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Multiplexed TEM Specimen Preparation and Analysis of Plasmonic Nanoparticles

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Abstract

We describe a system for rapidly screening hundreds of nanoparticle samples using transmission electron microscopy (TEM). The system uses a liquid handling robot to place up to 96 individual samples onto a single standard TEM grid at separate locations. The grid is then transferred into the TEM and automated software is used to acquire multi-scale images of each sample. The images are then analyzed to extract metrics on the size, shape, and morphology of the nanoparticles. The system has been used to characterize plasmonically-active nanomaterials.

Keywords

Nanoparticles; TEM; automation

1 Introduction

Over the last decade there has been significant progress in the synthesis of inorganic (Alkilany *et al.*, 2013; Rao *et al.*, 2012) and hybrid inorganic-organic nanoparticles (Moon *et al.*, 2013) in solution. Optimizing the synthesis conditions is often dependent on structural characterization of the nanoparticles using indirect and direct space imaging methods. Transmission Electron Microscopy (TEM) offers the highest resolution method for direct space imaging for structural characterization of nanoparticles. In addition to high-resolution, TEM also offers multi-scale imaging to characterize particle density, obtain statistical information regarding particle size and morphology distributions, and, if coupled to elemental analysis instrumentation, determine atomic composition information. However, specimen preparation for TEM imaging has traditionally been a very low-throughput assay

with single specimens prepared on individual TEM grids, which must be inserted and retracted from the instrument through the specimen airlock one at a time. Thus, imaging several tens to hundreds of nanoparticle synthesis conditions requires an equal number of TEM grids. Several methods for improving throughput have been proposed that use robotic grid exchange appliances to insert or remove TEM grids using a specialized specimen holder, where the entire specimen holder is inserted or retracted from the instrument (Potter *et al.*, 2004) (Coudray *et al.*, 2011; Hu *et al.*, 2010; Cheng *et al.*, 2007). When coupled to automated software for image acquisition (Suloway *et al.*, 2005) these systems eliminate the tedious and time consuming manual procedure of loading and unloading individual EM grids into the microscope. However, the entire procedure remains rather cumbersome and slow, requires specialized equipment be integrated with the microscope, and the continuous insertion and retraction of hundreds of samples in and out of the microscope adds mechanical stress to the system. Alternative options like the Autoloader™ provided with the Titan Krios™ (FEI) instrument are generally prohibitive based on the cost of the instrumentation. Together these factors have kept the cost/benefit ratio of these processes high enough to prevent wide spread adoption.

The restriction of using a unique TEM grid for every sample imposes a major bottleneck on imaging multiple samples efficiently and adds considerably to the cost per sample. The surface area of a typical EM specimen grid (~2 mm diameter of usable area) is such that there are potentially on the order of 100,000 high magnification (~50,000×) targets available for imaging. Even taking into account that the specimen may not be evenly distributed, the number of usable targets will always far exceed the number required for analysis. Indeed, the number is also greater than what can be feasibly acquired in any reasonable session at the electron microscope. Given that the size range of nanoparticles is typically on the order of ~100 nm, if the particles are reasonably distributed, ~ 10 images (25,000× magnification) will provide ~500-1000 particles with sufficient resolution to accurately extract the detailed structural characteristics of the particles. As a result, images for any one nanoparticle sample can be acquired from an area of the grid that only represents ~0.002% of the available real estate.

With ample surface area on any single TEM grid, it should be possible to accommodate multiple samples while retaining the ability to acquire sufficient numbers of images of each sample to enable structural analysis. We propose a high-throughput TEM pipeline where: (1) multiple nanoparticle specimens are transferred to a single TEM grid; (2) the grid is transferred to the microscope; and (3) automated imaging software (e.g. Leginon (Suloway *et al.*, 2005)) is used to acquire multi-scale images of each individual sample. This idea is outlined schematically in Figure 1. While this approach is conceptually straightforward, transferring multiple specimens to a single TEM grid poses several technical hurdles. With the usable area of standard TEM grids limited to ~2 mm in diameter, manual pipetting methods that are typically used for single sample transfer are not suitable for preparing multiplexed samples. This requires transferring very small sample volumes to very accurate locations on the grid making high precision micro-scale dispensing techniques a necessity. Furthermore, typical TEM grids consist of a thin (~5-50 nm) electron-transparent support film that is easily damaged by any mechanical impact or manipulation during specimen

transfer (Castro-Hartmann *et al.*, 2013). Therefore, the dispensing device must be delicately controlled while interfacing with the support film, or preferably be non-contact in nature. Finally, the individual specimens on the TEM grid must be well separated and accurately located to enable multi-scale imaging in order to register specimen conditions with downstream nanoparticle characterization.

