Influence of defocus on quantitative analysis of microscopic objects and individual cells with digital holography

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Abstract: Digital holography offers a unique method for studying microscopic objects using quantitative measurements of the optical phase delays of transmitted light. The optical phase may be integrated across the object to produce an optical volume measurement, a parameter related to dry mass by a simple scaling factor. While digital holography is useful for comparing the properties of microscopic objects, especially cells, we show here that quantitative comparisons of optical phase can be influenced by the focal plane of the measurement. Although holographic images can be refocused digitally using Fresnel propagation, ambiguity can result if this aspect is not carefully controlled. We demonstrate that microscopic objects can be accurately profiled by employing a digital refocusing method to analyze phase profiles of polystyrene microspheres and red blood cells.

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References and links

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1. Introduction

Quantitative phase microscopy (QPM) has seen much recent application to imaging of microscopic biological samples [1]. Targets have included assessment of microscopic objects such as growth of biological cells [2], changes in cell volume due to osmotic pressure [3], imaging of neurons [4, 5], and red blood cells [6]. These studies typically present quantitative comparisons across a population of cells and often use a digital refocusing approach to place the objects of interest at the focal plane. However, the success of the refocusing methods is usually evaluated with a qualitative metric in these studies, i.e., visual quality of refocused images, causing some uncertainty in the comparison of the obtained results [7–10]. To address this limitation, we now present quantitative comparisons of microscopic objects and examine how defocus error can influence these comparisons.

The dry mass (DM) metric, published concurrently by Davies and Wilkins [11] and Baber [12] is a fairly widely used means for characterizing the contents of microscopic objects, based on optical phase measurements. The dry mass of an object is defined as

\[ DM = \left( \int \int \phi(x,y) \, dx \, dy \right) / \alpha, \]

where \( \Delta \phi \) is the integrated phase measurement, and \( \alpha \) is the refractive index increment factor given as refractive index per mass. Dry mass is an attractive parameter for analysis via QPM since it does not explicitly depend on knowledge of the refractive index of the sample. However, the DM measurement is limited by knowledge of \( \alpha \) which is often taken as a constant, assumed value [13–15].

Several analyses of cell properties have been presented based on evaluating dry mass using quantitative phase imaging [16–18]. These studies of dry mass are ultimately limited by knowledge of \( \alpha \) and in some scenarios, it may be preferable to leave the analysis in terms of optical volume. For example, when analyzing dry mass of a dynamic cell sample, the selection of the true focal plane can influence the measurement as we will show below. This error was previously considered by Levin et al. [19] but only as an effect due to instrument drift rather than arising from a dynamic sample.

In our analysis below, changes in optical path length, \( \Delta OPL(x,y) = \Delta \phi(x,y)/k = \Delta n(x,y) \cdot h(x,y) \) are used to describe microscopic objects, rather than dry mass; here \( h(x,y) \) is the thickness (height) map of the object, \( \Delta n (x,y) \) is the map of refractive index and \( k = 2 \pi/\lambda \) is the wavenumber. The \( \Delta OPL \) parameter can be integrated to yield a volume metric, which we define here as Optical Volume (OV):

\[ OV = \left( \int \int \Delta OPL(x,y) \, dx \, dy \right) = \left( \int \int \Delta n(x,y) \cdot h(x,y) \, dx \, dy \right). \]

Note that if the refractive index of the object is assumed to be homogeneous, i.e., \( \Delta n(x,y) = \Delta \bar{n} \), then the optical volume is linearly related to the true volume of the object \( OV = \Delta \bar{n} \cdot V \).
The optical volume can then be scaled by the refractive index increment factor $\alpha$ to produce the dry mass measurement, $DM = OV / \alpha$. However, the $OV$ measurement offers the advantage that it does not rely on an assumed constant value of $\alpha$.

