2–5). (step 2) The two background-subtracted images SUBT_{TR} (I1) and SUBT_F (I2) were used to generate, pixel by pixel, a third image (I3), which represented the fluorescence ratio TR/F (I3 = I1/I2). (step 3) The binary generated in step one, B1, was applied as a mask to the ratio image (I3) to create a second edited ratio image (I4) in which the cell defined by the binary was the only grey area on the screen; all other regions of the screen were assigned a grey value of zero. (step 4) The second ratio image (I4) displayed the cytoplasm as a uniform grey, and the region over the nucleus as brighter grey, indicating the higher TR/F values in that region. This difference in brightness was used to generate a binary, B2, which identified the nucleus. (step 5) The binary generated by the cell fluorescence (B1) was combined with the binary defining the nuclear region (B2) to form a third binary image (B3), which marked regions of the cell which were exclusively cytoplasmic. (step 6) The background-subtracted images I1 and I2 were analyzed using the binaries B1 and B3 to obtain the fluorescence intensities TR_T, F_T, TR_{C1}, and F_{C1}, shown diagrammatically in Fig. 2. For example, TR_T was obtained by determining the av-

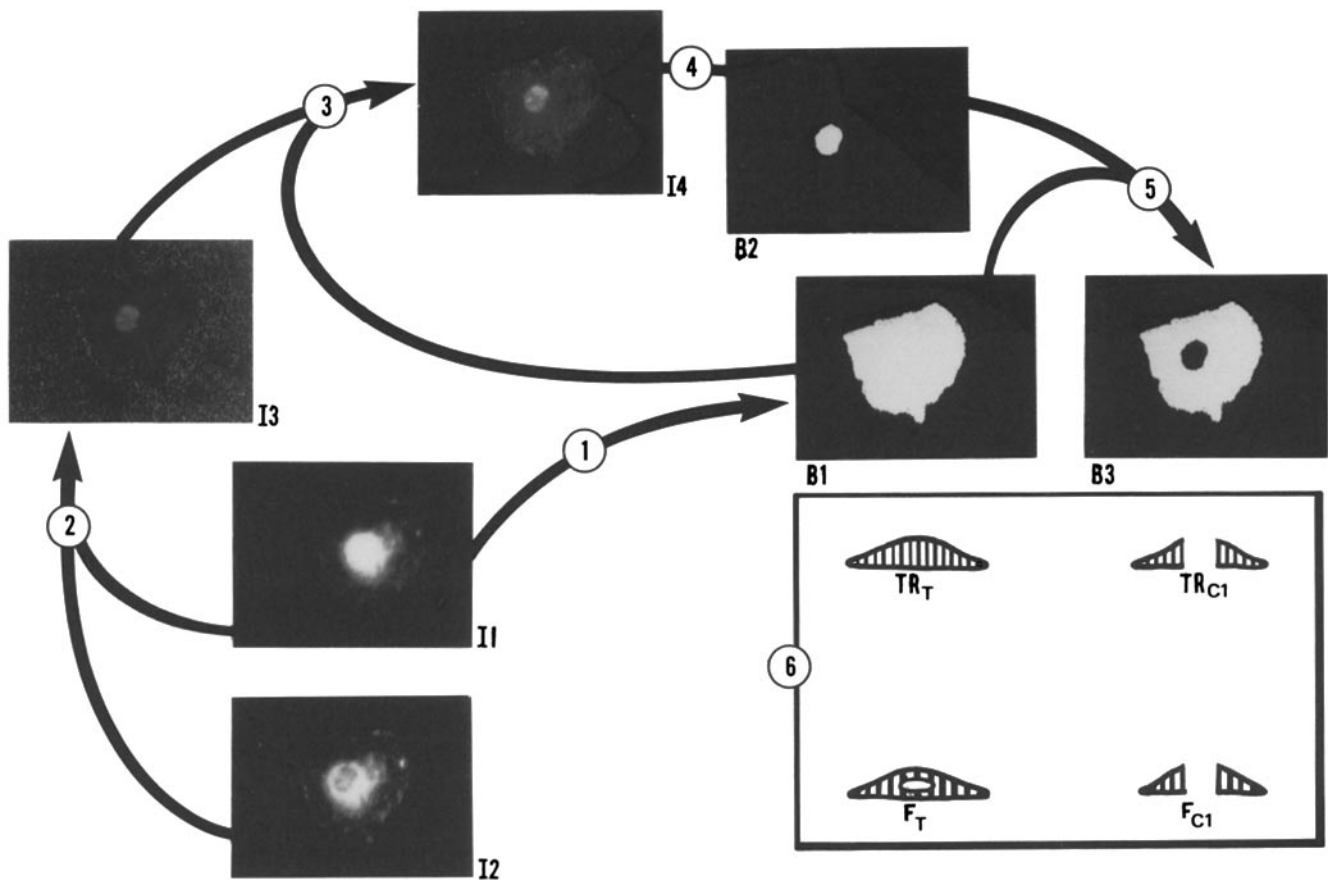


Figure 1. The method for obtaining nucleocytoplasmic volume ratio data. The boxes show digitized video images (I1–I4) or binary images (B1–B3) of a single macrophage scrape loaded with TRDx10 (I1) and FDx70 (I2). The steps summarized here are described in more detail in Methods. (a) Generate binary image (B1) from background-subtracted TRDx10 image (I1). (b) Generate ratio image (I3) by dividing the TRDx10 image (I1) by the FDx70 image (I2). (c) Apply the binary B1 as a mask to the ratio image I3 to obtain a second ratio image (I4) without background noise. (d) Generate binary of the nucleus (B2) using the ratio image I4. (e) Generate a binary of the cytoplasm (B3) by subtracting the nuclear binary (B2) from the total binary (B1). (f) Integrate fluorescence of I1 and I2 using the binaries B1 and B3 as masks.