Recently, we developed a picoliter to nanoliter scale, non-contact piezoelectric capillary (inkjet) dispensing system for transferring biological specimens to TEM grids as an alternative method for preparing samples for cryo-TEM (Jain *et al.*, 2012). Piezoelectric capillary dispensing provided precise spatial and temporal control of specimen loading into micro-scale regions on a single TEM grid while preserving biological macromolecular structure. This previous report described preparing a single biological specimen on a TEM grid for cryoTEM applications, but we also discussed the potential of using the same technology to deliver multiple non-biological specimens to a single TEM grid. Inorganic and dense hybrid nanoparticles comprising high Z-number atoms do not require rapid cryo-freezing to preserve the specimen and therefore transfer of multiple specimens to the grid is not time-constrained. An alternative approach to applying multiple specimens to a single grid using microarray technology has also been proposed (Castro-Hartmann *et al.*, 2013).

In this paper we describe a system that utilizes a commercially available liquid handling device to transfer and dispense 100 pL or more of each individual sample from a 96 well plate to defined locations on a silicon-based TEM grid. The grids are then transferred to a TEM and an automated system, Leginon (Suloway *et al.*, 2005; Potter *et al.*, 1999), is used to acquire multiple multi-scale images of each sample. To demonstrate the system we have used it to characterize plasmonically-active nanomaterials.

Nanoparticle characterization is often a limiting step in the engineering of new nanomaterials and sensors. Plasmonically-active nanomaterials, which show extraordinary size and shape dependent optical effects, are a case in point. Gold and silver nanoparticles, whose surface plasmon resonances can be tuned by altering the particle size and shape have great potential in sensing applications, including surface enhanced Raman spectroscopy (SERS) (Krafft & Popp, 2015; Vo-Dinh *et al.*, 2013; Nolan *et al.*, 2012; Rodriguez-Lorenzo *et al.*, 2012). However, the development of simple and robust nanoparticle synthesis methods requires facile tools to measure not only optical properties, but also particle size and shape. TEM is a preferred way to do this, but conventional TEM image acquisition and analysis can be labor and time consuming. By automating sample deposition, image acquisition, and image processing and analysis, we make it possible to include TEM as a routine method of sample analysis.

2 Materials and Methods

2.1 Overview

An overview of the process is illustrated in Figure 1. A standard 96 well plate containing individual nanoparticle samples was loaded in a liquid handling system. Multiple TEM grids were mounted on a specially designed support tray. The liquid handling robot systematically transferred each of the specimens from the 96 well plate to a specified position on the chosen

TEM grid(s) in the support tray. The grid was then transferred into the TEM and automated software was used to track each individual spotted sample on the grid and acquire a series of images at multiple magnifications (multi-scale) within each spot. The images can be viewed in a standard web browser and also transferred and reformatted to make them suitable for downstream processing and analysis.

2.2 Samples

Gold nanorods were prepared based on modifications of published protocols, as described previously (Nolan *et al.*, 2014). Briefly, for large rods (Smith *et al.*, 2009) (20×50 nm), gold seed was prepared by adding 5 mL of a 0.0005 M HAuCl_4 solution to 5 mL of an aqueous hexadecyltrimethyl ammonium bromide (CTAB) solution (0.2 M). To the vigorously stirred solution, 0.21 mL of 0.026 M ice cold NaBH_4 is added to produce a light-brown solution. Stirring was continued for 2 more minutes, after which the seed solution was kept in a water bath at 25°C for up to 2 hours. Then, 500 mL of a 0.2 M aqueous CTAB solution was mixed with 0.1 mL AgNO_3 at 0.004 M followed by addition of 500 mL of 0.001 M HAuCl_4 to form the growth solution. After vigorous mixing, 7 mL of 0.0788 M L-ascorbic acid was added, and the solution incubated in a 25°C water bath. After 10 min, the solution was removed from the water bath, 1.2 mL of the seed solution added, and the mixture stirred for 2 min. The solution was then placed back in the 25°C water bath for 18 hours. After fabrication, excess CTAB was removed by incubating rods at 4°C overnight and then removing supernatant and discarding precipitated CTAB.

