Differential Phase Contrast and Digital Refocusing in a Computational Reflection Interferometric Microscope for Nanoparticle Imaging

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Abstract: We develop a computational interferometric microscope with controllable illumination for detecting nanoparticles using reflection-based differential phase contrast (DPC) and digital refocusing algorithm. We validate improved nanoparticle detection on 100nm polystyrene spheres.
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1. Introduction

Nanoparticle detection and characterization is an increasingly important field for identifying and understanding the behaviors of viruses, proteins, and other biological particles at the nanoscale [1,2]. Existing optical methods for this purpose often rely on labeling techniques, such as fluorescence, that artificially alter the particle size and behavior of the nanoparticle. Thus, label-free imaging methods are desired for studying biological nanoparticles. This requirement creates significant barriers, however, as nanoparticles exist below the diffraction limit and cannot be detected or characterized with standard microscopy methods.

One such technology addressing this issue is the Single Particle Interferometric Reflectance-Based Imaging Sensor (SP-IRIS) [1,2]. This system consists of a homodyne, common-path reflectance interferometer with a high magnification objective that images nanoparticles under Köhler illumination. Through the use of partially coherent plane wave illumination, the resulting measured intensity at the detector is the interference of the scattered field from nanoparticles and the reference field,

$$I_{total} = |E_r|^2 + |E_s|^2 + |E_r||E_s|\cos(\theta), \quad (1)$$

where $|E_r|^2$ is the reference field intensity, $|E_s|^2$ is the pure nanoparticle scattered intensity, and the third term is the interference of the reference and scattered nanoparticle field with a phase shift θ. The nanoparticle's field E_s follows an R^3 signal decay defined by its polarizability, where R is the particle diameter [2]. Because the purely scattered field intensity decays by R^6, this field becomes negligible in (1) while the third term maintains the R^3 field decay and becomes the primary signal of interest in SP-IRIS systems. This technology further enhances this interference term by analyzing the nanoparticles on a layered substrate consisting of a thin silicon dioxide (SiO2) layer thermally deposited onto a silicon (Si) wafer that reflects the forward scattered nanoparticle signal to the detector. In controlling the SiO2 layer thickness, the phase shift between the scattered and reference fields can also be tuned such that the θ phase shift is maximized to increase constructive interference at the detector. These factors make SP-IRIS optimal for nanoparticle imaging and have enabled its use in detecting and characterizing proteins, vesicular stomatitis viruses and numerous other particles [1, 2].

While SP-IRIS provides reliable nanoparticle detection to 40nm length scales, this technique still suffers from achieving high nanoparticle contrast and easily capturing depth information [1]. Because the system detects nanoparticles based on light scattering, the images exhibit significant background from the reference field that can mask the presence of a nanoparticle on the substrate surface. In addition, the use of plane wave illumination provides the system with a large field-of-view but poor depth sectioning capabilities. Since nanoparticles can be distributed along the longitudinal axis, mechanical sample scans are required for finding a nanoparticle's height above the substrate [2]. Sample scanning can be inconvenient in real-time measurement applications such as monitoring protein binding kinetics and requires expensive high-precision mechanical scanning systems.

In this paper, we experimentally validate the use of Differential Phase Contrast (DPC) and digital refocusing techniques in an interferometric microscope in reflection. We show improvements of 100 nm polystyrene bead detection on a SiO2-Si substrate. We also present digitally refocused DPC sample measurements detecting out-of-focus nanoparticles without the need for mechanical sample scanning. This experimental validation illustrates the benefit of DPC in nanoparticle detection and opens the possibility for quantitative phase retrieval after a rigorous forward model of this system with off-axis illumination is developed.

2. Theory and Methods

DPC is a computational imaging technique developed for the detection of weakly scattering objects [3,4]. For DPC, a minimum of two images are obtained under off-axis illumination on opposing sides of an axis of asymmetry as shown in Figure 1a. The difference of these two images removes the image background and purely absorbing features while creating a linear phase gradient across the image that enhances the contrast for weakly scattering objects [4, 5]. The equation for obtaining a DPC measurement is given by

$$I_{DPC} = \frac{I^{+} - I^{-}}{I_{BF}} \quad , \qquad (2)$$

where I^{+} and I^{-} are the intensity images when the sample is illuminated from opposing sides of a defined axis of asymmetry and I_{BF} is the normal brightfield image, which can be obtained by the summation of all the images. This technique has previously shown utility on cells and other nearly transparent objects that primarily introduce phase shifts to an incident field in transmission microscopes [5].