erage pixel value for all pixels in I1 underlying the cell mask in B1, and then multiplying this average by the total number of pixels in that binary mask. Applying the binary B3 to that same image yielded TR_{C1} , the Texas red fluorescence for that selected region of cytoplasm.

Confocal Microscopy

Macrophages were scrape loaded with TRDx10 and FDx70, each at 10 mg/ml. Cells were observed and photographed using a laser scanning confocal system (MRC 500, Bio-Rad Laboratories, Richmond, CA), attached to an Axiophot microscope (Carl Zeiss, Inc.). The objective lens was a 63× Plan Apo (Carl Zeiss, Inc.). Confocal images were collected both as standard X-Y images and as X-Z images, which essentially provided side-view sections of the loaded cells.

Measurement of Cellular DNA Content

Cellular DNA was quantified using a fluorescence assay (6). Growing cultures of cells were suspended by trypsinization and counted using a hemocytometer. Known numbers of cells were lysed by freezing and thawing, and the DNA content of the lysates was determined by measuring the DNA-dependent increase in fluorescence of 100 ng/ml 4',6-diamidino-2-phenylindole in solutions containing 10 mM Tris, pH 7.0, 100 mM NaCl, 10 mM EDTA and 0.1% Triton X-100. Fluorescence was measured using a spectrofluorometer (Aminco 500c, SLM Instruments, Inc., Urbana, IL) set at excitation 360 nm, emission 450 and band width of 5 nm. Calf thymus DNA was used to generate a DNA standard curve.

Results

Rationale for the Method of Measurement

Fig. 2 shows the TRDx10 and FDx70 distribution inside a macrophage after scrape loading, displayed as an X-Z series (side views) from a laser scanning confocal microscope. TRDx10 occupies both nucleus and cytoplasm, and FDx70 is excluded from the nucleus. These images are presented again in Fig. 2, C–F to diagram the fluorescence data obtained by image processing.

$R_{N/C}$ was obtained from the Texas red and fluorescein images by measuring total cellular fluorescence for each probe and the concentration ratio of those probes in the cytoplasm. To explain the concept, we first consider a cell with no nucleus which is labeled with TRDx10 and FDx70. The two probes would have identical distribution throughout the cell. The ratio of total cellular Texas red fluorescence to total fluorescein fluorescence would be identical to the ratio of Texas red to fluorescein fluorescence measured in any subsection of the cell. Any size sampling area, from one pixel up to the whole cytoplasm, would report the same fluorescence ratio. However, the presence of a nucleus restricts

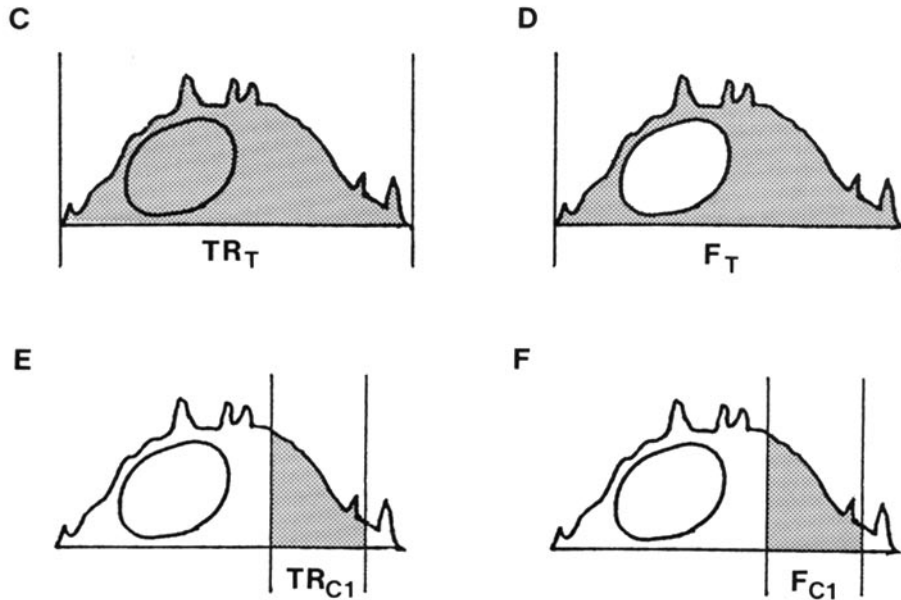
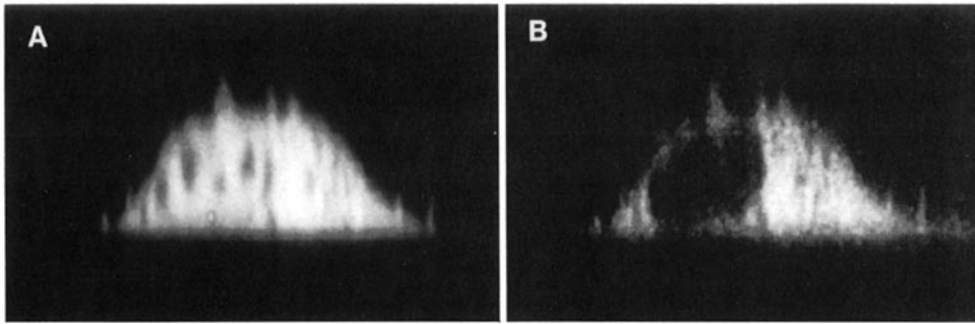