Smaller Au rods ($\sim 10 \times 25$ nm) were prepared as described (Ali *et al.*, 2012). Briefly, 666 mL of a 0.2 M aqueous CTAB solution was gently swirled with 333 mL HAuCl_4 and warmed to 27°C . Then 5 mL of 0.004 M AgNO_3 was added and the solution was gently swirled. Then 1.27 mL of 5 M HCl was added without mixing, followed by addition of 4.6 mL of 0.078 M ascorbic acid. Solution was gently swirled until color changed from yellow to clear and then 1 mL of 0.01 M NaBH_4 was added without mixing. The solution was then placed back in the 25°C water bath undisturbed for 18 hours. After fabrication, excess CTAB was removed by incubating rods at 4°C overnight and then removing supernatant and discarding precipitated CTAB.

2.3 Liquid Handling System

The Scienion SciFLEXARRAYER S3 (See supplementary movie SM1) is a commercial, fully programmable, computer controlled liquid handling system that transfers specific picoliter sample volumes utilizing Piezo Dispense Capillaries (PDCs). The PDCs are capable of dispensing volumes from 100-800 pL in a single droplet depending on the bore size and coating of the silica glass capillary tip. A computer controlled carriage moves a platform holding from 1 to 8 PDCs between targets, with a positional accuracy of $< 5 \mu\text{m}$. Both the $40 \mu\text{m}$ and the $60 \mu\text{m}$ bore tips (100-300 pL range for single droplets) were used for spotting on TEM grids as described in this paper. Larger volumes are achieved by dispensing multiple sequential drops. Relative humidity in the S3 cabinet was set at 40% which helped diminish the coffee ring effect of the drying droplets, while preventing rapid liquid loss due to evaporation. A commercially available machined aluminum plate (PELCO TEM Grid Holder Block, Ted Pella, Inc.) with wells for 81 standard EM grids was placed on

the slide platform to securely hold the TEM grids during dispensing. The S3 hardware and software used were standard.

2.4 TEM grid

While standard carbon coated copper grids could be adopted for this purpose, we have primarily used Silicon-nitride grids (SimPore TEMwindows Inc.). The grids (Figure 2b) consist of a 50 nm thick SiN film (Strierner *et al.*, 2007) supported by a 100 μm thick silicon wafer that have single, large open windows up to 500-1000 μm square. They provide a number of advantages: their silicon-based construction has proven to be more robust for sample deposition by PDCs, and the very wide viewing area allows great flexibility in sample volumes, density, variability, and spotting patterns over an unobstructed electron transparent substrate, making them easier to incorporate into a multiplex pipeline.

2.5 Transfer process

A 96 well plate, figure 2a, is placed on the SciFLEXARRAYER S3 slide platform and TEM grids are loaded onto the installed grid plate. Typically, two replicate grids were created as this replication step is extremely fast so that multiple replicates can easily be produced for a minimal extra time investment. Tasks are run on the system in a specific order using a scripting interface, which is configured to transfer the 96 samples one at a time onto the TEM grid. A first step in setting up the transfer is defining the spotting field. The operator enters instructions to tell the system which sample to transfer, and where to place it on a 10 $\times$ 10 raster pattern. Prior to spotting, an image of the mounted grid is acquired and compared to a previously acquired template. Targeting is then adjusted in the software so that the field is placed precisely on target. The spacing of the spots are typically 100 μm apart. The volume of each drop is approximately 240 pL and the user defines how many drops of each sample to deposit on the grid. Once the initial setup has been completed the transfer is executed as a fully automated loop until all of the selected samples have been transferred to the grid(s), figure 2c. For quality control, an image of the activated PDC is acquired before and after spotting and analyzed for drop volume and position. Also, an image of the spot is acquired and analyzed to determine its placement and position on the grid. The spot is declared in error if out of specification and the operator is prompted to skip or re-spot at the end of the run. Between each sample the PDC is cleaned by submersing into a reservoir with running DI water. The sequential steps in each loop are outlined schematically in figure 3 and demonstrated in supplementary movie SM1.