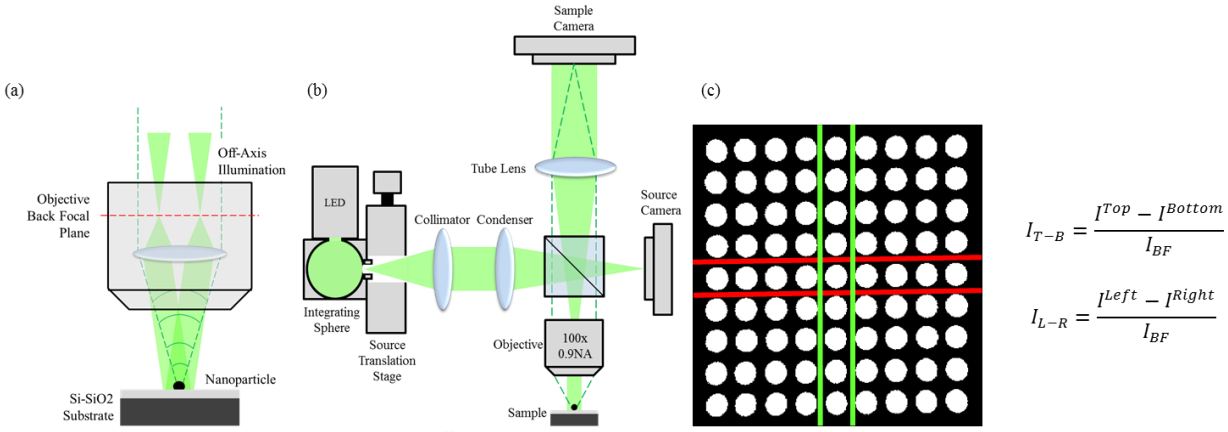

Figure 1: (a) DPC illumination strategy using off-axis illumination source positions (Green) at the objective BFP. Multiple illuminations are shown here to illustrate the effect of off-axis illumination on a sample, but single illumination positions were used during the experiment. (b) Simplified diagram of the computational reflection interferometric microscope. The source translation stage allows source movement to achieve off-axis sample illumination. (c) Discretized illumination grid with corresponding DPC equations. The images using illuminations within each line set were removed for the T-B (Red Lines) and L-R (Green Lines) to maintain symmetry for each DPC measurement.

For this experiment, the off-axis illumination was discretized to a square grid of point sources relayed to the back focal plane (BFP) of the objective as shown in Figures 1a and 1c, with individual images obtained at each off-axis illumination position. This procedure maintains nearly spatially coherent illumination for each image and allows arbitrary selection of the axis of asymmetry during post-processing. The axes were selected corresponding to the top and bottom (T-B) and left and right illumination (L-R) positions, and the summation of all sample images illuminated on a side of the axis were used as the off-axis intensities in the DPC calculation. Figure 1c shows the asymmetric axes and the corresponding equations used for DPC.

Discretizing the off-axis illumination with knowledge of the off-axis illumination angle further enables the use of the digital refocusing algorithm. Briefly, digital refocusing is a light-field based algorithm whereby refocused images corresponding to different axial positions are computationally synthesized using multiple images of a static object taken under various illumination angles [6]. To do so, a "shift-and-add" algorithm is implemented, in which the lateral shift is set by

$$\Delta u = \Delta z * tan(\varphi) \qquad (3)$$

where φ is the off-axis illumination angle, Δz is the desired defocus position with respect to the focal plane, and Δu is the distance to shift the image in *x* or *y* of the transverse plane. This method was investigated for evaluating whether multiple off-axis illuminations can replace mechanical sample scanning when identifying nanoparticles and determining their positions within an extended depth-of-focus (DOF) on a high-NA objective.

