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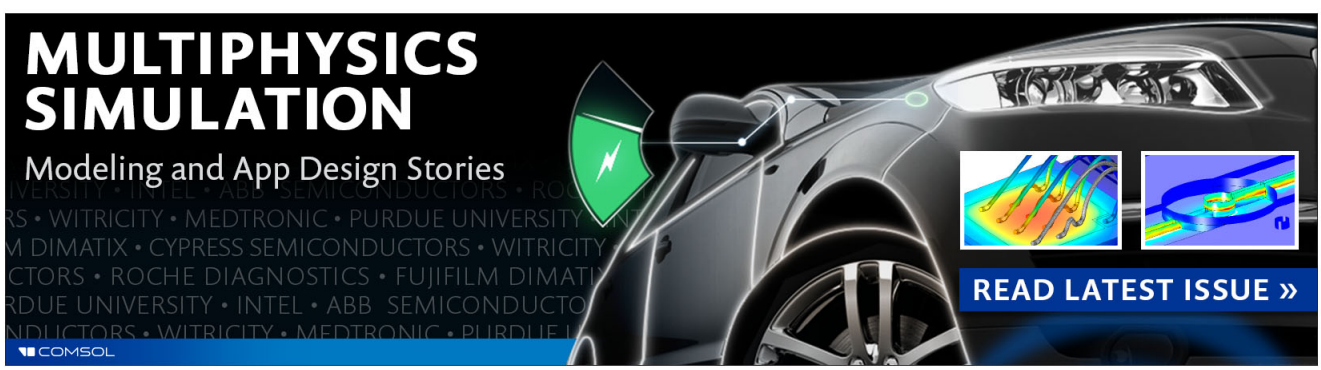

A method to determine the number of nanoparticles in a cluster using conventional optical microscopes

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We present a method that uses conventional optical microscopes to determine the number of nanoparticles in a cluster, which is typically not possible using traditional image-based optical methods due to the diffraction limit. The method, called through-focus scanning optical microscopy (TSOM), uses a series of optical images taken at varying focus levels to achieve this. The optical images cannot directly resolve the individual nanoparticles, but contain information related to the number of particles. The TSOM method makes use of this information to determine the number of nanoparticles in a cluster. Initial good agreement between the simulations and the measurements is also presented. The TSOM method can be applied to fluorescent and non-fluorescent as well as metallic and non-metallic nano-scale materials, including soft materials, making it attractive for tag-less, high-speed, optical analysis of nanoparticles down to 45 nm diameter.
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Advances in the controlled assembly of nanoscale building blocks, such as gold and silver nanoscale spheres, and quantum dots (QDs) have resulted in functional devices, such as nano-optoelectronic components, biophotonic nanosensors, and novel contrast probes for molecular imaging.[1–5] In the assembled structures, the photophysical properties of the cluster depend on the number and the shape of the nanoparticles and the distances among them.[6–19] For instance, the fluorescence lifetime of a quantum dot cluster depends on the number of the QDs, due to the energy transfer between them.[6] Surface enhanced Raman spectroscopy (SERS) signal in noble metal nanoparticle arrays depends not only on the properties of the building blocks, but also on the geometric characteristics of the whole array, such as array size.[9,12] The absorption band of gold nanoparticle aggregates is a function of the size and the different fractal structures of the gold nanoparticle aggregates and depends on electron-photon relaxation rate.[17] Therefore, it is very important to accurately count the number of the nanoparticles in a cluster in order to correctly understand the photophysical properties of clusters.

There are a few optical microscopy techniques to identify the number of the nanoparticles by measuring the optical properties of the nanoparticles such as the fluorescence lifetime, spectrum, and time-dependent intermittence.[6,13,20] However, all these optical microscopy techniques are restricted to the fluorescent nanomaterials. High-resolution imaging tools such as scanning electron microscope (SEM), transmission electron microscope, and atomic force microscope are capable of counting the number of the nanoparticles in a cluster.[11,14,21–23] However, they do not have the functionality to measure photophysical properties. Most photophysical properties of the nanoparticles are measured with optical microscopes.[6,8,22,23] Therefore, adding the functionality to determine the number of nanoparticles by the optical microscope gives a significant advantage to users and also helps them quantitatively interpret the photophysical properties of the nanoparticle clusters.