Figure 2. X-Z images of a macrophage scrape loaded with FDx70 and TRDx10 (10 mg/ml each). (A) TRDx10 labels both the cytoplasmic and the nuclear compartments, whereas FDx70 (B) is restricted to the cytoplasm. (C-F) The same images redrawn to demonstrate the regions of the cell sampled by image analysis, displayed as a side view of the cell for illustration. TR_T (C) and F_T (D) are total Texas red and fluorescein fluorescence, respectively. TR_{C1} (E) and F_{C1} (F) are the integrated fluorescence from a region of the image that is exclusively cytoplasmic.

FDx70 to cytoplasm because of its selective permeability to the dextrans. Consequently, the ratio of total cellular TRDx10 to FDx70 fluorescence will be higher than the ratio obtained in cytoplasmic regions. These ratios can be used to calculate R_C , the ratio of the cytoplasmic volume (V_C) to the nuclear-plus-cytoplasmic volume ($V_{(N+C)}$), and from this ratio one can obtain the aqueous volume ratio of nucleus to cytoplasm, V_N/V_C or $R_{N/C}$. It should be emphasized that al-

though our methods measured the fluorescence from as much of the cytoplasm as possible, it was not necessary to do this. It was only necessary to measure some portion of the cytoplasm that does not overlie the nucleus.

The total cell fluorescence intensities of Texas Red and of

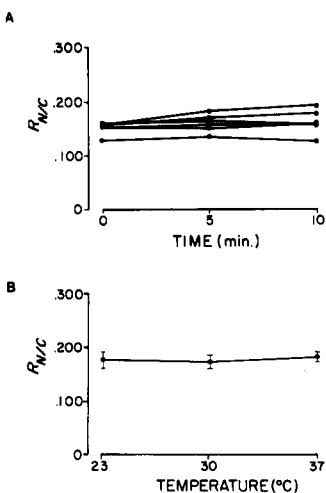


Figure 3. The effects of repeated measurements or of temperature on $R_{N/C}$ in macrophages. (A) Background-subtracted images from each of six cells were collected at 5-min intervals; $R_{N/C}$ was calculated later. (No trends in $R_{N/C}$ are evident.) (B) $R_{N/C}$ was measured in cells held 15–30 min at each temperature shown. Data are the mean and SEM; $n = 18$ (23°), 19 (30°), and 27 (37°C).

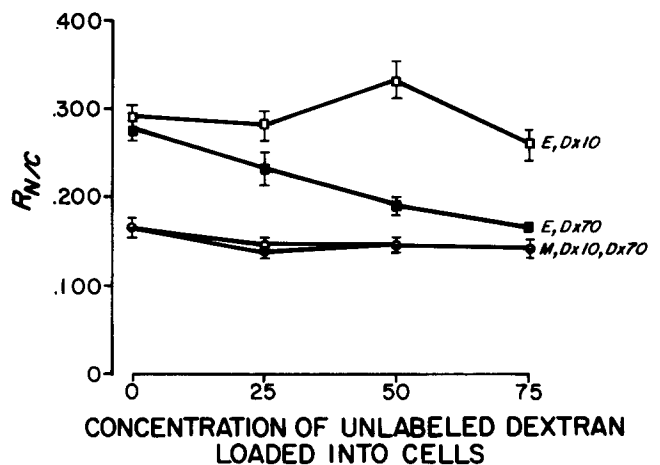


Figure 4. $R_{N/C}$ in macrophages (M) and endothelial cells (E) loaded with excess unlabeled dextrans. All cells were loaded with 3.5 mg/ml FDx70 and 3.5 mg/ml TRDx10. In addition, loading solutions contained 0, 25, 50 or 75 mg/ml Dx10 or Dx70. Final intracellular dextran concentrations are not known.