To analyze the influence of defocus on these parameters ($DM$, $OV$), we present quantitative phase analysis of several microscopic objects. First we analyze a technical microscopic object with well characterized properties and illustrate the limitations in accuracy that arise from defocus. By using a digital refocusing algorithm, we show that the accuracy of $OV$ (and thus $DM$) measurements can be preserved. This approach is validated with analysis of QPM images of a single red blood cell subjected to manual defocus and another subjected to a high shear flow. In both cases, the images are refocused to show that $OV$ and $DM$ can be used robustly for characterization in spite of defocus.

2. Methods

To demonstrate the influence of refocusing on analysis of microscopic objects, polystyrene NIST-certified microspheres with known refractive indices and sizes (Duke Scientific, $n_D = 1.5916$) were imaged by quantitative phase microscopy ($\lambda = 589$ nm, $\delta \lambda = 1.12$ nm). Figure 1 shows a schematic of the imaging system as well as typical phase and amplitude images of the microspheres. Four nominal sizes were used: $d = 4.000 \mu m \pm 1\%$ coefficient of variation (C.V.), $d = 6.007 \mu m \pm 1\%$ C.V., $d = 6.982 \mu m \pm 1\%$ C.V., and $d = 7.979 \mu m \pm 1.1\%$ C.V.. Each microsphere sample was washed with DI water twice and placed in vacuum overnight to fully dehydrate the spheres. The spheres were then diluted in immersion oil (Cargille, $n_D = 1.5150$) and loaded into rectangular capillary tubes (Vitro, $d = 0.3 \text{ mm}$) and allowed to settle to the bottom surface overnight. Each of the four samples were then imaged via QPM after manually focusing the sample; each interferogram contained 2-5 microspheres that were spatially separated, and 30 microsphere images were captured for each sample.

Interferograms were processed using standard methods for off-axis digital holography [20]: phase and amplitude images were recovered, referenced to background holograms captured of oil-only fields of view (FOV), and then fit to 5th-order polynomials to remove
any background phase and amplitude structure. The complex field obtained for each FOV was then refocused using a Fresnel transformation to digitally propagate the wavefront across a range of $-25 \, \mu m$ to $25 \, \mu m$ in $0.5 \, \mu m$ steps (100 total positions). In this approach, the complex field from a given plane is Fourier transformed to obtain an angular spectrum. A linear dispersive filter is then applied to obtain the angular spectrum for the field in another plane and then Fourier transformed back [21]. The propagation distance in the image plane was related to defocus in the object plane using the optical magnification of the system, determined by the focal lengths of the microscope objective and tube lens, $L_1$. The magnification was verified by analyzing the focused image of a resolution target. We note that the size of the reconstructed image does not change with propagation distance for this propagation method as discussed by Grilli, et al. [22]. However, the ability to accurately determine defocus distance in the object plane does depend on the accuracy of the system magnification. Once the field has been propagated, its amplitude was used to assess the true focal plane of the object by calculating the minimum of the amplitude variance.

A variety of methods for assessing the best-focus plane have been proposed by several groups [7–10]. We have selected minimization of amplitude variance as the metric for these studies after trying several of these approaches and comparing their performance for the objects we have imaged. As observed by Langehanenberg, et al., the amplitude variance metric provides a balance between the focus criterion’s sharpness in $z$ and the functional refocusing range when evaluating transmission-geometry phase images, and is computationally very light weight [8]. It should also be noted that in this referenced work, all four of the metrics compared side-by-side find the same best-focus plane within the error of measurement when refocusing individual cells.

Selection of the criteria for assessing the plane of best-focus in digitally propagated holographic images is not always straightforward. When algorithms are validated using simulated defocused data, it is straightforward to determine the correct focal plane. However when assessing measured holograms where the initial defocus distance is unknown, there is no quantitative validation that a “best focus” plane corresponds to the optimization of the focus metric apart from the visual quality of the reconstructed images. By using a metric that we can verify, here the $OV$ of a microsphere, we can obtain confirmation that our metric is valid.