2.6 TEM imaging

Data was acquired using a Tecnai F20 Twin transmission electron microscope operating at 200 KeV, using a dose of $\sim 15 e^-/\text{\AA}^2$. Images were automatically acquired at eucentric focus using a specialized application in Leginon (Suloway *et al.*, 2005), onto a Tietz F416 CMOS digital camera. The final high magnification images were acquired at a nominal magnification of 25,000 $\times$ and pixel size of 7.1 $\text{\AA}$. The specialized application allows for automatic tracking of each individual sample based on the sample distribution array type (e.g. 10 $\times$ 10). Imaging begins with an atlas of the entire grid acquired at a magnification of 120 $\times$ (~ 35 images) to track the position of the individual spotted samples (figure 4a). An

array of trial coordinates (row and column) is automatically constructed by Leginon and the user can then fit this grid to the array of spots. Individual positions can also be manually adjusted and/or rearranged in cases where the spots are offset or not at the expected raster spacing. At this point, each sample is now identified with a specific target area of the grid, allowing Leginon to automatically insert the sample identifier into the name of every subsequent image taken of that spot. With the spots mapped, an image of each spot is acquired at a magnification of 550 $\times$ (figure 4b). Areas of the spots that look promising for further imaging are then targeted (figures 4b, 4c). Four to five intermediate areas were selected within each spot, and images were acquired at a magnification of 2500 $\times$ (figure 4c, ~480 images acquired for all 96 spots). Within each of these four to five intermediate areas, final targets are selected for high magnification imaging at 25,000 $\times$ (figure 4d). For the experiments described here, an average of ~30 high magnification images at 25,000 $\times$ was sufficient for particle analysis of each sample (spot). However, this protocol can be used to acquire any number of high magnification images that may be necessary for downstream data processing. The total data set for 96 spots requires ~2500 high magnification images.

2.7 Analysis

Individual images for each well were processed and analyzed using Cell Profiler (www.cellprofiler.org) (Kamentsky *et al.*, 2011), an open-source package for automated image analysis. The image analysis pipeline included background estimation and subtraction, smoothing, and particle segmentation using the Otsu adaptive thresholding method. Features of individual particles, including diameter, major and minor axis lengths, aspect ratio, solidity, rectangular ratio, perimeter, and area were calculated and exported for single particle population analysis in FCS Express Image (De Novo Software). Gates were set on solidity and rectangular ratio to remove particles that corresponded to background artifacts, unsegmented clumps of particles, and particles that fell at the edge of the image and were thus truncated. This gating typically removed 20-33% of the total particles, with the remainder being single, well segmented nanoparticles. Histograms of major and minor axis lengths and aspect ratio were generated for each sample.

3 Results

3.1 Multiplex sample preparation

Various procedures were developed to ensure robust operation of the pipeline. Pre-programmed cleaning cycles were performed every 5 samples to prevent tip clogging and maintain accuracy/precision. Experience showed that it was a challenge to achieve consistent spotting volume and accuracy when delivering a single drop per target; it appears that the instrument is better adapted to deliver 3 or more drops per target. The instrument delivers 96 spots in ~4 hours (~2.5 min/sample), and this time is minimally impacted by duplicating the grid preparation (or further replication), requiring less than 1 second per cycle for each grid replicate. The typical spot volume used was 240 pL and the spot spread was 50 $\mu\text{m} \pm 20 \mu\text{m}$. When a single sample was spotted multiple (96) times, the spot positional drift was about ~5% of the spot diameter, see Figure 2c. When multiple different samples were spotted, the spot positional drift was much larger, ~25% of the spot diameter and the spot positional accuracy was much lower. Thus, as expected, the most accurate

spotting and positional accuracy are achieved when all samples are very similar in composition. This is due to optimization of the settings for depositing a particular sample; it is possible to tune the PDCs for more accurate positioning of spots on each target.