fluorescein, respectively, were obtained from the background-subtracted images $SUBT_{TR}$ and $SUBT_F$ as described in Materials and Methods (Fig. 2, C and D). These values are described by the following equations:

$$TR_T = k_{TR} \cdot [TRDx10] \cdot V_{(N+C)} \text{ and} \quad (1)$$

$$F_T = k_F [FDx70] \cdot V_C, \quad (2)$$

where k_{TR} and k_F are constants describing the fluorescence yield of the fluorophores and the various amplification parameters of the electronics, and where $[TRDx10]$ and $[FDx70]$ are the intracellular concentrations of TRDx10 and FDx70, respectively. Similar equations can be written to describe TR_{Cl} and F_{Cl} , which represent the Texas red and fluorescein fluorescence from a selected volume of cytoplasm, V_{Cl} :

$$TR_{Cl} = k_{TR} \cdot [TRDx10] \cdot V_{Cl} \text{ and} \quad (3)$$

$$F_{Cl} = k_F [FDx70] \cdot V_{Cl}. \quad (4)$$

Rearranging Eqs. 3 and 4 yields

$$\frac{TR_{Cl}}{V_{Cl}} = k_{TR} [TRDx10] \text{ and} \quad (5)$$

$$\frac{F_{Cl}}{V_{Cl}} = k_F [FDx70]. \quad (6)$$

Substituting into Eqs. 1 and 2 the expressions for k_{TR} $[TRDx10]$ (Eq. 5) and $k_F [FDx70]$ (Eq. 6) obtains

$$TR_T = \frac{TR_{Cl}}{V_{Cl} \cdot V_{(N+C)}} \text{ or } \frac{TR_T \cdot V_{Cl}}{TR_{Cl}} = V_{(N+C)} \text{ and} \quad (7)$$

$$F_T = \frac{F_{Cl}}{V_{Cl} \cdot V_C} \text{ or } \frac{F_T \cdot V_{Cl}}{F_{Cl}} = V_C. \quad (8)$$

Then combining Eqs. 7 and 8 yields

$$R_C = \frac{V_C}{V_{(N+C)}} = \frac{F_T \cdot TR_{Cl}}{F_{Cl} \cdot TR_T}. \quad (9)$$

R_{N+C} is obtained by

$$R_{N/C} = \frac{1 - R_C}{R_C} = \frac{TR_T F_{Cl} - TR_{Cl} F_T}{TR_{Cl} F_T}. \quad (10)$$

Therefore, by obtaining the measured values TR_T , F_T , TR_{Cl} , and F_{Cl} one can calculate the volume ratio V_N/V_C , or $R_{N/C}$.

Reliability of the Measured Values

Once the method was devised, it was necessary to examine both the natural and the experimental variation. In one experimental scheme, $R_{N/C}$ was measured in the same cell three times, once every 5 min. Fig. 3 A shows the measured values for each of six macrophages, with connecting lines showing the related points. These measurements showed no trend, upward or downward, in the $R_{N/C}$ values, indicating that the method of collecting data did not appear to alter the measured ratios. In a larger sample of macrophages in which each cell was measured once, the mean $R_{N/C}$ was 0.179, with a standard deviation of 0.034 ($n = 22$, SEM = 0.007). As shown below, these values were different from those measured for other cell types.

The observation that the volume ratio remained constant throughout successive measurements indicated that fluorescence photobleaching, a process that affects fluorescein dex-

tran more than it does Texas red dextran, was not altering the measured ratios. Since the method is ratiometric, it corrects for differences in the absolute fluorescence intensities of the two fluorophores. Moreover, the ratiometric images indicated that the ratio TR/F was nearly uniform throughout cytoplasmic regions. A small increase in the ratio was detectable at the edges of the thinnest margins of well-spread cells (Fig. 1 I4). We presume that this was due to fluorescein photobleaching and slower diffusion of FDx70, since this always appeared as an increase in the fluorescence ratio regardless of the concentration ratio of fluorophores loaded into cells (data not shown). This potential artifact was corrected by the method of integrating the fluorescence from as much of the cytoplasm as possible (Fig. 1 B3).

Macrophages were measured at different temperatures by mounting coverslips with scrape-loaded cells into Sykes-Moore chambers, putting these chambers into a temperature-controlled stage, and collecting ratio images. Incubation of cells at 23°, 30°, or 37°C for 15–30 min had no measurable effect on $R_{N/C}$ (Fig. 3 B). All subsequent measurements were made at room temperature (23°C).

It was possible that the fluorescent dextrans, loaded into the cells at 3.5 mg/ml each, were themselves altering the true nucleocytoplasmic volume ratio. To examine this possibility we loaded cells with TRDx10 and FDx70, each at 3.5 mg/ml, plus an excess of unlabeled dextran. Fig. 4 shows that in macrophages, scrape loading an excess dextran of up to 75 mg/ml had little if any effect on $R_{N/C}$. In endothelial cells, which exhibit higher $R_{N/C}$ values, unlabeled Dx10 had little effect on $R_{N/C}$, but high concentrations of unlabeled Dx70 lowered the measured ratios significantly. The mechanism of this effect is not clear, although the observation might be explained by osmotic effects of the larger dextran in the cytoplasm essentially drawing water from the nucleus into the cytoplasm. Nonetheless, the effect of Dx70 is slight enough that one would not expect the true ratio to be altered by the 3.5 mg/ml FDx70 used to make the measurements.

The Relationship of $R_{N/C}$ to Other Cellular Dimensions

There is no reason to think that if cells regulate the nucleocytoplasmic ratio they do so by measuring a volume ratio. They could regulate ratios other than nuclear to cytoplasmic volume, such as nuclear volume to total cell volume, or nuclear surface area to total cell surface area. We therefore performed experiments to determine which dimensions cells hold most directly proportional, i.e., which dimensions are isometric.

