Single Cell Volume Measurement by Quantitative Phase Microscopy (QPM): A Case Study of Erythrocyte Morphology

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Key Words

Quantitative phase microscopy • Erythrocyte • Osmolality • Refractive index • Tonicity • Rat • Optical thickness • Image analysis

Abstract

The measurement of the volume of intact, viable cells presents challenging problems in many areas of experimental and diagnostic science involved in the evaluation of cellular morphology, growth and function. This investigation details the implementation of a recently developed quantitative phase microscopy (QPM) method to measure the volume of erythrocytes under a range of osmotic conditions. QPM is a computational approach which utilizes simple bright field optics to generate cell phase maps which, together with knowledge of the cellular refractive index, may be used to measure cellular volume. Rat erythrocytes incubated in imidazole-buffered solutions (22°C) of graded tonicity were analysed using QPM (n=10 cells/group, x63, 0.8 NA objective). Erythrocyte refractive index (1.367) was measured using a combination of phase and morphological data obtained from cells adopting spherical geometry under hypotonic conditions. Phase-computed volume

increased with decreasing solution osmolality: 42.8 ± 2.4 , 48.7 ± 2.3 , 62.6 ± 2.3 , $90.8 \pm 7.7 \mu\text{m}^3$ in solutions of 540, 400, 240, and 170 mosmol/kg respectively. These volume changes were associated with crenated, bi-concave and spherical morphological states associated with increasing tonicity. This investigation demonstrates that QPM is a valid, simple and non-destructive approach for measuring cellular phase properties and volume. QPM cell volume analysis represents a significant advance in viable cell experimental capability and provides for acquisition of 'real-time' data - an option not previously available using other approaches.

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Introduction

The measurement of the volume of intact, viable cells presents challenging problems in many areas of experimental and diagnostic science involved with the investigation of cellular morphology, growth and function.

A variety of 'interventional' techniques have been previously used to provide estimates of the volume of single cells. These include electrophysiological methods requiring electrode attachment or impalement including

capacitance measurements by patch clamping [1] and intracellular ion activity by ion-sensitive microelectrodes [2]. Methods for measurement of the volume of substrate attached cells by scanning ion conductance microscopy, using micropipettes to traverse the cell have also been devised [3]. These techniques are technically demanding, difficult to implement for more than a small number of cells, have limited spatial resolution [3] and are not amenable to repetition.

The mean cell volume for cell populations may be assessed indirectly using Coulter counting [4], flow cytometry [5] or radioisotope labelling techniques [6]. While these procedures have the advantage of high through-put, they require presentation of cell specimens in suspension, and also do not permit the tracking of individual cell volume changes experimentally [7].

Imaging methods generally provide less disruptive and less interventional means for assessing cell volume. For many investigations, the use of perimeter tracing or extrapolation from two-dimensional measurements may suffice for an indication of cell size or relative change in size, particularly when cell morphology is known, stable and simple [8, 9]. More sophisticated optical methods have been developed which provide absolute volumetric determinations including interferometry [10], optical sectioning by light microscopy [11] and by laser scanning confocal microscopy [1], light scattering detection [12, 13], quantitative fluorescence microscopy [14, 15], video-enhanced contrast optical microscopy [16], light microscopy with spatial filtering [10] and three-dimensional fluorescence imaging [17]. Many of these methodologies necessitate complex microscope configurations and/or laborious imaging and computational procedures. Others involve the introduction of light-sensitive fluorophores into the cell, which ultimately impact on viability and permit data acquisition for only a limited period before photobleaching degrades the signal. Thus, the development of a simple, efficient and non-interventional procedure which allows measurement of single cell volume has clear and extensive application potential.