Our experience was that replicate grids were highly reproducible. Given the minimal time and cost deficit for producing replicates, it seems advisable to always make duplicate or triplicate grids in case of random misfires or off center targets that cannot be indexed.

3.2 Imaging

Automated imaging of the multiplex grid was carried out using Leginon (Suloway *et al.*, 2005). This required the development of a new application node and other feature enhancements, and currently requires a combination of both manual and automatic steps. Leginon already had the ability to label and automatically take multi-scale images for multiple grids into a single data collection session (e.g. screening). Extending this concept, a new application node was created to label multiple samples on a single grid and to take advantage of the sample organization already present in the microtiter plate. The node expects the samples to be placed in an array of rows and columns and that the grid be oriented in the holder such that the first sample corresponding to well A1 on the plate is positioned on the lower left. Orientation of the grid was aided by the use of fiducials at specific locations on the grid. Different types of spotting arrays have been programmed and can be selected from the interface (e.g. 10×10 vs. 8×12). After the grid is aligned the user selects the type of array and identifies the location of each of the sample spots on the grid. The application uses the selected positions to automatically label each drop with the coordinates of the well from which it originated. Since each of the samples react differently with the PDC nozzle surface, the spots can be offset from their expected positions in the array beyond the tolerances of the automatic labeling routine. In these cases, the spot names can be manually changed and re-ordered before the data collection run is started. After the sample identifiers are correctly set, Leginon will add the corresponding label to the names of all subsequent images acquired within that targeted area. The new Leginon application node increases the efficiency of the imaging process dramatically by eliminating the labor involved with manually tracking and organizing the thousands of images produced to image all the samples.

The initial setup to target and image the sample spots at low resolution is a set routine that requires approximately 45 minutes of manual operation. As images of the spots become available, selection of final magnification targets can begin before the spot images are completed. Overall, it takes approximately 4 hours of work at the microscope to completely set all targets and any number of high magnification images can be taken to achieve statistically meaningful results. For this study, the data collection consisted of ~28 high magnification images per specimen (2688 total images) and required 56 hours total microscope time (including time to select all targets) to complete (~50 images/hour).

Automated imaging in Leginon proceeded without incident with only a few exceptions. The combination of the gold nanorod samples and silicon nitride grids sometimes resulted in beam induced charging effects exhibited by the low magnification images (<5000X). A pre-exposure setting was implemented and this eliminated the charging effects while having no

effect on the high magnification images of the sample. We also observed that the target positions would drift as a result of the constant cycling between a wide range of magnifications over an extended period of time. This resulted in missing 2 spots out of the 96 deposited on the grid and also in some high magnification images being devoid of particles. To address this, the data collection was split into two major steps: 1) low and intermediate magnification targeting was completed first and this was then followed by 2) final magnification image collection. This limited the range of the switch between magnifications in each period of time at the microscope, which resulted in more stable behavior of the instruments and enabled all 96 spots to be successfully imaged.

Notably the targeting was also affected by the drying of the drops spotted on the grid in a so-called coffee-ring effect (Deegan *et al.*, 1997). Without blotting, particles suspended in a liquid that dries on a solid surface will accumulate where the original edge of the drop is pinned. This complex phenomenon is dependent on many factors including particle shape (Yunker *et al.*, 2011). As the liquid evaporates, capillary flow occurs outward toward the edge of the drop and the particles deposit. A majority of the nanorod samples dried in this manner and required that targeting be done along the edge of the ring. Each sample also spreads somewhat differently, limiting the use of a fixed template to assign the targets automatically. While this required additional time to manually queue targets for the final magnification images, the number of images needed was readily available around the rings and did not otherwise inhibit the imaging or data analysis.

