Published in final edited form as:

Anal Chem. 2016 December 20; 88(24): 12001–12005. doi:10.1021/acs.analchem.6b03661.

A noise reduction method for quantifying nanoparticle light scattering in low magnification dark-field microscope far-field images

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Abstract

Nanoparticles have become a powerful tool for cell imaging, biomolecule and cell and protein interaction studies, but are difficult to rapidly and accurately measure in most assays. Dark-field microscope (DFM) image analysis approaches used to quantify nanoparticles require high-magnification near-field (HN) images that are labor intensive due to a requirement for manual image selection and focal adjustments needed when identifying and capturing new regions of interest. Low-magnification far-field (LF) DFM imagery is technically simpler to perform but cannot be used as an alternate to HN-DFM quantification, since it is highly sensitive to surface artifacts and debris that can easily mask nanoparticle signal. We now describe a new noise reduction approach that markedly reduces LF-DFM image artifacts to allow sensitive and accurate nanoparticle signal quantification from LF-DFM images. We have used this approach to develop a “Dark Scatter Master” (DSM) algorithm for the popular NIH image analysis program ImageJ, which can be readily adapted for use with automated high-throughput assay analyses. This method demonstrated robust performance quantifying nanoparticles in different assay formats, including a novel method that quantified extracellular vesicles in patient blood sample to detect pancreatic cancer cases. Based on these results, we believe our LF-DFM quantification method can markedly

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Supporting Information

The Supporting Information file contains supplemental experimental methods and data. Supplemental methods include the image processing workflow and macro development, the EV isolation protocol and details of clinical sample collection. Supplemental tables include macro parameters, patient demographics, correlations of different immunoassay image data with target concentration, DSM reproducibility data and a comparison of far- and near-field nanoparticle quantification methods. Supplemental figures include a diagram of the ImageJ macro process, the correlation of raw and processed image data with immunoassay target concentration, the DSM ROC curve for pancreatic cancer diagnosis and graphs depicting the contribution of the three RGB channels to blank, background and nanoparticle signal.

Author Contributions

D.S. designed the study and conducted most of the experiments.

The authors declare no competing financial interest.

decrease the analysis time of most nanoparticle-based assays to impact both basic research and clinical analyses.

Keywords

Dark field microscope; quantification; image processing; ImageJ; Immuno-assay; Extracellular Vesicles

INTRODUCTION

HN-DFM imagery is commonly used to detect and characterize nanoparticles,^{1–6} and has been applied in cell imaging,^{5,7–9} biomolecular quantification,^{10,11} and interaction studies.^{12–14} Several approaches have been tried to enhance HN-DFM sensitivity,^{15–17} but serious drawbacks restrict HN-DFM to research applications, including: 1) sampling concerns, since most users select and analyze representative images, which can introduce observer bias, rather than analyzing a single compound image created from tiled fields covering the entire sample area; 2) intensive manual adjustments, since even minor stage movements can degrade HN-DFM images beyond auto-focus correction; and 3) image erosion, since shifting between objectives used to identify (~10x) and capture (100x) regions of interest (ROI) can introduce bubbles in the lens immersion oil.

LF-DFM imagery can be performed on optical microscopes available in most laboratories with only minor adjustments and has none of the intrinsic barriers to automation associated with standard HN-DFM imagery, but is susceptible to lighting, sample and slide imperfections can significantly impact its signal-to-noise ratio. Several groups have used nonlinear DFM microscopy approaches where two lasers and far-field optics have been used to increase scatter intensity; however, these approaches analyze small areas (< 2 micron) and require additional expensive equipment, making it impractical for use in most clinical labs.^{18,19} We now describe the development of a LF-DFM quantification macro for the popular NIH-developed image analysis program ImageJ (<https://imagej.nih.gov/ij/>) that addresses these issues. Our Dark Scatter Master (DSM) algorithm uses a subtraction-based filtering approach to remove scattering artifacts while retaining nanoparticle signal too weak to distinguish from background signal by conventional methods.^{20,21}

This paper proposes a quantification and processing scheme for far-field DFM imaging with low-power objective lens, which is high throughput and compatible with large scale automation to facilitate bioresearch and clinical application. Proof-of-principle direct and sandwich immunoassays with LF-DFM readouts demonstrated robust standard curve linearity for DSM-processed image data, unlike unfiltered LF-DFM data, which was informative only in direct binding assays. This approach was also found to be useful in directly quantifying extracellular vesicles (EVs) in human serum, finding that these LF-DFM analysis of human serum EV concentrations could be used to distinguish patients with pancreatic cancer from patients without malignant disease, exemplifying the utility of this method in a novel clinical application. The LF-DFM quantification method we describe permits rapid, sensitive nanoparticle quantitation in a variety of bioassay types, and can be

readily modified to permit automation of sample handling, image capture and data analysis to facilitate its adoption for research and clinical applications.