Optical techniques which recover phase information from cellular samples have the capacity to provide such a measurement. Light propagated through transparent specimens, such as live cells, can be subject to significant phase shift (even when amplitude modulation is minimal), the extent of which depends on the thickness of the specimen. Phase microscopy methods are frequently used to exploit this optical phenomenon to enhance the visualization of cellular materials for qualitative viewing purposes (ie differential interference contrast, DIC). The

potential of quantitative phase recovery as a means to achieve cellular volume rendition using simple optical microscopy has not been previously explored.

This investigation details the utilization of a recently developed quantitative phase microscopy (QPM) method [18-20] to generate cell volume measurements. Rodent erythrocytes were selected as the prototype cell for this investigation as the ability to manipulate cell geometry and volume by modification of the osmotic environment is predictable and has been well documented [21, 22-28]. Furthermore, the erythrocyte internal composition is relatively uniform and unencumbered by nuclear material. We demonstrate that cell volumetric analysis by QPM is feasible and practicable using bright field microscopy under prescribed conditions where cellular refractive index may be determined or assumed. The more general utility of the methodology developed, for a range of cell types, is discussed.

Materials and Methods

Erythrocyte Collection

Erythrocytes were harvested from trunk blood collected from heparinized male Sprague-Dawley rats killed by exsanguination under pentobarbitone anaesthesia (50 mg/kg). Spun cells were transferred to standard imidazole-buffered solutions (22°C) of defined osmolality and graded tonicity (170, 240, 400, 540 mosmol/kg) used for clinical haematological analysis. The buffer stock solution was composed (% w:v specified) of imidazole 6.8, NaCl 11.7, HCl 4.1 and deionised water. Osmolality was adjusted by dilution with de-ionised water and the final osmolality of incubation solutions was determined by vapour pressure osmometry (Wescor 5520 VAPRO, Wescor Inc, Utah, USA). The refractive index of each solution was determined using an Abbe refractometer (Edmund Industrial Optics, New Jersey, USA). Erythrocytes were incubated in one of these four buffers for 10 minutes prior to imaging.

Bright Field Imaging and Phase Map Calculation

Using QPM [18], a cell specimen phase map is produced by applying a computational algorithm to three unfiltered bright-field images: an in-focus and a pair of positive and negative de-focus images (Figure 1). The phase values calculated for each position in the phase map are determined by the magnitude of phase shift ($\Delta\phi$) induced in light traversing the specimen [29]. The magnitude of the phase shift measured at any point

Fig. 1. Schematic representation of the QPM phase map generation. The phase map is calculated from an in-focus and a pair of equidistant de-focus (positive and negative, 0.5 μm) bright field images of rat erythrocytes. While there is little visually discernible difference in the appearance of the bright field images, the phase map generated shows clear evidence of centrally modulated optical thickness.

depends on optical thickness, which is defined as the product of the specimen thickness (t) and refractive index (n):

$$\Delta\Phi = \frac{2\pi}{\lambda} (n_{\text{cell}} - n_{\text{medium}}) t \quad (\text{Equation 1})$$

where n_{medium} is the refractive index of the medium surrounding the cells and λ is the wavelength of the light used to calculate the phase image (in this case 580nm).

Bright field images of erythrocytes were captured using a monochrome 1300 x 1030 pixel Coolsnap FX CCD camera (Roper Scientific) mounted on a Zeiss Axiovert 100M inverted microscope utilising a Zeiss Plan-Neofluar (x63, 0.8 NA) objective. To ensure optimal specimen illumination, Köhler illumination conditions were established for each optical arrangement. In-focus, and equidistant positive and negative de-focus images were acquired (using a de-focus distance of 0.5 μm) to calculate a phase map for each cell. This was achieved using a piezoelectric positioning device (PiFoc, Physik Instrumente, Karlsruhe, Germany) for objective translation. The de-focus distance was determined in relation to the objective numerical aperture and depth of field [20]. Bright field images were subsequently processed to generate phase maps using QPM software (v2.0 IATIA Ltd, Australia). Phase map generation involved software-automated calculation of the rate of change of light intensity between the three bright field images. The derivation and validation of this computational methodology under the conditions specified has been previously demonstrated [19, 20]. Post-acquisition image manipulation procedures described below were performed with IDL V5.6 (Interactive Data Language, Research Systems, USA).