Here, we demonstrate the applicability of the through-focus scanning optical microscopy method[24–26] to count the unknown number of nanoparticles in a cluster. The TSOM method is based on an analysis of a set of out-of-focus optical images containing the scattering signals (4-D data[25]). A TSOM image extracted from the 4-D optical image set is sensitive to changes in the 3-D shape of a target, even down to the sub-nanometer level.[25] Therefore, the TSOM technique can be used to determine the number of nanoparticles clusters, because a change in the number of nanoparticles leads to changes in the dimension of clusters. In addition, the TSOM method does not require complex modifications to the hardware of conventional optical microscopes. Simple through-focus scanning of targets is all that is required. In this paper, we demonstrate the determination of the number of nanospheres in monomer, dimer, trimer, and tetramer polystyrene clusters through optical simulations and experimental measurements.

The following procedure was used to generate the monomer, the dimer, the trimer, and the tetramer clusters of polystyrene nanospheres. A droplet of 15 μL containing polystyrene nanospheres (with 2.50E + 12 nanospheres per cc) was dropped onto a clean silicon wafer. After 10 min, the silicon wafer was washed with pure deionized water followed by gentle blow-drying with clean, dry nitrogen gas. This produced low-density clusters of random number of particles. The wafer was analyzed using an SEM to identify the locations and to determine the number of particles in the clusters. The identification of the locations of 15 for the monomers, 12 for the dimers, 8 for the trimers, and 5 for the tetramers was facilitated by fiducial marks. The mean size of the nanoparticles was measured to be 93 nm with a standard deviation of 3 nm. The identified clusters were then analyzed using the TSOM method. The TSOM data were acquired using a bright-field, reflection mode optical microscope (objective magnification = 100×, collection numerical aperture (NA) = 0.85, illumination NA = 0.15, camera pixel

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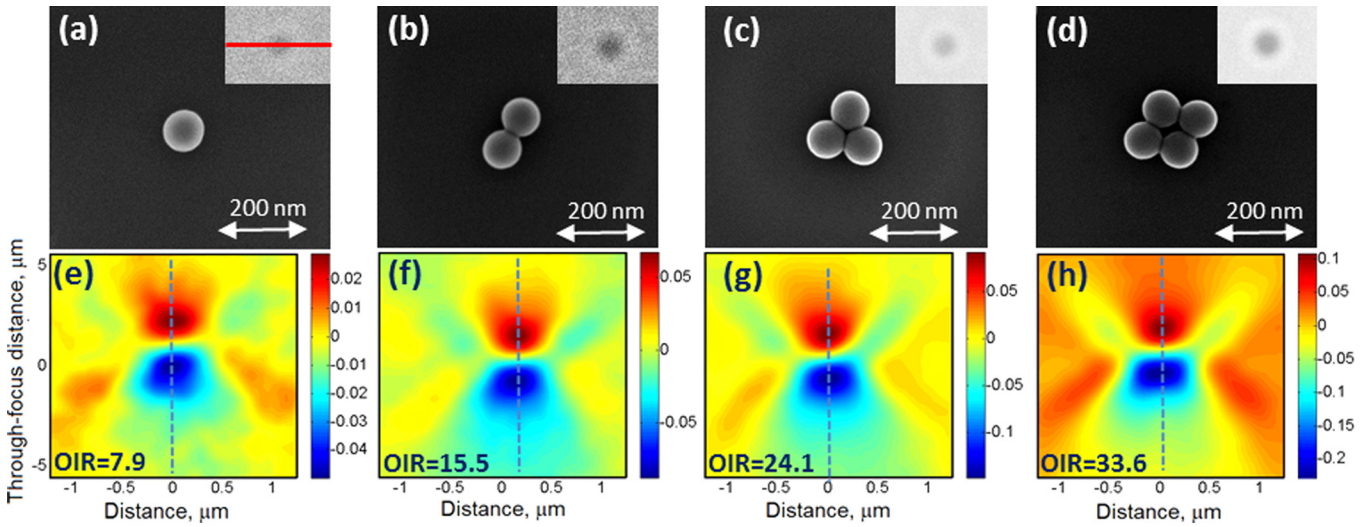