Fig. 2. Digital refocusing of a single microsphere image. (a) $xz$-slice of amplitude focus; (b) representative phase images of microsphere at multiple propagation distances; (c) amplitude variance as a function of propagation distance, minimum variance location indicated with arrow; (d) measured microsphere volume vs. focal distance, red line indicates change in volume measurement with defocus distance, dashed lines and blue regions indicate the actual microsphere population distribution, $\pm \sigma$, $2\sigma$, $3\sigma$. 
3. Application to technical samples

Figure 2 illustrates our chosen refocusing procedure as applied to an image of a single 8-μm diameter microsphere. This microsphere has been focused over an expanded z-range of ± 100 μm to illustrate the effects of defocus. As this microsphere image was initially hand-focused, the amplitude cross-section image (z = 0 μm) appears to be well-focused with minimal wavefront variation. The corresponding phase image also appears to be spherical while the defocused phase images quickly lose fidelity. Examining the amplitude variance of the FOV, the minimum variance occurs at z = −2.46 μm. The variance also monotonically increases on either side of the minimum-variance location up to a range of ~ ± 50 μm. When hand-focusing an object prior to image acquisition, it is fairly easy to place the object within ~ ± 20 μm of the true focus; once the microsphere is manually defocused to ± 100 μm, the image quality is extremely poor, and diffraction rings indicate an incorrect focal plane. Because the errors of hand focusing are in-practice very small, this automated refocusing method has the potential to be performed much more efficiently than is presented here without risk of identifying local minima that do not correspond to a best-focus plane.

To examine the effects of defocus on volume and dry mass calculation, the phase image of the 8-μm microsphere in Fig. 2 is analyzed at each defocus distance over the axial range. After phase image referencing and polynomial subtraction, the optical volume (OV) over the displayed region is calculated according to Eq. (2). The physical volume of the microsphere is then calculated using the RIs of both the index-matching oil and polystyrene (Δn = 0.0766). The physical volume can be directly related to the mass of the microspheres using the density of polystyrene (ρ = 1.05 pg / fl). It is important to note that the refractive index increment α which is used for dry mass determination in cases when microscopic objects are composed of soluble materials in varying concentrations; polystyrene is insoluble in water at room temperature and therefore exists as a constant-density so it is not appropriate to use α here.

![Graph showing measured volume changes with defocus](image)

Fig. 3. Volume prediction plots for four microsphere populations. Left: blue dots and bars indicate population mean and standard deviation. Right: Individual measurements for 7-μm diameter microspheres. Red circles indicate measurements of hand-focused images, blue circles indicate digitally-refocused measurements. Dashed lines and blue regions indicate the actual microsphere population distribution, ± σ, 2σ, 3σ. Metrics are reported as means ± standard deviations.

Figure 2 shows that the measured volume changes significantly with defocus position: for every micron of defocus, the measured volume changes by 2.2% (slope of the tangent line at the minimum-variance axial position). Once the microsphere is refocused further than a distance of ± 10 μm, phase wrapping artifacts begin to further degrade the accuracy of the
volume measurement. The NIST-certified microsphere population has a diameter distribution of 1% C.V., which corresponds to a volume (and dry mass) distribution of 3% C.V. Each blue region in Fig. 3(d) represents ±σ. The hand-focused microsphere has a calculated volume of 281.7 fL, which is 5.9% (~2σ) higher than the specified population average volume of 266.0 fL; once digitally-refocused, the microsphere’s volume is measured to be 266.6 fL, within 0.22% of the specified population average volume. This change in volume translates directly into a change in measured dry mass. The differences obtained in this experiment, illustrate that even a slight defocus of 2.46 μm, which is well within the variation of manual focus, can introduce significant error in volume as well as dry mass measurement.

Comparison of the measured volume of the four refocused microsphere populations (n = 30 each) against the reference volumes demonstrates the accuracy of volumetric measurements by QPM (Fig. 3). The dashed line indicates perfect agreement with the nominal volume, while the blue error bars indicate the range of all microspheres measured. The root-mean-square (RMS) errors of all volume measurements is 5.24% and showed no trend with object size. Closer examination of the 7-μm microsphere population (Fig. 3 right) reveals that the hand-focused population mean volume is +11.2% higher than the true population mean with a coefficient of variation of σ = 3.2% (specified CV = 3%). Digital refocusing reduces the population mean volume error to +1.9% and reduces the CV (σ = 2.75%) to less than the specified CV. As noted above, for a well characterized material such as polystyrene, the volume measurements and associated errors translate directly to dry mass through a simple scaling factor (i.e., density) and thus the error analysis is directly applicable to dry mass calculations. We note that the QPM volume measurements after digital refocusing by these methods may in fact be more accurate for individual microspheres, however the variation of microsphere sizes within each population limits our ability to quantify the true bounds.