The space labeled by fluorescent dextran does not include the volume occupied by membranous organelles. In most circumstances membranous organelles are collapsed and occupy a small percentage of the total cell volume (14), but they can be enlarged via endocytosis to increase total cell volume. We measured $R_{N/C}$ in macrophages whose volume had been increased either through phagocytosis or pinocytosis. Macrophages were scrape loaded with fluorescent dextrans and allowed to phagocytose 7- μ m-diameter latex beads before measurement of $R_{N/C}$. The accumulated data from three sets of experiments (Fig. 5) indicated that as the endocytic compartment was expanded with increasing numbers of beads per cell, $R_{N/C}$ decreased. Linear regression of the data indicated a weak relationship between $R_{N/C}$ and the number of beads per cell (slope = -0.0062; $b = 0.180$; R^2

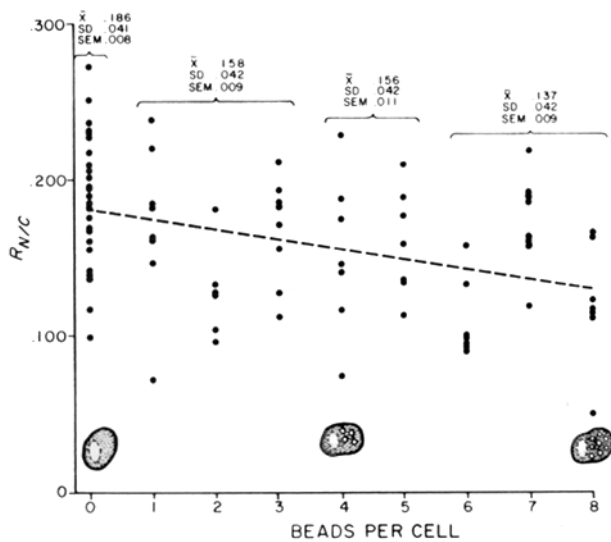


Figure 5. The effect of phagocytosis on $R_{N/C}$ in macrophages. Scrape loaded cells were allowed to phagocytose 7- μm diameter latex beads before measurement of $R_{N/C}$. All data points from three sets of experiments are shown ($n = 87$). The dashed line represents the linear regression of the data. In addition, $R_{N/C}$ values are shown for clusters at 0, 1-3, 4-5, and 6-8 beads per cell. The cellular condition is shown in the drawings, with stippled regions indicating the distribution of FDx70.

= 0.39; $n = 87$), but comparison of the unfed cells with those containing six to eight beads showed a significant difference ($P < 0.001$) in $R_{N/C}$. This indicated that expanding the vacuolar compartment, which probably increased total cell volume, actually decreased the volume ratio of nucleus to cytoplasmic space.

Another way to enlarge the endocytic vacuolar compartment of macrophages is to allow extensive pinocytosis of sucrose. Sucrose accumulates undegraded in macrophage lysosomes, eventually expanding that compartment into many phase-bright vacuoles (4, 17). We compared $R_{N/C}$ in normal and sucrose-vacuolated macrophages. Macrophages accumulated abundant sucrose vacuoles, yet they exhibited no significant difference in $R_{N/C}$ ($P > 0.25$). We measured the total cell volume in populations of sucrose-vacuolated mac-

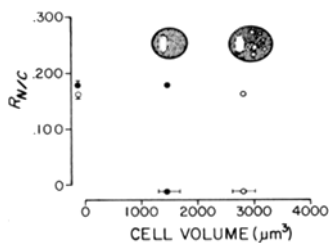


Figure 6. Macrophages vacuolated by pinocytosis of sucrose increase their total volume nearly twofold, with only a slight decrease in $R_{N/C}$. To determine $R_{N/C}$, cells were incubated overnight in medium containing 20 mg/ml sucrose and then were scrape loaded and measured; measurements

were taken for control cells $n = 22$ and for vacuolated cells $n = 28$. To determine cell volumes, control (●) or sucrose-vacuolated (○) macrophages were resuspended from their dishes by chilling, then the diameters of these rounded cells were measured using an ocular micrometer. Total cell volume was calculated for each cell before determining mean and SEM; for both sets of measurements $n = 83$. The drawings of cells indicate the condition of the nearby points; stippling indicates the distribution of FDx70.

Table I. $R_{N/C}$ in Mononucleate and Binucleate Endothelial Cells

$R_{N/C}$	Mononucleate	Binucleate
\bar{x}	0.194	0.179
SD	0.055	0.045
SEM	0.013	0.010
n	18	20

rophages and untreated controls and found that vacuolation nearly doubled cell volume (Fig. 6). This, together with the measured $R_{N/C}$ values after phagocytosis (Fig. 5), indicated that the volume of the nucleus was not held proportional to total cell volume (cytoplasmic volume plus included organelles), but rather was more closely linked to cytoplasmic volume (V_C) or to nuclear-plus-cytoplasmic volume ($V_{(N+C)}$).