FIG. 1. Typical SEM images of (a) monomer, (b) dimer, (c) trimer, and (d) tetramer polystyrene nanoparticle clusters. Insets show corresponding optical images at the best focus positions. (e) Through (h): corresponding TSOM images.

size = 32 nm, illumination wavelength = 405 nm (narrow band-pass filtered LED light source), through-focus step size = 200 nm, and total focus range = 16 μm). The TSOM analysis was done using the software developed at NIST. This software does the following to the data: normalizes the image intensity as given in Ref. 24, subtracts the background noise, selects the intensity profiles from the through-focus optical images, as shown in Fig. 1(a) inset, constructs TSOM image, interpolates, and smoothens. The normalization process eliminates the effect of image intensity variations.

Figure 1 shows SEM images of typical monomers, dimers, trimers, and tetramers along with the TSOM images (normalized as described in Ref. 24). Conventionally, the best-focus optical images are obtained at the highest contrast focus position. The best-focus optical images obtained in such a manner are shown as insets in the SEM images (Figs. 1(a) to 1(d)).

As expected, the number of nanoparticles in the clusters cannot be determined using only best-focus optical images due to the diffraction limit. However, the optical intensity of the TSOM images increased with the number of the particles in the clusters as can be observed by the color scale bar. One way to quantify this optical content is the optical intensity range (OIR), defined as the absolute difference between the maximum and the minimum intensity, and multiplied by 100.[24] The OIR values show an increasing trend with increased number of the particles in the clusters (Fig. 1).

Another way to visualize the optical content of the TSOM images is to draw the intensity plots along the dotted lines shown on the TSOM images (Fig. 1). The resultant plot is shown in Fig. 2(a) for the different clusters. Here, again we see an increasing trend in the optical signal. In this case, it so happens that the absolute difference in the optical content (peak-to-valley) matches with the OIR values.

To confirm the validity of the trends observed from the measurements, we also simulated optical TSOM images under the exact experimental conditions (illumination NA = 0.15, collection NA = 0.85, wavelength = 405 nm, through-focus step size = 200 nm, and image pixel size = 32 nm) using a commercially available optical simulation program that solves Maxwell's equations using finite-difference-time-domain method (domain size = 3.0 μm × 3.0 μm × 0.5 μm, element size = 5 nm, and nanoparticle clusters located at the center and on top of the 100 nm thick Si substrate). In the simulation program, the domain size repeats itself laterally, and the air at the top and the Si substrate at the bottom are matched by perfectly matched layers outside the domain size. The simulation data were processed in the same manner as that of the

