LIGHT SCATTERING VS. MICROSCOPY FOR MEASURING AVERAGE CELL SIZE AND SHAPE

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ABSTRACT Light-scattering photometry is compared with electron and light microscopy as a source of information about the average size and shape of cells in populations. Examined are the effects of limited instrument resolution, necessary experimental procedures, and cell heterogeneity. Information theory is used to survey the relative amounts of information provided by photometric and microscopic measurements. Then in model exploratory experiments, cell size and shape and changes therein are determined both by microscopy and by photometry for spherical and spheroidal cells. Scattering theory is used to calculate photometrically observed light from cell parameters. It is found that if certain other appropriate information about the morphological property of interest is available, then visible light photometry is the preferred method for obtaining quantitative information. It has good absolute sensitivity (0.01–0.10 μm resolution), its results are relatively unaffected by sample heterogeneity, it is nondestructive and compatable with many other techniques, it requires no sample preparation, and it provides its information in real time.

INTRODUCTION

The recently recognized widespread existence of structure-function relationships at the cellular level suggests increased importance of methods for measuring cell dimensions. Morphological properties can be quite dynamic; some are more closely coupled to physiological processes than had been supposed. Many biological cells and large component parts are now known to spontaneously undergo changes in size, shape, and structure, most of which are reversible. These morphological changes usually lead to large changes in readily observed light-scattering properties. Indeed, most of them were first detected by photometric methods.

One such conformational change is the increase in cell volume caused by ordinary growth. Others involve changes in the size or shape of cells or large structures while total dry weight remains constant. They occur in response to respiratory processes in mitochrondria (1), photosynthetic processes in chloroplasts or cells (2), nerve activity in axons (3), contraction of muscle fibers (4), the initiation of hemostasis by platelets (5, 6), and volume changes that follow changes in the osmotic environment of the cell (7).

Light and electron microscopy have generally been our only sources of information about cell size, shape, and structure. However, several new ones that use the electrical, fluorescent, and light-scattering properties of cells have begun to provide useful information about cell size and shape (8–10).

Some studies have advocated the use of scattering techniques as sources of morphological information on the basis of exploratory theoretical calculations only (11–17). Then to show that such suggestions are realistic, certain optical properties of cells have been measured and compared with those theoretically calculated from independently measured morphological

properties (18–24). Despite interesting cell-to-cell differences, the results clearly demonstrate that many observable optical properties of typical cell suspensions are relatively simple functions of basic morphological properties such as average cell size and shape. Hence, it is now realistic to expect the first-order scattering properties of biological cells to approximate those of simple particle models.

A logical step in the development of light-scattering techniques for determining morphological properties of cells is to try them in realistic model experiments. We presently carry out several such experiments in which cell size and shape and changes therein are measured by photometry and by microscopy. The results are compared in terms of practical effective resolving powers.

INFORMATION THEORY

Information theory (25–28) offers an overview of the potential of an analytical technique. We use it to quantitate the information provided by microscopic and photometric measurements. The information, H, contained in the results of such a measurement, a "message," can be measured in bits. A bit is defined in terms of the probability, P, that a certain situation exists before and after the message (experimental result) is received: $H = \log_2 (P_{\text{after}}) / (P_{\text{before}})$.

A photometer accurate to ± 1% of full scale supplies approximately six bits of information per reading (27). To estimate the information content of a microscope image, we examined another image, a picture in the catalogue of a laboratory supply house. It was printed in a grid of discrete elements, 0.2 × 0.2 mm. Each element may take one of eight degrees of darkness from completely white to completely black. It then can contain three bits of information. A 1-cm^2 portion of this picture, 2,500 grid elements, can contain 7,500 bits of information. Most microscope images contain information of the same order as that of 1 cm^2 of this picture.

The photometer reading (6 bits) is found to be no match for the microscope image (7,500 bits) in terms of total information. A picture is worth a thousand words! Indeed, the microscope is without challenge in terms of the total amount of information that it can supply about cell morphology.

On the other hand, only a small fraction of the information of a microscopy image actually relates to absolute cell size of shape, or changes therein. Only those grid points close to the cell edges significantly contribute. In practice, the recognition of the cell boundary normally uses less thant three bits per element. Furthermore, information about the absolute location of a cell edge is not relevant; only the relative positions of the different parts of the image of a cell boundary are of interest. The vast majority of the information in the image is wasted in simple determinations of cell size and shape. The useful fraction of this information is clearly a small one.