Cultures of bovine aortic endothelial cells often contain binucleate cells, the frequency of which we found could be increased by brief incubation of cultures (~ 30 min) at room temperature. This cooling was done at various times before bead loading. We measured mononucleate and binucleate endothelial cells and found an insignificant difference in $R_{N/C}$ (Table I; $P > 0.30$). This comparison supports many previous observations which indicate that cytoplasmic volume is proportional to the total size of the nuclear compartment (3, 13). It is also consistent with the proportionality we

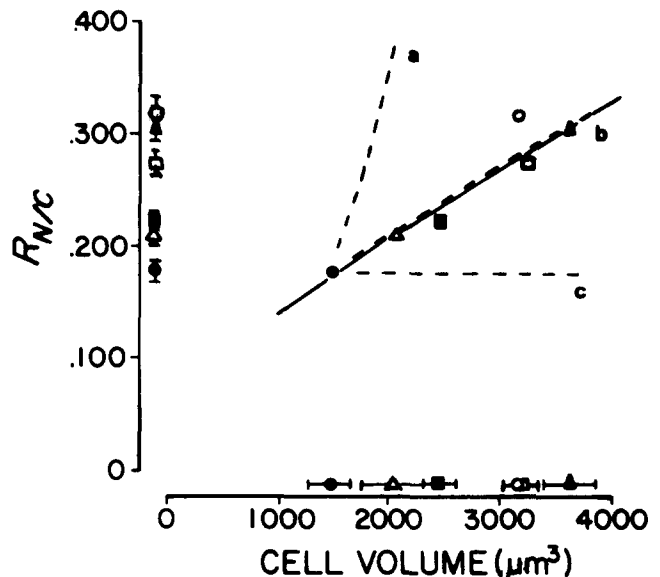


Figure 7. $R_{N/C}$ is higher in larger cells. $R_{N/C}$ was measured in well-spread macrophages (●; $n = 22$; same data as in Fig. 6), endothelial cells, BAEC (■; $n = 74$), or BAED (Δ; $n = 81$); CV-1 cells (○; $n = 49$); PtK₂ cells (□; $n = 25$), and fibroblasts (▲; $n = 30$). The left-hand edge of the plot shows the mean and SEM. Volumes were measured as in Fig. 6. Data are the mean and SEM, where $n = 83$ (macrophages) or 81 (all other cell types). The solid line indicates the regression analysis of the data ($V_{(N+C)} = 44.2 V_N^{0.65}$). The dashed lines indicate the curves which would be obtained if (a) cell volume were proportional to nuclear diameter ($V_{(N+C)} \propto V_N^{1/3}$), (b) cell volume were proportional to nuclear surface area ($V_{(N+C)} \propto V_N^{2/3}$) or (c) cell volume were proportional to nuclear volume ($V_{(N+C)} \propto V_N$). Curves a-c all originate from the point determined for macrophages.

Table II. DNA Content of Cultured Cells

Cell type	$R_{N/C}$	DNA per cell pg
Macrophage	0.179 ± 0.007	8.6 ± 0.6
BAED	0.209 ± 0.006	4.9 ± 0.9
PtK ₂	0.272 ± 0.010	15.4 ± 1.2
Fibroblasts	0.306 ± 0.011	13.8 ± 0.6
CV-1	0.318 ± 0.011	13.7 ± 0.5

observed between cytoplasmic volume and nuclear surface area (see below).

The aqueous $R_{N/C}$ was different in different cell types; in general $R_{N/C}$ was greater in larger cells. Fig. 7 shows the calculated cell volumes (\pm SEM) for macrophages, PtK₂ cells, CV-1 cells, 3T3 fibroblasts, and two isolates of bovine aortic endothelial cells, obtained by measuring diameters of spherical cells following resuspension. The six cell types comprise distinct groupings of total cell volume. $R_{N/C}$ in these cells, obtained in separate experiments, are shown as well. The plot $R_{N/C}$ vs. cell volume (Fig. 7) shows that larger cells have disproportionately larger nuclei. That is, the relationship between nuclear volume and total volume is allometric.

Further analysis of these data revealed more about the allometric relation. Using R_c and the measured cell volume for each cell type, and assuming that the measured cell volume in these nonvacuolated cells was not significantly greater than the nuclear-plus-cytoplasmic volume (i.e., $V_T \approx V_{(N+C)}$), we calculated the mean nuclear volumes ($V_N = V_{(N+C)}[1 - R_c]$). We then applied the data to the equation

$$V_{(N+C)} = aV_N^b, \quad (11)$$

and determined that $a = 44.2$ and $b = 0.653$ ($R^2 = 0.99$).

The dashed lines in Fig. 7 show the plots that would be obtained if cell volume were proportional to nuclear diameter (line *a*), nuclear surface area (line *b*) or nuclear volume (line *c*), originating from the point determined for macrophages. The solid line shows the curve determined by the linear regression analysis ($V_{(N+C)} = 44.2 V_N^{0.65}$). The similarity of this curve to line *b* indicates that cytoplasmic-plus-nuclear volume scales isometrically with nuclear surface area.