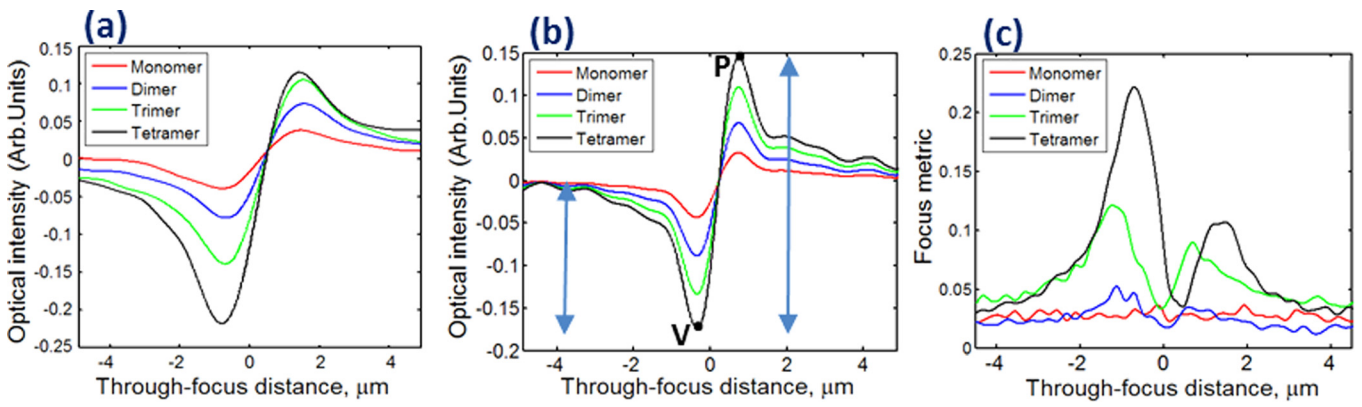

FIG. 2. Intensity profiles (along the dotted lines shown in Figs. 1(e) to 1(h)) for (a) measurements, and (b) simulations. (c) Through-focus focus metric analysis done on the same measured data.

measured data (except the background noise subtraction), i.e., normalizes the image intensity, selects the intensity profiles, constructs TSOM image, interpolates, and smoothens. These results are plotted in Fig. 2(b). Even though there are some differences between the measurements and the simulations, the overall trend matches very well as a function of the number of particles in the clusters and thus provides confidence in the process.

Here, we show the advantage of using the through-focus data in the form of TSOM images. Conventionally, an optical image is acquired at the best-focus position. This best-focus position is indicated by letter V (valley) in Fig. 2(b), for the tetramer cluster. The strength of the largest optical signal at this focus position for the tetramer cluster is indicated by an arrow mark on the left side in the same figure. However, if the TSOM method is used, the maximum signal strength from the same tetramer cluster under the same conditions nearly doubles as shown by an arrow mark on the right side (Fig. 2(b)). This is because in the TSOM method both the valleys (V) and the peaks (indicated by letter P) are taken into consideration for the analysis, even though they are separated by several microns of difference in the focus position. This is not possible in the conventional method of analysis where only a single, best-focus image is used. The increased signal strength in the TSOM method increases signal-to-noise ratio, sometimes substantially and enables robust analysis. For example, if the same measured data presented in Fig. 2(a) is analyzed using the through-focus focusmetric (TFFM) method,[27] the results are as shown in Fig. 2(c) (in the TFFM method, contrast in an optical image is plotted as a function of the focus position). The monomer TFFM optical signal is at the same level as the noise and hence cannot be recognized. For dimer, the TFFM signal is barely above the optical noise. Hence, both the monomers and dimers are hard to analyze using the TFFM method. However, both the monomer and the dimer can be analyzed using the TSOM method as its signal-to-noise ratio is much higher.

With that in mind, we plotted the OIR values from the TSOM images as a function of the number of the nanoparticles in the clusters, for both the measurements and the simulations, as shown in Fig. 3. For the measurements, the mean values were plotted along with their standard deviations. The following observations can be made from this plot. The OIR values increase nearly linearly with the number of particles. This property can be useful to determine the number of particles in clusters. There is a good agreement between the simulations and the measurements. The standard deviation of the monomers, the dimers, and the trimer is nearly identical and relatively small compared to the differences in the OIR values of the different clusters. The tetramer clusters have a relatively large standard deviation. We will discuss the reason for this below. However, it still does not overlap with the OIR value of the trimer.

During the SEM analysis of the particles, almost always the monomer, the dimer, and the trimer cluster shapes were found to be as shown in Figs. 1(a) to 1(c). However, we observed different shape configurations for the tetramer clusters (shown as inset in Fig. 3). During the TSOM measurement, the different shaped tetramer clusters