3.3 Application to gold nanorods

The context for this study was the synthesis of gold nanorods with a red localized surface plasmon resonance (LSPR) for use as surface enhanced Raman scattering (SERS) tags. The LSPR of gold nanorods is determined by the particle aspect ratio, with Au spheres (aspect ratio = 1) producing a green-excited resonance and larger aspect ratios resulting in longer resonance wavelengths. We prepared a plate of gold nanorods generated using different reaction conditions aiming to produce nanoparticles with spectrally-defined localized surface plasmon resonances. The plate contained 48-different nanoparticle preparations, plus 4 wells containing 100 or 200 nm polystyrene beads to serve as orientation landmarks on the plate. We spotted these samples on to two separate grids and imaged multiple fields in each well. The images were processed and analyzed using Cell Profiler to identify, count and measure individual nanoparticles. The resulting data, which included more than 40 features for each particle, including the major and minor axes lengths, aspect ratio, area and perimeter, was exported to FCS Express Image, which was used to display and analyze the particle population distributions.

The particle numbers analyzed for each sample, as well as the mean major and minor axis lengths for each sample in the two replicate grids are presented in Figure 5. While there was variation in the total number of particles measured for each well between grids, the key morphological measurements of major and minor axis lengths and aspect ratio showed excellent grid to grid reproducibility. We also measured the extinction spectrum for each sample, and the extinction maximum increased with increasing aspect ratio (figure 5c), as expected (Orendorff *et al.*, 2006).

The samples analyzed in this study were produced to optimize the production of red- and near- infrared(NIR)-resonant Au nanorods used in SERS tags (Nolan *et al.*, 2012). Many protocols for nanorod synthesis have been reported (Lohse & Murphy, 2013), one of which is a two-step procedure involving the reduction of Au salt to form a small Au seed followed by the surfactant-directed growth at a defined face of the particle to form a rod (Nolan *et al.*, 2014; Smith *et al.*, 2009). An example of such a nanoparticle preparation is presented in Figure 6 (panels a and d). These rods have a strong red-resonance and were, on average 46×28 nm, with an aspect ratio of 1.6 and a relative standard deviation of ~25% (Table 1), with less than 15% of the particles having a spherical shape ($AR < 1.2$). These spherical particles are not resonant with red excitation, and thus would produce SERS tags that would be "dark" upon excitation with red light.

A second protocol for the synthesis of Au nanorods is a one-step, "seedless" protocol that can produce smaller rods (Ali *et al.*, 2012). One example of such a preparation, presented in Figure 6 (panels b and e), produced red resonant rods that were, on average, nearly half the size (24×17 nm) as the rods described above, but with a similar aspect ratio (Table 1). Approximately 20% of the particles in this preparation had an $AR < 1.2$, and thus would be non-productive SERS tags.

A variation on this protocol increased the $AgNO_3$ in the seedless reaction mix, favoring the formation of longer, thinner rods. Figure 6c shows the extinction spectrum for such a preparation, which has NIR resonance at ~800 nm. The results of TEM analysis are shown in Figure 6f, and the population mean values in Table 1. The population averages show that these rods are slightly longer than the red resonant rods in preparation B, but inspection of the single particle distributions (Figure 6f) reveals that a significant fraction (35%) of the particles are spherical in shape and thus not plasmonically active in the NIR. If we consider only the non-spherical particles in Table 1, we measure an average aspect ratio of ~3.0, consistent with NIR resonance. Thus, automated TEM provides significant information for engineering of optimal SERS tags that would not have been obvious from the bulk spectral analysis alone, and will enable screening for synthesis conditions that favor the growth on these longer rods while minimizing the growth of non-NIR resonant spheres.

4 Discussion

We have developed a system to prepare multiplexed samples onto a single TEM grid. Inkjet dispensing is particularly well suited for this application given its non-contact nature and ability to tune the volume dispensed in the picoliter to nanoliter range. We demonstrate that up to 96 individual samples can be transferred automatically onto a single TEM grid from a standard well-plate. A critical aspect of the inkjet method we describe here is the grid itself, with silicon-based TEM grids offering more film rigidity and larger window areas for more straightforward TEM imaging. Other benefits of silicon-based TEM grids are that the film properties and the micro-scale structural features can be precisely fabricated using cleanroom technologies.