4. Application to biological samples

To demonstrate the effect of defocus on phase measurement parameters in a biological sample, analysis of a single red blood cell is presented. Images were acquired at 7 various focal planes over a range of ~200μm (Fig. 4). The top row displays the amplitude images recovered from the holograms; this is representative of what a microscope user relies on as a visual guide while attempting to focus the sample prior to acquisition. The bottom row shows the phase images corresponding to each amplitude image above. The center image was judged to be the “best focus plane” by eye when acquiring these images. Each of the manually defocused holograms is refocused over a range of ±150 μm. Each cell’s amplitude variance shows a distinct global minimum, indicating the distance of original defocus. After refocusing each hologram, the resulting phase images are similar. The slight variations in shape are due to the dynamic nature of RBCs where slight membrane fluctuations, internal distribution changes of hemoglobin and other biomolecules, and potential fluid transport across the membrane introduce differences in the morphology of the refocused RBCs across A-G.

We characterize the RBC at each focal depth using a phase derived parameter, OV. Prior to refocusing, the measured OV shows large variation, ranging from 5.15 to 7.03 fL (σ = 13.55%). After refocusing, the measured OV’s range from 5.54 to 5.70 fL (σ = 0.99%), indicating high precision and repeatability of these measurements. There is a slight negative trend in refocused OV from A to G, suggesting that the refocus distance may affect the calculated volume. However, this effect only has a minor influence on the measured parameter.

To illustrate the effect of defocus on the parameters of a dynamic biological sample, QPM images were acquired at 100 frames-per-second (fps) for an RBC subjected to a high flow. Media 1 shows a movie of an RBC adhered to a glass substrate and then subjected to a change in flow rate from 100 μl/min to 4000 μl/min. As can be seen, the RBC rapidly changes shape
when the change in flow occurs and recovers somewhat when the flow is again reduced. Each recorded frame in this movie sequence has been digitally focused using our algorithm.

The effect of defocus on quantitative analysis can be seen by calculating the \( OV \) for each frame in the movie, as plotted in Fig. 5. The blue data points show the \( OV \) calculated directly from each image without using the refocusing algorithm. As can be seen, the \( OV \) apparently increases by 6.56% and remains at this level for the duration of the high flow rate. When the flow rate is lowered, the \( OV \) appears to drop back to nearly the original level but remains 1.66% higher than seen prior to the increased flow. As discussed above, \( OV \) is related to dry mass of the cell via a simple scaling factor. Neither \( OV \) nor \( DM \) should be affected by the change in flow as presumably no material is entering or leaving the cell. Rather the high flow causes the cell to deform and shift focal planes. As it flattens, the focal plane changes and the \( OV \) measurement appears to change due to defocus.

Upon application of our refocusing algorithm, we see that the \( OV \) measurements are restored to produce a result that is consistent across the entire time course of the experiments, spanning the initial increase in flow and the return to lower flow rate (Fig. 5, red data points). Prior to the change in flow, a refocus distance of approximately 3.4 \( \mu m \) is determined by the algorithm and the associated \( OV \) measurement decreases by 0.21 fL. When the flow is increased, the defocus distance changes sharply, increasing to up to 13.0 \( \mu m \) but upon application of the refocusing algorithm, the \( OV \) measurement remains constant within \( \sigma = 0.02 \) fL (\( \sigma = 0.28\% \)), shown by the red band in Fig. 5. Once the flow is reduced, the defocus distance decreases to nearly the initial value but remains slightly elevated. The use of the refocusing algorithm results in a consistent \( OV \) measurement during and after the decrease in
flow. As noted above, the OV measurement is simply related to DM using a simple scaling factor and thus the trends and analysis shown here are directly applicable to that metric as well.