EXPERIMENTAL SECTION

LF-DFM Immunoassay performance

Antibody-conjugated nanoparticles were purchased from commercial vendors and modified as described in Supporting Information. LF-DFM immunoassays were performed using 1 μL multi-well slides functionalized with protein A/G (Arrayit) and blocked 2h at 25°C with 1 μL /well Pierce Protein-Free Blocking Buffer (Thermo Scientific). Primary immunoassays slides were incubated with the indicated amounts of nanoparticle-labeled antibody (1 μL /well) for 2h at 25°C. Secondary immunoassay slides were incubated overnight at 4°C with 1 μL /well capture antibody solution (0.13 mg/mL), blocked 2h at 25°C with 1 μL /well blocking buffer, incubated overnight at 4°C with 1 μL /well antigen solution (target antibody or EV serum), and then incubated for 2h at 25°C with nanoparticle-labeled detection antibody. After the final antibody incubation step, slides were washed for 10 min at 25°C with 0.01% Tween-20 in PBS (PBST, pH 7.0), then deionized water, and then air-dried and imaged by LF-DFM. All wells were aspirated and washed with 1 μL /well PBS between loading steps. All analyses used 8 replicates/sample, unless specified otherwise. Intra- and inter-assays coefficients of variation (CVs) were assessed from of eight replicates of the indicated samples. Analyses were performed using the middle anti-CD9-AuNRs concentration standards in primary (0.06 $\mu\text{g}/\mu\text{L}$) and secondary (0.18 $\mu\text{g}/\mu\text{L}$) binding assays performed on three different days.

Antibody modification of gold nanoparticles

Carboxyl-functionalized nanoparticles were modified by mixing 40 μL of gold nanospheres (AuNPs; Ocean NanoTech GPH-50-20, 9×10^{-10} M) or nanorods (AuNRs; Nanopartz C12-25-650-TC-50, 7×10^{-9} M) with 20 μL PBS (pH 7.4) buffer and 20 μL carbodiimide crosslinking solution (2 mg/mL 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; 1 mg/mL sodium N-hydroxysulfosuccinimide in PBS) (Sigma) and incubating for 10 min at 25°C. Modified AuNRs and AuNPs samples were mixed with 80 μL coupling buffer and 20 μL antibody solution (0.5 mg/mL), shaken for 2h at 25°C, and then mixed with 2 μL quenching buffer. Conjugated AuNRs and AuNPs particles were then 3x washed by 10 min centrifugation at 4,000 g, aspiration and resuspension in 200 μL washing buffer, then re-suspended in 40 μL storage buffer and kept at 4°C until use. All carbodiimide reaction buffers were purchased from Ocean Nanotech.

DFM image capture and processing

LF-DFM images were acquired with a 1/220 s exposure at consistent lighting and magnification on an Olympus IX81 microscope equipped with a 10x objective and a dark-field condenser ($1.2 < \text{NA} < 1.4$) using an Olympus DP70 digital camera with a 10x objective by an investigator blinded to sample identity. Samples were analyzed using a predefined process in which software-defined parameters were used as input for native ImageJ commands to ensure run-to-run consistency. Our ImageJ macro first located the region of interest (ROI) for each well by defining a contour threshold (C_t) that fit the high intensity

boundary of the image, applying the ImageJ native commands “Fit Circle to Image” and “Scale” to obtain a centrally aligned circle selection (*Cs*) mask without threshold edges (Figures 1 and S1) which was saved in the ImageJ ROI manager. All analyses used predefined contour threshold and scaling parameters (Table S1).

Color filtering, thresholding and measurement area

To generate filtered image (*Fi*) files in which scattering artifacts had been subtracted from nanoparticle signal over the entire field, DFM images were split into red, green, and blue channels using the ImageJ command “Split Channels”, and the blue channel was subtracted from the red and green channels to identify AuNPs and AuNRs signal, respectively (Figure 1), and to remove artifacts due to uneven illumination (Figure 2).^{22,23} Next, a threshold filter mask (*Tf*) was generated that removed *Fi* pixels beyond the predefined high and low image thresholds (Table S1), and merger of the *Tf* and *Cs* masks defined the final measurement area (*Ma*).