Information theory accounts for the reputation of microscopy as the overall prime source of general information about cell morphology. A good cytologist indeed learns to use a substantial part of the microscope's information in one way or another. However, the specific information about cell size and shape contained in a photomicrograph is clearly limited. In fact, this information may be no greater than that of a set of photometer readings.

APPROACH

The usefulness of a tool for measuring cell size, etc., depends on the resolving power of the instrument, the size of likely errors, the time required for measurements, the nature of the sample, the difficulty of the sample preparation, etc. Model experiments that account for some of these factors are presently used in appraising the techniques.

The visible light microscope (LM) resolves $\simeq 0.1$ μm; the scanning electron microscope (SEM) $\simeq 0.01$ μm. The sample cells are usually fixed for careful examination with the LM, i.e., with stain or glutaraldehyde; only occasionally are the live cells used. Examination by SEM requires that the cells be first dehydrated, then coated with a conducting layer. This process kills the cells and it may also alter their dimensions. A microscope is normally used to examine only a few cells, i.e., 1–100. In this case sampling errors can be important if the cells are not identical.

It is assumed that scattered light can be measured accurately to within 1% of full scale. When transmittance is measured to obtain extinction (absorbance, optical density, $E = \log_{10}[T^{-1}]$), it is assumed that $E \simeq 1.0$ and that the uncertainty in the observed value, $\triangle E$, is $\pm$ 0.01. This corresponds to an uncertainty in the transmittance of 0.2%. The ordinary photometer examines $\sim 10^6$ live cells simultaneously so that sampling errors are unimportant.

Our model cells are spherical or spheroidal, i.e., oblate ellipsoids of revolution of axes a, a, and av. The nominal cell volume, V, is 5.8 μm^3, v is either 0.4 or 1.0, and the relative refractive index is $n = 1.04$. The wave length, λ, of the light in the medium is 0.5 μm. These parameters approximate those of blood platelets in plasma (23, 29).

Our model cell populations are homogeneous or heterogeneous in either size, shape, or refractive index. On the basis of measurements of yeast cells and platelets of natural cell populations, we use model cell populations heterogeneous in size with a coefficient of variation of cell volume, σ_V/V, of 0.45; that of cell diameter is 0.13. For cells heterogeneous in shape, $v = 0.40$ on the average and $\sigma_v = 0.18$. For cells heterogeneous in refractive index, nominally $(n-1) = 0.040$ and $\sigma_{n-1} = 0.018$. These model populations are more heterogeneous than many natural ones.

If cell concentration is known, transmittance, T, of a nonabsorbing population of live cells can be used to obtain information about average cell size or shape. Transmittance is given by $T = e^{-NLR_{ext}}$ where N is the number of particles per unit volume of suspension, L is the path length of the light beam in the sample, and R_{ext} is the extinction cross section of a single cell due to total scattering at all angles and to absorption. Alternately, one may measure the angular dependence of scattering, $I(\theta)$, which is proportional to the differential scattering cross section, $\sigma(\theta)$. This is not to be confused with the Gaussian error function, σ. R_{ext} and $\sigma(\theta)$ of spheroidal cells were calculated (30–32) with the Anomalous Diffraction Method I for R_{ext} and the Rayleigh-Debye Method I and $\sigma(\theta)$. For the spheroidal cells, R_{ext} and $\sigma(\theta)$ are the suitably weighted averages over all possible orientations. For heterogeneous populations they are also averaged over all population classes.

MICROSCOPIC ASSAYS

The tasks of determining average cell size and shape and changes therein are not among those for which microscopy is the best suited. Indeed, those who use microscopes routinely seldom

use them specifically to obtain this type of information. However, despite its limitations, the microscope has been the only potential source of such information. We now assess the expected influence of normal experimental errors on microscopic measurements of cell size and shape.

The diameter of a sphere of volume 5.8 μm^3 is 2.23 μm. To microscopically measure it, one locates opposite edges. With a light microscope, each is located to within ± 0.1 μm. If the errors are random, the net uncertainty (33) in the measured diameter will be $\sigma = (0.1^2 + 0.1^2)^{1/2} = 0.14$ μm. Limited microscope resolution introduces a fractional uncertainty in cell diameter of 0.14/2.33 or 6.3%, that in the cell volume of 20%. For the SEM that resolves 0.01 μm, these figures are, respectively, 0.6 and 2.0%.