3. Experimental Results

A modified SP-IRIS imaging system with off-axis illumination was developed (Figure 1b). This system uses a 530nm LED light source (LED Engin, LZ4-00G108) in an integrating sphere to remove the LED image and a

200um pinhole to maintain partially coherent illumination on the sample. This source is mounted on an XY translation stage (Thorlabs, ST1XY-D) and placed in a 4f configuration with a 0.9, 100x Nikon Objective (Nikon, MUE13900) to relay the pinhole image to the objective's BFP for Köhler illumination. Two cameras are used to monitor the illumination source position for determining the off-axis illumination angle (Pointgrey, BFLY-PGE-50A2M) and capture the nanoparticle sample image (Pointgrey, GS3-U3-41C6M). 81 sample images were obtained while scanning the illumination source through a 9 x 9 grid (Figure 1c). Each image was corrected for dark noise and processed using the DPC equations described above. For digital refocusing, the approximate illumination angle was obtained based on the circular source image's distance from the center of the microscope objective BFP, and an algorithm for finding the exact angles based on self-calibration in [7] was implemented to improve the image shift accuracy.

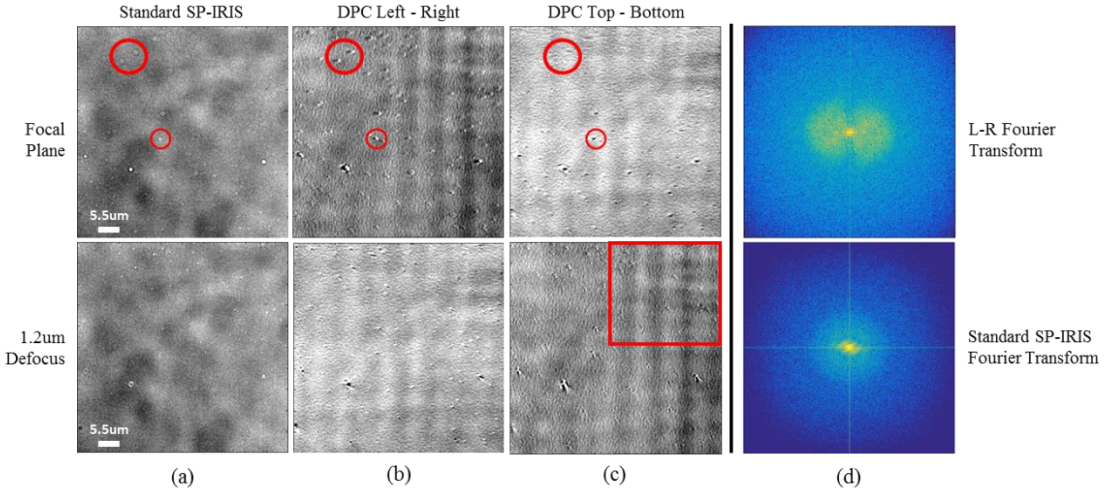

Figure 2: Standard SP-IRIS images of nanoparticles under on-axis illumination in (a), after performing T-B DPC (b), and L-R DPC (c) for both in-focus and defocused images. The red circles highlight example nanoparticles. The defocus shown in (a) shows a physically defocused image after moving the sample stage by 1.2um while (b) and (c) show nanoparticle images after digital refocusing to 1.2um. Additional nanoparticles with digital refocusing are highlighted in (c). (d) shows the Fourier transform for the in-focus L-R DPC image compared to the single on-axis illumination SP-IRIS transform. The DPC image matches the expected asymmetric objective transfer function as discussed in [5].

The DPC nanoparticle measurements compared with the single illumination SP-IRIS images are shown in Figure 2. Both DPC measurements in 2b and 2c exhibit a visual improvement of contrast in the number of nanoparticles present at the focal plane and after digital refocusing to 1.2um above the sample. Nanoparticles at defocus planes in the standard SP-IRIS image that are invisible in 2a are readily apparent in the defocused images (Figure 2c). The increased nanoparticle contrast in the DPC images over SP-IRIS suggests DPC provides a simple method for extracting nanoparticles typically lost in the strong background signal of SP-IRIS images, and the additional nanoparticles in the refocused images suggest mechanical scanning may not be necessary for identifying nanoparticle height above the substrate across an extended DOF. Furthermore, the Fourier spectrum images present in Figure 2d show that the reflection-based DPC exhibits the same asymmetric transfer function described in [5] despite being used in reflection versus transmission mode. This result supports the use of DPC in reflection microscopy and suggests that quantitative phase retrieval methods may be used for quantifying the phase shifts and improving nanoparticle resolution.

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