Nanoparticle quantification

LF-DFM images were next analyzed to determine mean nanoparticle intensity per image

area, where mean intensity was defined as $I_m = \frac{\sum_{i=1}^p I_i}{p}$, using the intensity (*I*) of each pixel (*p*) in the *Ma*. To compensate for filter-induced intensity losses, *Ma* nanoparticle signal responses (*R*) was defined as $R = \sqrt{L \cdot I'}$, where *L* is the mean *Oi* intensity ($L = I_m^{O_i}$) and *I* is the mean *Fi* intensity ($I' = I_m^{F_i}$). *Cs* ∩ *Oi* intensity averages (*IA*) were also calculated for comparison.

Clinical samples and extracellular vesicles purification

Serum samples were obtained after obtaining written informed consent from patients at Houston Methodist Hospital (IRB no. 0213-0011) with corresponding patient demographic and clinical data reported in Table S2.

Data analysis

GraphPad Prism 5.0 software was used to analyze and graph all data, where p-values < 0.05 were considered statistically significant. Limit of detection (LOD) and quantification (LOQ) values were defined to be 3x and 10x the standard deviation of the assay blank, respectively.

RESULT AND DISCUSSION

DSM effectively removes DFM image artifacts

LF-DFM images are highly sensitive to surface imperfections, salt crystals and debris introduced during assay performance, and our data routinely contained image artifacts due to scratches, particulates, mixing voids and uneven sample illumination (Figure 2a). Scratches were primarily caused by liquid addition and aspiration, particulates were introduced by salt precipitation and during air drying, mixing voids were caused by hydrodynamic reflections and uneven illumination arose from condenser and focus imperfections, which are difficult to address. We found that LF-DFM signal was ~3 fold higher in the blue (B) vs. red (R) or

green (G) channels of RGB images of blank wells, such that R-B or G-B subtraction almost completely removed all DFM signal (Figure S4). Similar results were obtained with AuNR immunoassay wells where R-B subtraction removed >98% of background red channel signal in negative control wells without added antigen, and allowed robust detection of AuNR signal at increasing antigen concentrations (Figure S5). Subtracting this signal from red channel AuNRs signal or green channel AuNPs signal reduces artifacts due to most physical imperfections²⁴ as well as those resulting from uneven illumination (Figure 2c), while thresholding excludes areas affected by signal artifacts from the analysis area (Figure 2c and d).

DSM quantitation of nanoparticle signal from LF-DFM images

Analysis of LF-DFM AuNRs signal in assays where protein A/G was used to capture AuNRs-conjugated-antibody found that DSM R values strongly correlated with input antibody concentration, performing better than IA values (Figure 3a, Table S3). A sandwich immunoassay (SIA) using excess target antibody target and increasing amounts AuNRs-labeled detection antibody revealed similar correlation, albeit with reduced DSM R signal (Figure 3b), likely reflecting decreased binding efficiency in the absence of excess detection antibody. Reduced AuNRs signal in this assay completely ablated the IA correlation, indicating robust IA differences were required to quantify unprocessed LF-DFM AuNRs signal (Figure 3b). Similar results were observed in assays using AuNPs-labeled antibody (Figure S2), which scatter green light, even though CCD cameras are more sensitive to red light across a broad spectrum.²⁵ Results from assays with varying target and constant detection antibody concentrations (Figure 3c) also followed this pattern, and revealed limit of detection and quantitation values highly similar to those detected in direct binding assays (Table S3).

All measures of LF-DFM nanoparticle signal correlated with probe concentration in direct binding assays, but IA , L and I' values, unlike DSM R values, did not consistently correlate with input protein concentration in sandwich immunoassays (Table S3). LF-DFM background thus negatively impacted quantitation from raw signal (IA) and/or individual components (L and I') of our algorithm without inhibiting DSM quantification. Similar intra- and inter-assay variation was observed for direct and sandwich immunoassays (Table S4) and assays could be stored at room temperature and read at later dates without loss of assay signal or increased variability (Table S5).

HN-DFM imagery is commonly used to quantify individual nanoparticles,^{5,7,8} but uses conditions and analyses very different from those employed in our assay. Nanoparticle applications proposed for cell imaging,^{7,26–34} also contain noise that would confound direct analysis of total nanoparticle signals.^{29–32,34} Most studies thus employ either absorption spectrum methods^{29,33} or counting approaches that use high magnification, near field images^{4,5,30} for nanoparticle quantification.

To compare our results with NF-DFM results, we developed a near-field DSM (NDSM) script (See Supporting Information) mimicking a process described by Xu *et al* to count target nanoparticles. Comparison of DSM and NDSM performance on the same sample wells found that NDSM results were less sensitive (Table S6) and had worse linearity, due to

inconsistent signal vs. concentration increases (Figure S6), and much greater coefficients-of-variation, due to uneven nanoparticle distribution among replicates (Figure S7).