The measurement of the average size of cells in a heterogeneous population ($\sigma_V/V = 0.45$) requires random sampling. If only a single cell were measured, the best estimate of average diameter would be 2.23 ± 0.29 μm, the uncertainty being due to sampling errors. The net uncertainity in diameter due to both sampling errors and limited resolution of a LM then becomes $(0.29^2 + 0.14^2)^{1/2} = 0.32$ μm; that for the SEM would be $\simeq 0.29$ μm.

If n cells were measured instead of one, the effects of sampling errors would be reduced by the factor $n^{-1/2}$. If $n = 100$, the sampling uncertainty would be 0.029 μm. The net uncertainty of the LM measurement becomes $(0.029^2 + 0.14^2)^{1/2} = 0.143$ μm; that for the SEM is 0.037 μm. In addition, systematic errors relating to sample preparation could be expected. Even for large n, we estimate that the net practical minimum uncertainty in average sphere diameter as measured with an SEM is at least 0.04 μm, 0.2 μm as measured with LM.

If the sample cells were spheroidal rather than spherical, both the major and minor axes would have to be measured to evaluate cell size and shape. This is more difficult. For instance, oblate cells like platelets and erythrocytes tend to settle onto the slide during microscope examination. Then they present only flat surfaces to the viewer; cell thickness cannot be seen and measured. This problem can be circumvented (23), but only with some effort.

If changes in cell size or shape are to be measured microscopically, they must be obtained from the difference in the measured properties of separate random samples taken before and after the conformational change. The same cells cannot usually be used for both measurements because fixation for microscopic examination kills them. Hence, a conformational change can be determined only by evaluating a small difference between two large and independently measured quantities. This is a classically difficult problem. The minimum uncertainty in the change in diameter would be the above estimated uncertainties times $2^{1/2}$: 0.06 μm for the SEM, 0.28 μm for the LM.

PHOTOMETRIC ASSAYS

Absolute Particle Size (V)

Small-angle scattering depends strongly on cell size. The scattered light varies with angle more or less in accord with the well known equation for single slit diffraction (34). Hence, scattered light measured at two or more angles can then be used to determine cell size.

One such method (32) evaluates size from the ratio of the intensities of the light scattered at 5 and 10°. The ratio I(10°):I(5°), σ(10°):σ(5°), was calculated as a function of cell size for randomly oriented spheroids. Size could be expressed in terms of volume, V, or radius, a.

However, for spheroids, we shall express cell size as the diameter ($2a_s$) of a corresponding sphere of equal volume. For a homogeneous population, I(10°):I(5°) was found to vary with cell size as shown in the left part of Fig. 1. For corresponding spheres of diameters of up to 3.8 μm, I(10°):I(5°) is found to be a single-valued function of θ. Included in this figure are scale sketches of the two cell models.

A particle parameter, q, may always be expressed as a linear function of the photometric quantity, r, over a limited range, all other factors being constant:

$$a = \alpha r + \beta, \tag{1}$$

where α and β are constants. Then,

$$\alpha = \Delta q / \Delta r. \tag{2}$$

Let q be cell diameter and r be I(10°):(5°). From the indicated increments in Fig. 1, $\Delta q = 1.40 - 1.80$ μm, whereas $\Delta r = 0.534 - 0.360$, so that $\alpha = -2.3$ μm. If each intensity were measured to within 1%, the two could be resolved if they differ by 1.4%. Then, since $r = E \simeq 1.0$, $\Delta r = 0.014$ and $\Delta q = -0.032$ μm. By resolving a difference in light levels of 1.4%, the photometer distinguishes between populations of oblate spheroids of axes of 3.026 and 1.212 μm and of axes 3.069 and 1.229 μm. Differences in spheroid dimensions of 0.02–0.04 μm are thus photometrically resolved.

The calculations were repeated for cell populations heterogeneous in either cell size, shape, or refractive index (model populations of Approach). R_{ext} of each heterogeneous system is the simple average of the values of five classes of cells. The results are shown as unconnected