![Digital refocusing of a single red blood cell image subjected to high flow rates. Top-Left: Optical volume of RBC with flow on and off (rates indicated on plot). Blue data points are OV calculations from raw images and red data points are from digitally refocused images. Images of cells at points A-E on plot are shown at the right with corresponding time indicated. Bottom-Left: Defocus at each time point as determined by the output of the digital refocusing algorithm (Media 1).](image)

5. Discussion

The presented analysis demonstrates that image defocus is a potentially significant source of error when characterizing microscopic objects with digital holography. Images of polystyrene microspheres were analyzed as technical objects with well-defined and well-characterized properties. As shown, the introduction of intentional defocus produced significant changes in measured parameters, including OV and DM. Even when using a manual focus that is apparently optimized for the measurement, an error of nearly 6% was observed. While this is not an exceptionally large error, it does place bounds on the precision of analysis based on this measurement. Given the high sensitivity of optical phase measurements and the ability to undertake precision quantitative analysis, it would appear that errors of this magnitude should be avoided. Indeed in the analysis of populations of polystyrene beads using QPM, we see...
that not only is the mean value significantly skewed by using manually focused images but that the distribution of the measurements is also broadened. The influence of defocus can thus be significant when seeking to find the differences between two populations of microscopic objects or even when characterizing a single population using multiple hand-focused fields of view.

Imaging of biological objects such as RBCs can present challenges, because their RI and three-dimensional structure are not known a priori and often must be assumed to implement a quantitative analysis. Our first experiment with RBCs showed that intentional defocus also introduced significant errors in $OV$ measurements, producing an error of 13.55% over a range of 100 $\mu$m of defocus. Although this level of defocus would likely not be encountered with manually focused images, we see that the two imaged points closest to the optimal focus plane (points D,E in Fig. 4) would result in errors of $\pm$ 10%, a significant error. In experiments where large significant changes in RBC properties are expected, this may not be a significant error. However, in experiments where more subtle changes in RBC’s are encountered, such a large error may obscure a significant change from being observed.

The experiments with the RBC subjected to flow illustrate how defocus can cause a subtle change that may significantly skew experiments. In these experiments, the optical volume obtained from manually focused images show an approximately 10% change due to the introduction of flow. However, upon using the refocusing algorithm, the $OV$ measurement remains within a fraction of a percent of the original value ($\sigma = 0.28\%$). The defocus distance was seen to change by upwards of 8 $\mu$m through these experiments. While it is unlikely that the flattening of the cell caused it to change focal planes by this magnitude, the introduction of the high flow rate could have easily caused this displacement by flexing the coverglass on which the cell was resting. These results illustrate the significance of refocusing digital holography images when conducting quantitative analysis. Without the defocusing algorithm, the systematic change due to the sample chamber could have been attributed to a large change in the cell properties. Upon correction, the $OV$ and in turn the $DM$ of the RBC remained consistent, matching expectations that changes in flow should not change cell volume or mass.

The metric used here, optical volume, only differs from dry mass by a scaling constant but offers significant utility. The dry mass of a cell can only be calculated if the refractive index increment, $\alpha$, is well known and presumed to remain constant throughout experiments. Thus, any errors introduced by incorrect values of $\alpha$ would result in systematic errors in cell experiments but would leave any observed trends intact. On the other hand, if changes in material properties of cells were to occur during experiments, it is not clear that the value of $\alpha$ would indeed stay constant as some materials do change their refractive index with density [13–15]. Further experiments to assess the validity of assuming the value of $\alpha$ to be constant are warranted.

6. Conclusions

We have demonstrated that defocus of QPM images can result in errors in measurements of derived parameters describing microscopic objects. By using a refocusing algorithm, measurements of optical volume were shown to be preserved even with significant focusing efforts. These results demonstrate that consistent measurements of individual RBCs can be obtained by properly refocusing images, independent of a user’s ability to manually place them in a focal plane.

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