Experimental Approach

Given that the magnitude of the measured phase shift is the product of the specimen thickness and refractive index (Equation 1), it can be predicted that when the specimen refractive index can be estimated, the measurement of the phase shift provides a means by which cell thickness (and therefore volume) is determined in a variety of experimental contexts. Thus, the first stage of this investigation was to find a nominal value for erythrocyte refractive index. Subsequent experiments involved the manipulation of osmotic environment to examine the phase-derived erythrocyte morphometric and volumetric data. Using phase image segmentation and integration analysis techniques, erythrocyte shape and size in incubation solutions of graded tonicity (hypo- to hyper-tonic) was evaluated.

Results

Erythrocyte refractive index determination by phase measurement

Erythrocyte morphology and volume regulatory mechanisms have been extensively studied [21-24, 28]. The morphological transitions exhibited by this cell type in graded osmotic environments are well documented. In hypotonic conditions erythrocyte expansion to spherical geometry may be maintained as a stable morphology if the osmolyte level is titrated to be just sufficient to prevent lysis. Phase analysis of erythrocytes in this stable spherical state was used to obtain an estimate of cell refractive index. As defined in Equation 1 above, if phase shift, specimen thickness and medium refractive index

Fig. 2. Rat erythrocyte morphology in buffer solutions of differing osmolality. For each Panel (A, B & C), the upper image is the bright field in-focus view, the centre image is the calculated phase map, and the lower plot shows the line profile from the phase map obtained at the line position indicated (shown as normalised, relative phase shift values). Panel A. Hypotonic (170 mosmol/kg) solution. Line profile plot through cell diameter shows phase modulation indicative of spherical morphology. Panel B. Near-isotonic (240 mosmol/kg) solution. Line profile plot through cell diameter shows phase modulation indicative of bi-concave morphology. Panel C. Hypertonic (540 mosmol/kg) solution. Line profile plot through cell diameter shows phase modulation indicative of crenated morphology.

Fig. 3. Cellular volume measurements using QPM. Edge detection and segmentation applied to a bright field image (Panels A & B) and a phase map (Panels C & D) obtained from a single erythrocyte (240 mosmol/kg). (Achroplan, x63, NA 0.80). Panel A. Bright field (in-focus) image of rat erythrocyte. Panel B. Attempted segmentation of bright field image. The poor contrast between the cell and the background renders edge detection unsuccessful. Panel C. QPM calculated phase map of same erythrocyte. Panel D. Binary image generated by segmentation of the phase map where clear delineation between the cell and background may be achieved. Thickness measurements obtained at every point identified to be within cell boundary are integrated to calculate volume.

(n_{medium}) are measurable, then a value for refractive index (n_{cell}) may be determined. For a spherical erythrocyte, maximum depth can be inferred from measurement of maximum width - a dimension which is easily obtained microscopically under bright field conditions.

In hypotonic buffer (170 mosmol/kg), erythrocyte spherical morphology was confirmed by phase map evaluation. For erythrocytes incubated in this buffer, a line profile of phase values across the cell diameter typically generated a hyperbolic plot, as shown in Figure 2A. Under these conditions the difference in refractive

index between the cell phase maxima and the incubating buffer was 0.027 ± 0.003 , corresponding to a erythrocyte diameter measurement of $6.0 \pm 0.7 \mu\text{m}$. Using the buffer refractive index measured by refractometry to be 1.3400 ± 0.003 , the value for erythrocyte refractive index obtained by solving Equation 1. was 1.367 ± 0.003 . This general

Fig. 4. Single erythrocyte volume measurement by QPM. Panel A. Mean ($\pm$ sem) erythrocyte volume measured in buffer solutions of different tonicity (n=10-13 erythrocytes per solution). Panel B. Phase derived rendered volume representation of erythrocyte in 170 mosmol/kg solution (spherical). Panel C. Phase derived rendered volume representation of erythrocyte in 240 mosmol/kg solution (bi-concave). Panel D. Phase derived rendered volume representation of erythrocyte in 540 mosmol/kg solution (crenated).

value for erythrocyte refractive index was then applied in experimental situations where phase shift could be measured, to obtain a value for thickness at any cellular position (using Equation 1) in erythrocytes exhibiting less regular geometry.