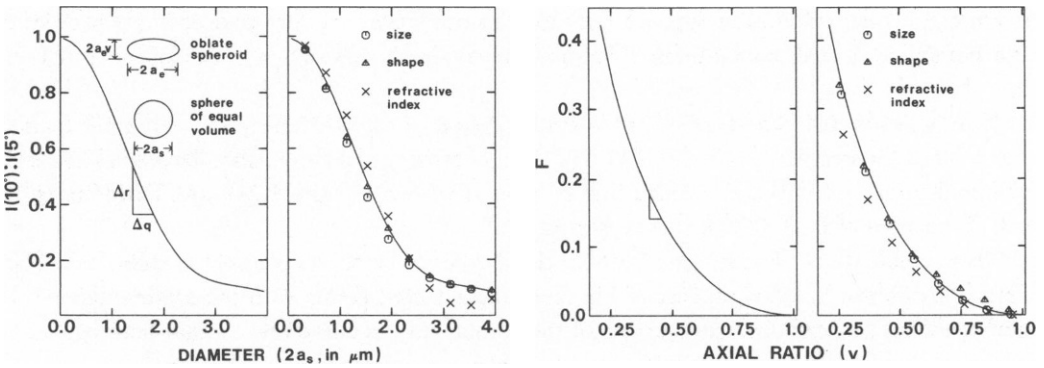

FIGURE 1 The ratio of scattered light intensities at 10 and 5° as a function of cell size; a tool for size determination. Cell size is measured in terms of the diameter of a sphere of equal volume. The smooth curves are for homogeneous suspensions of randomly oriented oblate spheroids ($V = 5.8$ μm^3, $\nu = 0.4$), the unconnected points are for suspensions each heterogeneous in the indicated property. Also shown are scale drawings of the oblate spheroid of axes $2a_s\nu$ and $2a_s$ and of a sphere of equal volume of diameter $2a_s$.

FIGURE 2 The fractional change in extinction of a suspension ($V = 5.8$ μm^3) on turning off the stirrer as a function of cell shape axial ratio. Initially the cells in a stirred suspension are oriented with their flat surfaces perpendicular to the beam. On turning off the stirrer, Brownian motion causes them to become randomly oriented and this changes extinction (23). The smooth lines are for a homogeneous suspension, the points for suspensions heterogeneous in the indicated properties.

points with the original curve for a homogeneous population in the right part of Fig. 1. Most of the points are found to fall close to the curve. Hence, heterogeneity has only a small effect on such determinations of average particle size.

This finding differs from the widely held view that sample heterogeneity obscures the information contained in scattered light. Indeed, heterogeneity can be a serious source of error in molecular weight determinations of small-to-intermediate size particles, i.e., macromolecules. Similarily the usefulness of large-angle scattering by large particles, such as cells, is also sensitive to population heterogeneity (15). However, the methods of this study for large particles are based primarily on small-angle scattering. This is presently found to be insensitive to sample heterogeneity.

Absolute Particle Shape (ν, Axial Ratio)

The average cell shape, ν, is determined from the influence of stirring of the suspension on optical extinction (16, 23). Measured is the light transmitted across the diameter of a cylindrical vessel. The suspension therein is stirred with a magnetic bar to create a vertical vortex. While cells in a static suspension are randomly oriented by Brownian motion, stirring produces another orientation distribution with a different average value of R_{ext}. The effect is quantitated in terms of F, the fractional change in extinction on turning off the stirrer:

$$F = |E_{\text{unstirred}} - E_{\text{stirred}}| / E_{\text{stirred}}.$$

With suspensions of blood platelets ($\nu = 0.4$), we observed F = values of 25–40%. Smaller effects were also found with erythrocytes that are similar in shape but larger (23).

Both the degree of orientation of the cells in the flowing (stirred) suspension and the effects of the shape of R_{ext} increase with asphericity. We assume that ν is independent of the rate of stirring and that orientation depends on ν to the same extent as does that of blood platelets. For homogeneous cell populations, F is found to vary with ν as shown in the left part of Fig. 2.

If $\Delta\nu = 0.408$–0.458 and $\Delta E = 0.177$–0.141, then $\alpha = -1.39$. Hence, if $E \simeq 1$, a resolvable $\Delta E = 0.014$ corresponds to $\Delta\nu = 0.019$. The photometer distinguishes between 5.8 μm^3 spheroids of axes 3.030 and 1.212 μm as compared with 2.980 and 1.249 μm. Differences in cell dimensions of 0.03–0.05 μm are resolved.

These calculations were repeated for the three types of heterogeneous cell populations. The results are shown in the right side of Fig. 2 as unconnected points with the original curve for homogeneous suspensions. Comparison of the points and the curve reveals that heterogeneity has only a small influence on most such photometric measurements of cell shape.