We measured cellular DNA for five of these cell types and found that, in general, cell types with higher $R_{N/C}$ contained more DNA per cell (Table II). The higher values of DNA per cell obtained in CV-1, PtK₂ and Swiss 3T3 cells is most likely due to aneuploidy occurring after prolonged culture. The low correlation coefficient between $R_{N/C}$ and DNA per cell ($R^2 = 0.80$) did not warrant further analysis.

Discussion

This method for measuring the $R_{N/C}$ should be applicable to a variety of cultured cells. It measures $R_{N/C}$ best in cells which are readily loaded with fluorescent dextrans and which spread well, or remain spread after loading. The method requires that one fluorophore has access to both nucleus and cytoplasm, and that the other is restricted to cytoplasm. The probes must also be freely diffusible in cytoplasm, with no binding to cytoplasmic or nuclear components. Fluorescent dextrans satisfy these criteria (7, 10,

11). It remains possible, however, that there are weak interactions between dextrans and cytoplasmic or nuclear components, and these could introduce some bias into the measurements.

Having characterized the method of measuring $R_{N/C}$ we can ask experimentally how this ratio is regulated. A first step, taken here, is to ask how the dimensions of different compartments scale with one another. Two compartments can be said to scale isometrically if, over a range of sizes, the dimensions of one are directly proportional to the other. To put this another way, if one dimension is y and the other is x , their relationship can be expressed as $y = ax^b$. If x and y scale isometrically, then $b = 1$. If x and y vary disproportionately, b may be some number other than one, and the relationship is called allometric. If variation of x has no effect on y , then $b = 0$ and $y = a$, a constant value unrelated to x .

If nuclear volume scaled isometrically with total cell volume (i.e., nucleus plus cytoplasm plus included organelles), then enlargement of total cell volume, either through phagocytosis of beads or vacuolation with sucrose, should have increased nuclear volume, and $R_{N/C}$, substantially. The measured total cell volume of the macrophages, $1493 \mu\text{m}^3$, is equivalent to that of 8–9 7- μm diameter latex beads. Therefore, phagocytosis of eight beads should have nearly doubled cell volume, and, if nuclear volume scales isometrically with total cell volume, should have increased $R_{N/C}$. Similarly, vacuolation with sucrose, which increased cell volume nearly twofold, should also have increased $R_{N/C}$. Since R_{N+C} decreased after phagocytosis and changed very little in vacuolated cells, we conclude that nuclear volume is not isometric with total cell volume.

Because we do not know if or how much nuclear-plus-cytoplasmic volume changes after vacuolar expansion, either with sucrose or latex beads, we cannot refine this conclusion. We can say that nuclear volume does not scale with total cell volume, and that the more likely relationship is between nuclear and cytoplasmic volume or between nuclear volume and nuclear-plus-cytoplasmic volume. However, we cannot yet determine that it is one of these two alternatives. In visualizing mechanisms for monitoring cell dimensions, it is easier to imagine ones that monitor the sizes of the nuclear and cytoplasmic spaces, as these represent contiguous and communicating compartments. The decrease in $R_{N/C}$ after phagocytosis of beads indicates that something else is involved, perhaps a relationship between $R_{N/C}$ and cell shape.

The plot of $R_{N/C}$ vs. cell volume in six cell types (Fig. 7) indicated an allometric relationship between cell size and nuclear size. The fact that $R_{N/C}$ increased with increasing cell volume suggested that some lower order dimension of the nucleus, e.g., diameter or surface area, scales isometrically with cell volume. Certainly nuclear volume and total volume are not isometric. When we calculated nuclear volume from our data and examined its relationship to total cell volume, the correlation between nuclear surface area and total cell volume appeared.

Our essential conclusions are demonstrated by Figs. 6 and 7. If cell volume is increased by expanding the vacuolar compartment (Fig. 6), then $R_{N/C}$ —the volume ratio of nucleus to cytoplasm—does not change. In contrast, if cell volume is increased by increasing the cytoplasmic volume, which could be done by comparing different kinds of cells (Fig. 7), then $R_{N/C}$ increases allometrically and indicates a propor-

tionality between nuclear surface area and cytoplasmic volume or total cell volume.

Doubling the number of nuclei per cell did not alter $R_{N/C}$ significantly (Table I). This result appears inconsistent with the hypothesis that cell volume scales isometrically with nuclear surface area: the larger binucleate cells should have higher $R_{N/C}$ values. The discrepancy may be explained by the way that the nuclear compartment has been enlarged. If the total nuclear surface area is increased by enlarging a single, spherical nucleus, then nuclear volume will increase as the $3/2$ power of the surface area. However, if the total nuclear surface area is increased by doubling nuclear number, then nuclear volume will simply double as well. Therefore, regardless of which nuclear dimension is isometric with cell volume, increasing nuclear number should not change $R_{N/C}$. Nonetheless, the constancy of $R_{N/C}$ in mononucleate and binucleate cells lends weight to the proposal that the nucleocytoplasmic ratio is a regulated proportionality.

We have observed in preliminary experiments that $R_{N/C}$ decreases when cells differentiate and stop dividing, which suggests that the relationships we have observed pertain mostly to growing and dividing cells. A mechanism that maintains in growing cells an isometric relationship between nuclear surface area and cell volume would limit the possible range of cell sizes.