Phase effects of osmotic manipulation indicative of different erythrocyte morphological states

Erythrocyte morphology in solutions of different osmotic composition was examined by phase map analysis. As detailed above, hypotonicity of the incubation solution (170 mosmol/kg) rendered cells spherical and the associated phase line profile plots exhibited central maxima (Figure 2A). Under osmotic conditions closer to isotonic (240 mosmol/kg) the erythrocyte morphology indicated biconcavity, associated with a distinctly annular phase map yielding a characteristic dual peak phase line profile (Figure 2B). Although the bright field erythrocyte images from which the phase maps were derived were suggestive of a 'dimpled' morphology, only in the phase map representation was the biconcavity clearly delineated.

In solutions of higher tonicity (400 & 540 mosmol/kg) erythrocyte geometry appeared less uniform. The 'crumpled' appearance of the cell visualized under bright field conditions was associated with edge irregularity and loss of biconcave disk structure consistent with crenation. These features were accentuated in phase map representations (as shown in Figure 2C) with indicators of biconcavity or sphericity absent from the phase line profile.

Phase calculated erythrocyte volume

Volumetric analyses of erythrocytes incubated in solutions of varying tonicity were performed using QPM. This process involved the use of Equation 1 to solve for thickness at each pixel position within the cell, based on the measured extent of phase shift combined with the pre-determined value for refractive index. This process required accurate delineation of cellular boundaries, in order that the phase contribution from the incubation solution could be excluded. As depicted in Figure 3, standard techniques of nearest neighbour gradient thresholding (IDL V5.5) applied to erythrocyte bright field images (Figure 3A) did not generally allow successful edge detection for the complete cell perimeter (Figure 3B). However similar techniques applied to phase maps (Figure 3C) derived from the same cells provided for very effective thresholding and segmentation (Figures 3D) of the cell-delineated areas. To determine the cell-specific phase shift for the cell delineated image area, a background subtraction was performed. An average phase value was obtained for all pixels deemed extracellular and this was subtracted, effectively setting the background to zero. Integration of the cell thickness values calculated for all pixels deemed intracellular generated a cell volume measurement. The mean refractive indices of the solutions of different osmotic composition were not different (1.3400).

Figure 4A presents volumetric data obtained for cells in hypotonic (170 mosmol/kg), near isotonic (240 mosmol/kg) and hypertonic (400 & 540 mosmol/kg) environments.

As expected, an inverse relationship between osmolality and erythrocyte volume was observed. At 240 mosmol/kg, with tonicity approaching physiologic conditions, the mean cell volume was $62.6 \pm 2.3 \mu\text{m}^3$. Three dimensional rendering (Figures 4B-D) of erythrocyte volume in these physiological conditions illustrates the pronounced extent of concavity, with the central portion of the disk of minimal thickness. Cell swelling in hypotonic solution increased volume by 45%, while crenated cells exhibited a shrinkage of about 32% (at 540 mosmol/kg) relative to the volume measured at 240 mosmol/kg under conditions nearest physiological tonicity.

Discussion

In this investigation an innovative, non-destructive method for measuring viable cell volume using the newly developed technique of quantitative phase microscopy (QPM) has been implemented. Measurement of cellular volume by QPM involves the mathematical manipulation of data extracted from a set of three bright field images acquired under simple optical conditions. A phase map is generated, and with knowledge (or assumption) of a value for cell refractive index, the phase data embedded in the map are used to determine volume by an integration process. With this technique it is not necessary to introduce dyes or probes for cellular visualization and there is no requirement for any specific incubation medium. Cellular specimens may be serially imaged to perform phase map calculations without functional or structural detriment.