Changes in Cell Size

Cell growth normally increases extinction. To illustrate the measurement of growth without cell division, V was assumed to increase while ν, n, and N remained constant. Extinction of a homogeneous suspension was calculated as a function of V which increases due to growth. The results are shown in the left part of Fig. 3. The increments of V and E indicate that $\alpha = 4.6$ μm^3. Then if E were to increase 1.4%, $\Delta r = 0.014$ so that $\Delta q = 0.065$ μm^3. This change in extinction means that the major axis ($2a$) increase from 3.026 to 3.060 μm and thickness from 1.212 to 1.226 μm. The photometer resolved changes in cell dimensions of 0.01–0.03 μm.

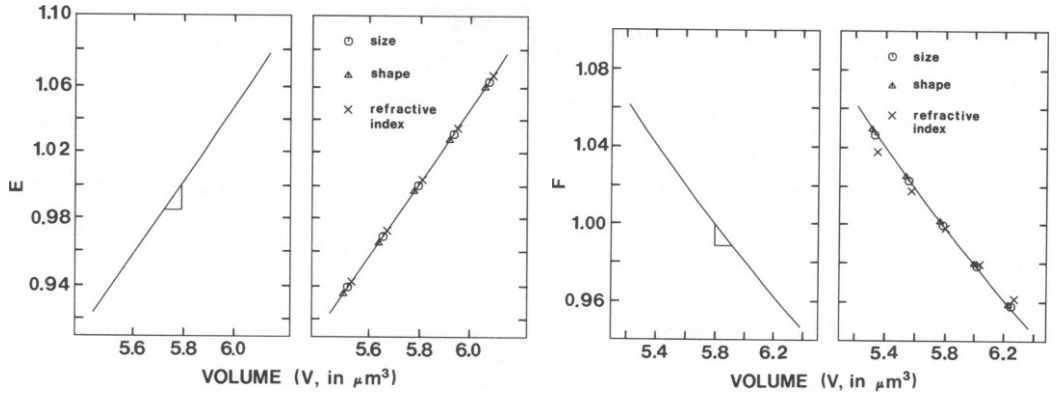

FIGURE 3 Extinction as a function of cell size during growth without division; a tool for observing size. The smooth lines are for a homogeneous suspension; the points are for suspensions of cells heterogeneous in one of the cell properties.

FIGURE 4 Extinction as a function of cell size when dry weight and shape remain constant, a tool for determining changes in size due to the uptake or extrusion of medium ($[n - 1] \sim V^{-1}$). The smooth lines are for a homogeneous suspension, the unconnected points are for suspensions heterogeneous in the indicated properties.

These calculations were repeated for heterogeneous cell populations. Each cell class within a population was assumed to grow by the same percentage of itself. The predicted extinctions for heterogeneous populations are shown as connected points in the right part of Fig. 3 with the original curve for a homogeneous system. The points fall close to the curve for a homogeneous suspension indicating that this method is insensitive to heterogeneity.

In another type of change in cell conformation, the cells swell or shrink because medium flows in or out of the cell. Dry weight and shape remain constant while cell size and refractive index change: $(n - 1) \sim V^{-1}$. The influence of such conformational changes on extinction and scattering has been studied theoretically (11, 12, 15) and experimentally (19, 20).

To determine how well the transmittance photometer can resolve such a volume change, E was calculated as a function of V for a homogeneous suspension of spheroids. The results are shown in the left part of Fig. 4. It is found that $\alpha = -10.5$. In this case, a photometer that resolves a change in E of 0.014 can resolve a volume change of 0.146 μm^3, i.e., when volume increases from 5.800 to 5.946 μm^3. The major axis ($2a$) increases from 3.030 to 3.055 μm and the minor axis ($2av$) from 1.212 to 1.222 μm. The photometer resolves changes in cell dimensions of 0.01 – 0.03 μm.

These calculations were repeated for suspensions heterogeneous in cell size, shape, and refraction. E was found to vary with average cell volume as indicated by the unconnected points in the right side of Fig. 4. The points fall close to the curve for a homogeneous system meaning that heterogeneity has only a small influence on such photometric measurements of changes in particle volume.