Over a wide range of sizes, eukaryotic cell size scales isometrically with the amount of DNA in the cell (2, 3, 5). Diploid cells are twice the size of haploid cells, and multinucleate cells have proportionally large cell volumes. We measured the quantity of DNA in the various cell types and found that the larger cells, with their higher $R_{N/C}$ values, contained more DNA per cell. It is possible that nuclear surface area is related to cellular DNA content. However, it may also be that cell type-specific condensation of chromatin against the nuclear lamina affects nuclear surface area and $R_{N/C}$. We are currently examining these possibilities.

The importance of the karyoplasmic ratio was raised by Wilson (18), and his consideration of the subject provides significant precedent to our findings. First, he cites Boveri (page 730), whose "extensive measurements led him to the unexpected result that in the sea urchin, with increasing chromosome-number the nuclear volume increases more rapidly than the cytoplasmic, in such a manner that it is the surface of the nucleus and not its volume that is directly proportional to the number of chromosomes." This is consistent with our finding that the cell types with higher average DNA per cell have higher $R_{N/C}$ values. Second, Wilson credits Conklin (page 731), who showed "that nuclear volume varies not with the total volume of the cytosome but only with that of its active protoplasm. This is proved by eggs centrifuged during cleavage, in which a sharp separation

takes place between the heavier yolk and the lighter protoplasm, so that their volumes may readily be determined. In such eggs, during cleavage, it may readily be seen that the largest nuclei appear in blastomeres that contain the largest amount of active protoplasm, irrespective of their total size." This, too, is compatible with our conclusions.

This paper is dedicated to Professor John T. Bonner on the occasion of his retirement.

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References

1. Adolph, E. F. 1931. *The Regulation of Size as Illustrated in Unicellular Organisms*. Charles Thomas & Co., Baltimore, MD. 236 pp.
2. Bennett, M. D. 1972. Nuclear DNA content and the minimum generation time in herbaceous plants. *Proc. R. Soc. Lond. B.* 181:109-135.
3. Cavalier-Smith, T. 1985. Cell volume and the evolution of eukaryotic genome size. *In The Evolution of Genome Size*. T. Cavalier-Smith, editor. John Wiley & Sons Inc. New York. 105-184.
4. Cohn, Z. A., and B. A. Ehrenreich. 1969. The uptake, storage and intracellular hydrolysis of carbohydrates by macrophages. *J. Exp. Med.* 129:201-225.
5. Horner, H. A., and H. C. MacGregor. 1983. C Value and cell volume: their significance in the evolution and development of amphibians. *J. Cell Sci.* 63:135-146.
6. Lee, A. M., J. T. Thornthwaite, and E. M. Rasch. 1984. Picogram per cell determination of DNA by flow cytometry. *Anal. Biochem.* 137:221-226.
7. Luby-Phelps, K., D. L. Taylor, and F. Lanni. 1986. Probing the structure of cytoplasm. *J. Cell Biol.* 102:2015-2022.
8. McNeil, P. L., and E. Warder. 1987. Glass beads load molecules into living cells. *J. Cell Biol.* 88:669-678.
9. McNeil, P. L., R. F. Murphy, F. Lanni, and D. L. Taylor. 1984. A method for incorporating macromolecules into adherent cells. *J. Cell Biol.* 98:1556-1564.
10. Paine, P. L., L. C. Moore, and S. B. Horowitz. 1975. Nuclear envelope permeability. *Nature (Lond.)*. 254:109-114.
11. Peters, R. 1983. Nuclear envelope permeability measured by fluorescence microphotolysis of single liver cell nuclei. *J. Biol. Chem.* 258:11427-11429.
12. Prescott, D. M. 1956. Relation between cell growth and cell division. II. The effect of cell size on cell growth rate and generation time in *Amoeba proteus*. III. Changes in nuclear volume and growth rate and prevention of cell division in *Amoeba proteus* resulting from cytoplasmic amputations. *Exp. Cell Res.* 11:86-98.
13. Smith, G. 1903. *Actinosphaerium Eichorni*. A biometrical study in the mass relations of nucleus and cytoplasm. *Biometrika* 2:241-254.
14. Steinman, R. M., S. E. Brodie, and Z. A. Cohn. 1976. Membrane flow during pinocytosis. A stereological analysis. *J. Cell Biol.* 68:665-687.
15. Swanson, J. A. 1989. Fluorescent labeling of endocytic compartments. *In Fluorescence Microscopy of Living Cells in Culture*. Methods in Cell Biology. Vol. 29. Y.-L. Wang and D. L. Taylor, editors. Academic Press Inc. New York. 137-151.
16. Swanson, J. A. 1989. Phorbol esters stimulate macropinocytosis and solute flow through macrophages. *J. Cell Sci.* 94:135-142.
17. Swanson, J. A., B. Yirinec, E. Burke, A. Bushnell, and S. C. Silverstein. 1986. Effect of alterations in the size of the vacuolar compartment on pinocytosis in J774.2 macrophages. *J. Cell Physiol.* 128:185-201.
18. Wilson, E. B. 1928. *The Cell in Development and Heredity*. MacMillan Co., New York. 1232 pp.