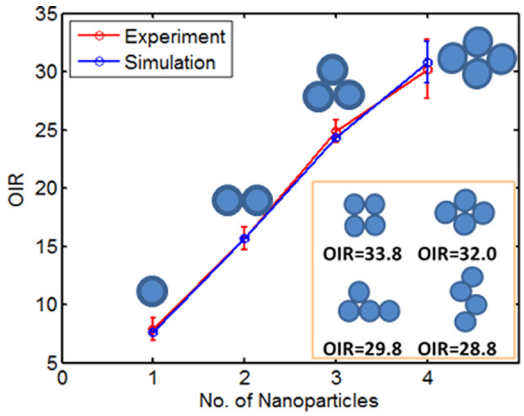

FIG. 3. A plot of the OIR values from the measurements and the simulations for the different clusters of polystyrene nanoparticles. The inset shows different shape configurations observed for the tetramer clusters using SEM and their simulated OIR values.

showed a variation in the OIR values more than the standard deviation observed for the monomer, the dimer, and the trimer clusters. To determine if this is due to different shape configurations, we simulated the TSOM images for the tetramer shape configurations shown in Fig. 3. The calculated OIR values from the simulations showed a variation consistent with the variation observed experimentally, and hence, we concluded that the more-than-normal standard deviation observed for the tetramer clusters is due to different shape configurations. For this reason, we also plotted for comparison in Fig. 3 the standard deviation of the tetrameter cluster simulations having different configurations. Both the measured and the simulated standard deviations show similar behavior.

We propose the following procedure for determining the number of particles in a cluster based on the above observations. Measure the OIR value of a cluster of an unknown number of particles and compare its value to the OIR values from simulations. The number of particles in the cluster can be determined based on the best match. If optical simulations are not available, an alternate method can be followed. In this method, a library of measured OIR values needs to be generated *a priori* with known numbers of particles in the clusters under the same measurement conditions. Once this is done, similar to the above method, the OIR value of a cluster can be compared to this experimental library and the number of particles can be determined based on the best match.

In this work, we presented a procedure to determine the number of nanoparticles in a cluster containing up to four particles using 93 nm polystyrene particles. The same procedure can be used for other materials and sizes as well, provided sufficient optical signal strength is available. At present, we recommend two methods to enhance the optical signal strength of nanoparticles: use lower illumination NAs and shorter illumination wavelengths. It has been reported that gold nanoparticles down to 3 nm (nominal) can be imaged (using visible wavelengths) simply by reducing illumination NA (Ref. 28) (using an inverted microscope in transmission mode). This suggests that the method proposed in this paper could be applied for nanoparticles down to 3 nm.

In the current study, we also analyzed the monomers using 520 nm illumination wavelength, which produced a lower OIR value of about 4.5, while the 405 nm illumination wavelength (from the above results) produced an OIR of 7.9, showing that lower wavelengths produce stronger optical signal. Using optical simulations, we found that monomer nanoparticles down to about 45 nm size could be analyzed using the TSOM method if a wavelength of 305 nm were used (under the experimental optical conditions used in the current study). This was determined using a noise base OIR of 1.0.[24]

Here are some caveats for this method to work successfully. The distance between the clusters is recommended to be above five times the illumination wavelength to minimize optical interference. Nanoparticle size and shape should be fairly uniform. The background should be optically smooth. The number of particles in a cluster is recommended to be four or less. Above four particles, it may be challenging to apply this procedure as the OIR is sensitive to not only the number but also to the configuration of the particles.

There are several advantages of the TSOM method.[25] The advantages relevant to this application area are that it can be applied to any type of nanoparticle material, whether metallic or non-metallic, fluorescent or non-fluorescent, dry nanoparticles on a substrate or wet nanoparticles suspended in a transparent liquid. It can be readily implemented using any conventional optical microscope without complicated hardware modifications.

In summary, we presented a procedure to determine the number of nanoparticles in a cluster using the optical TSOM method. The OIR values of the experimental results are in good agreement with the simulation results. The OIR values increased proportionally with the number of the nanoparticles in a cluster. The unknown number of particles in a cluster can be determined by comparing its OIR value with that of the OIR values obtained using either simulations or measurements. Since the TSOM technique is based on an analysis of the scattering signals from the targets, it can be applied for the evaluation of clusters composed of any type of material, under dry or wet conditions, including soft materials. It can also be used to study either fluorescent or non-fluorescent materials.

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