Changes in Cell Shape

When the shape of the cells changes, the extinction of the suspension usually does also (16, 23, 35, 36). To calibrate a method based on this effect, E of a homogeneous suspension

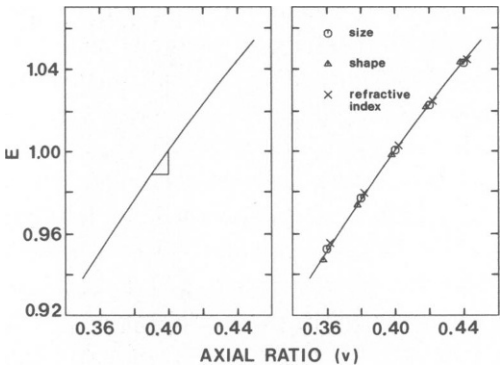

FIGURE 5 Extinction as a function of cell shape, v; a tool for measuring changes in particle shape while cell volume and dry weight remain constant. The smooth curves refer to homogeneous suspensions, the unconnected points to heterogeneous ones ($\bar{V} = 5.8\ \mu m^3$).

was calculated as a function of v, constant cell volume V, and dry mass. The results are shown in the left part of Fig. 5. The indicated increments of E and v give $\alpha = 0.91$. Hence, a photometer can resolve cells of equal volume when $v = 0.400$ and 0.413: i.e., their respective dimensions are 3.026 and 2.994 μm and thicknesses ($2av$) are 1.210 and 1.235 μm. The photometer resolves changes in cell dimensions of $\simeq 0.03\ \mu$m.

These calculations were repeated for heterogeneous suspensions. The right side of Fig. 5 shows the results of such calculations for heterogeneous populations as unconnected points and also the original curve for a homogeneous population. The points fall quite close to the line, thus indicating that cell heterogeneity has a negligible influence on the photometric measurement of systematic changes in shape.

DISCUSSION

For simplicity we have limited considerations to one cell size and two shapes. The question then arises, could similar calibrations be expected for cells of other sizes?

Scattering theory (37) tells us that the extinction cross section, which is involved in four out of five of our model experiments, is controlled by two factors: $R_{ext} = A\,K_{ext}$, where A is the projected area presented to the beam by the cell and K_{ext} is the extinction efficiency of the cell. K_{ext} is controlled by the phase shift of light passing through the cell. This is in turn controlled by cell thickness and refractive index. Differences in cell dimensions, etc., calculated here reflect the combined effects of these parameters on A and K_{ext}. The proportionate effects of differences in cell size or shape on A are independent of absolute cell size and should be the same for cells of all sizes. On the other hand, the effects of a difference in particle parameters on K_{ext} vary slowly with particle size. As a result, the various methods illustrated above could be expected to have somewhat different absolute resolving powers for cells of different sizes and shapes. Related to this is the fact that somewhat different absolute resolutions were obtained above with the different methods. However, all are of the same order.

Other studies (11, 12, 15, 19–21) have provided an in-depth view of the optical effects of volume changes with no change in dry weight (Fig. 4). While the extent of such optical changes is found to depend on original particle size and shape, those papers indicate that the

present parameters ($V = 5.8\ \mu m^3$) should yield representative optical changes. Hence, the present results should be typical of that for most biological cells.

In an experimental/theoretical study (22) of the angular dependence of scattering by *E. coli* cells, ordinary heterogeneous cell populations were found to produce well-defined scattering minima at angles controlled by the uniform minor axes of the prolate spheroids. As a variation on the above method for determining cell size, those results can be interpreted as experimental measures of average cell width. Then the scattering photometer in those experiments resolved $\simeq 0.1\ \mu m$. This is of the order of what was found theoretically above.

Other experimentally observed changes in cell size (18, 20, 21) and shape (23) have also been shown to obey light-scattering theory. These sets of results can be interpreted as providing experimental confirmation of predicted photometric resolutions of $< 0.1\ \mu m$.

A measure of resolving power is suggested by the well-known criteria for small or point-particle scattering. A particle is generally said to be a "small", i.e., a "Rayleigh" scatterer, if it is no larger than $\simeq \lambda/10$. For visible light this corresponds to particles of dimensions of $0.05\ \mu m$. This is the approximate resolution level found above.

Several experimental studies in this lab (18, 20–23) observed interesting optical effects with suspensions of biological cells. Microscopy and other techniques were used to independently determine cell parameters. The results were put into conventional light scattering equations to account for the observed light.

These studies admittedly did not have the benefit of the best cytological expertise or equipment. However, these limitations usually were not the important ones. The reliability of the independent characterizations of the cells appeared to rest mainly on problems like those described above. To account for the observed optical properties in those studies, we tacitly assumed microscopy, etc., to be the primary and reliable sources of physical truth. Photometry was the technique to be proven. However, when conflicts arose, it frequently seemed that it would have been more appropriate to reverse the logic and consider photometry as the primary source of information about physical facts and microscopy to be the technique being tested. The above results support this idea.

The present study is a "theoretical" one; all "experiments" were carried out on paper rather than in the laboratory. However, the impetus for it originated with real practical problems encountered in the laboratory.

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