From Figure 4A, a value for mean rat erythrocyte volume at isotonic conditions (290 mosmol/kg) may be interpolated - approximately $58 \mu\text{m}^3$. This value is somewhat higher than the previously reported values of $41 \mu\text{m}^3$ [30] and $55 \mu\text{m}^3$ [31] measured under physiologic conditions. These earlier values were determined using methodology combining haematocrit and cell count data, and are surprisingly discrepant. It is likely that the direct measurement technique applied in the present study involving individual cells provides an inherently more accurate measurement.

Volume measurements in this study were conducted on cells after 10 minutes incubation in various hypo- and hypertonic solutions. Comparing the highest and lowest levels of tonicity tested, erythrocytes exhibited a more than 100% increase in mean volume. After 10 minutes it would be expected that the cellular regulatory volume responses would have largely offset the initial acute solvent flux associated with solution transition. However,

it is possible that slower time course response components may not have been complete and these volume estimates do not entirely represent the steady state [21, 32]. Whilst the goal of this investigation was not to evaluate these temporal responses *per se*, it is evident that QPM offers a valuable tool for the study of regulatory volume behaviour at the single cell level.

The erythrocyte was selected for this investigation as a 'prototype' cell which exhibits convenient spherical geometry under defined hypotonic conditions, thus facilitating optical analysis. These investigations show that QPM-based erythrocyte screening may also have clinical application in detecting abnormal cell geometry and volume. Erythrocyte deformability is an important determinant of rheological behaviour, and is a direct consequence of spheroidicity [28] - a structural attribute which can be readily examined using QPM. Evaluation of the morphological impact of cytotoxic anti-neoplastic therapeutic agents on erythrocyte morphology in particular [33] would also be amenable to QPM.

The QPM methodology described has general application potential for cell volume measurement in a variety of cell types including epithelial, muscle, neural and stem cells [34-36]. QPM may be applied to cell suspensions, cultures and sections with appropriate specification of optical and imaging conditions. Consideration and validation of the QPM methodology in relation to magnification selected, depth of field of objective and specimen dimensional aspects has been previously documented [20]. The absolute accuracy of the volume measurement relies on the reproducibility and precision with which the refractive index may be determined for any particular cell type. In the present study, the refractive index estimate made on the basis of geometrical assumption, could be reasonably validated by the examination of phase line profile plots. Some error may be introduced as the refractive index measured under hypotonic conditions may not strictly apply to cells in iso- and hypertonic conditions. The maximal error in volume calculation produced by assuming a similar index for all these osmotic states is not more than 2%, and likely substantially less. The value for rat erythrocyte refractive index reported in this study (1.367) is somewhat lower than a value previously determined (1.375) by immersion 'refraction-matching' methods for human erythrocytes [37]. Species differences and/or temperature differences (22 °C versus 37 °C) may at least partially account for this modest discrepancy. In the present study, refractive index determination was not the primary goal. We have previously described a methodology involving combined

QPM and confocal 'optical slicing' techniques which enables a more rigorous measurement of refractive index where this is required [35]. In many experimental circumstances an absolute volume determination is not required, and relative volume changes are more relevant. In these situations, as Equation 1 specifies, there is a linear relationship between phase and volume, and a nominal or assumed constant value for refractive index is sufficient for volume tracking purposes.

In summary, this investigation has described the implementation of QPM in the non-destructive, non-interventional measurement of viable cell volume using the osmotically challenged erythrocyte as a prototype experimental system. The validation of QPM as a method for cell volume analysis represents a significant advance

in real-time experimental capability and yields data not previously available using other approaches. The relative simplicity and economy of the optics required, combined with the ease of implementation, enhances experimental feasibility and scope.

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