We demonstrated the utility of the system for characterizing plasmonically-active nanomaterials. A total of 48 different samples were imaged and analyzed. Morphological

features extracted from the images of the single particles correlated well with other measured properties of the specimens. Replicate experiments indicate that the measurements have excellent reproducibility. The TEM analysis also provided significant new information for engineering of optimal SERS tags that would not have been obvious from the bulk spectral analysis alone.

The transfer of the samples to the grid is completely automated other than a short required setup time. The imaging of the grid still requires some manual targeting as targets are distributed in a coffee ring and are not of a consistent size (or shape). However, it is certainly feasible to develop algorithms that would automate several of the manual targeting steps. It would also be possible to speed up the time required for image acquisition by limiting the time spent on monitoring specimen drift, limiting the number of times the software adjusts defocus and reducing the number of targets selected for imaging. The goal in doing this would be to reduce the time required for imaging a 96 well plate to less than 24 hours.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Overview of the process for multiple sample imaging on a single TEM grid: starting from a 96-well plate, picoliter sized drops of individual samples are transferred to an EM grid, the grid is transferred to the electron microscope for automated image acquisition and collected image sets are analyzed to determine individual sample morphology.

Figure 2.

Sample transfer to a TEM grid. **a.** 96-well plate with various nanorod samples. Variation in color between the wells is due to the different light scattering properties by nanorods of different lengths. **b.** Silicon grid in custom grid holder, with 1 mm $\times$ 1 mm and 50nm thick silicon nitride membrane. **c.** Sample drops deposited on the silicon nitride membrane, viewed under light microscope.

Figure 3.

The piezo capillary dispense head (P) is first washed (W) then moved (1) to the sample which is aspirated (A). Then the PDC is moved (2) back to the wash station and washed. Then the PDC is moved (3) to the target and spotted (S), then moved (4) back to the wash station. The cycle is repeated for all samples.

Figure 4.

Multiscale imaging of each spot using a specialized Leginon application. All images are automatically named and tracked in a database back to spot of origin. **a.** An atlas of the grid is acquired (small section shown) using 35 images at 120 $\times$ magnification to locate the spots and assign coordinates to a positional grid. The spots used for the following images are boxed. **(b,e,h).** Each selected spot is automatically imaged at 550 $\times$. Areas for further imaging are selected. **(c,f,i).** Selected areas are automatically imaged at 2500 $\times$. Boxed sections from **(b,e,h)**, respectively, are shown. Locations that may provide high magnification images of the sample are targeted and can be imaged at several magnification scales. **(d,g,j).** Images of each location are taken automatically. Shown are the boxed areas from **(c,f,i)**, respectively, at 25,000 $\times$. The nanoparticles are well dispersed and can be measured individually.

Figure 5.

Analysis of replicate grids prepared from a microwell plate containing 48 different Au nanoparticle preparations. **a.** Particle numbers analyzed for all wells after automated image segmentation. **b.** Median major and minor axis lengths for all wells as determined by automated image analysis. **c.** Mean aspect ratio for each sample plotted vs its extinction maximum as measured by UV/vis spectroscopy.

Figure 6. Characterization of Au nanorods. **a-c.** Optical extinction spectra of three nanoparticle preparations. **d-f.** Single particle TEM measurements (left: bivariate plot of major and minor axis lengths; middle: aspect ratio distribution; right: example particle images) for three nanoparticle preparations. **a.** Large red resonant rods. **b.** Small red-resonant rods. **c.** Small near-IR-resonant rods. The individual particle images are rescaled to fit the display box, so scaling is orientation-dependent.

Table 1

Mean particle dimensions determined by TEM

Prep	Particles	Major Axis length (nm)	Minor Axis length (nm)	Aspect Ratio
A	399	46.3±13.0	27.8±8.5	1.7±0.4
B	1099	26.4±8.3	16.6±5.6	1.6±0.5
C	789	26.7±9.9	14.4±4.6	2.1±1.1
C _{AR>1.2}	511	30.2±9.7	12.2±4.8	2.9±0.9