DSM quantitation of extracellular vesicles in serum for cancer diagnosis

Extracellular vesicles (EVs) shuttle factors that reflect their originating cell type and can regulate the function of both adjacent and distant cells. Circulating EVs have great potential for noninvasive cancer detection,³⁵ but their clinical use is hindered by the lack of simple EV analysis methods, as current assays require labor-intensive purification steps prior to quantification,³⁶ and are clinically impractical as they are slow, complex, low-throughput and expensive. We thus examined whether DSM analysis conferred sufficient sensitivity to allow quantification of AuNRs-probe-based SIA assays of EV samples, using the EV membrane markers CD63 and CD9, respectively, as capture and detection antibodies.^{37–40} Our results indicated a strong linear relationship between DSM response and EV concentration (Figure 4b).

This approach was used to directly analyze serum EV concentrations from patients with and without pancreatic cancer (PC) to determine if EV signal could distinguish between these populations. Our results found that serum samples of PC patients had significantly more EVs than those of control subjects (Figure 4c).⁴¹ Receiver operating characteristic (ROC) curves analysis indicated that DSM-quantified serum EV level was an excellent classifier (area under the curve of 0.939) for differentiating pancreatic cancer patients and their controls (Figure S3), suggesting that a rapid, non-invasive assay that can be performed with inexpensive equipment already available in clinical laboratories may be useful in cancer screening for pancreatic cancer, and perhaps other cancer types.

Conclusions

In the current study we present evidence that LF-DFM images can be used to quantify nanoparticle signal in direct binding and SIA assays, through the use of a custom ImageJ macro that corrects for the high background noise and uneven illumination by color subtraction and threshold masks. This approach allows nanoparticle-based quantitation to be used in routine bioassay quantification, which offers several advantages over conventional assays, as the example LF-DFM nanoparticle assays: 1) require very little sample, which is often an issue when analyzing limited mouse or clinical blood samples; 2) are endpoint assays, which prevents readout saturation, allows maximum signal intensity to be adjusted by input parameters, and permits slide archiving and reanalysis; 3) utilizes relatively inexpensive equipment that available to most research and clinical laboratories; and 4) is amenable to automation of the sample-handling, image capture and data analysis steps to allow high-throughput analyses with minimal direct human intervention.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

This research was supported in part by US National Institute of Allergy and Infectious Diseases grant R01AI113725-01A1 and R01AI122932-01A1, and John S. Dunn Foundation award. Authors would like to thank Dr. Jianhua Gu and Dr. Kai Liang for their invaluable advice.

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Figure 1.

Image processing algorithm of the DSM macro for ImageJ. The C_s selection area mask is the centered circular area enclosed by the contour threshold, while the T_f selection area mask is the area falling within the predefined pixel intensity range (white pixels).

Figure 2.

Images and corresponding 3D profile of image pixel intensity in an immunoassay well before and after image processing, demonstrating the noise reduction effect of these steps. (a) Image and 3D intensity profile of a representative high concentration immunoassay well revealing multiple common defects that produce DFM image artifacts, including scratches, salt crystals, mixing voids and uneven lighting (equal to Figure 1: $Oi \cap Cs$ image). (b) R, B and R-B images of the $Oi \cap Cs$ image. Note that signal from scratches, salt/debris deposits and lighting irregularities are highly visible (white areas) in the blue channel and thus absent (black areas) in the R-B image (equal to Figure 1: $Fi \cap Cs$). (c, d) Image and 3D intensity map after threshold application for (c) R-B and (d) the original $Oi \cap Cs$ image data (equal to $I' (Fi \cap Cs, Tf)$ and $L (Oi \cap Cs, Tf)$, Figure 1), revealing elimination of the dark field signal artifacts in these images and 3D maps (black and red areas, respectively).

Figure 3.

Correlation of LF-DFM image intensity average (IA) and Dark Scatter Master (DSM) R signal with nanoparticle-labeled antibody concentration in (a) direct binding assays with variable AuNRs probe concentrations, or sandwich immunoassays (SIA) with (b) constant target/variable AuNRs probe or (c) variable target/constant probe concentrations. Data points represent mean $\pm$ SEM; $N=8$ /sample. Listed p -values indicate the odds that the associated linear regression line is non-zero.

Figure 4.

(a) EV quantification scheme. (b) Linear correlation between the quantification response from DSM and exosome concentration. (c) DSM-quantified EV concentrations in serum samples of pancreatic cancer and control patients. Data are presented as mean $\pm$ SEM, N=7/group; **p=0.002 by two-sided Mann-Whitney U-test.