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TITLE:

Performing Spectroscopy on Plasmonic Nanoparticles with Transmission-Based Nomarski-Type Differential Interference Contrast Microscopy

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KEYWORDS:

localized surface plasmon resonance, single particle spectroscopy, polarization, DIC, wavelength, nanoscale, Nomarski

SUMMARY:

The goal of this protocol is to detail a proven approach for the preparation of plasmonic nanoparticle samples and for performing single particle spectroscopy on them with differential interference contrast (DIC) microscopy.

ABSTRACT:

Differential interference contrast (DIC) microscopy is a powerful imaging tool that is most commonly employed for imaging microscale objects using visible-range light. The purpose of this protocol is to detail a proven method for preparing plasmonic nanoparticle samples and performing single particle spectroscopy on them with DIC microscopy. Several important steps must be followed carefully in order to perform repeatable spectroscopy experiments. First, landmarks can be etched into the sample substrate, which aids in locating the sample surface and in tracking the region of interest during experiments. Next, the substrate must be properly cleaned of debris and contaminants that can otherwise hinder or obscure examination of the sample. Once a sample is properly prepared, the optical path of the microscope must be aligned, using Kohler Illumination. With a standard Nomarski style DIC microscope, rotation of the sample may be necessary, particularly when the plasmonic nanoparticles exhibit orientation-dependent optical properties. Because DIC microscopy has two inherent orthogonal polarization fields, the wavelength-dependent DIC contrast pattern reveals the orientation of rod-shaped plasmonic nanoparticles. Finally, data acquisition and data analyses must be carefully performed. It is common to represent DIC-based spectroscopy data as a contrast value, but it is also possible to present it as intensity data. In this demonstration of DIC for single particle spectroscopy, the focus is on spherical and rod-shaped gold nanoparticles.

INTRODUCTION:

Since the 1980s, differential interference contrast (DIC) microscopy has largely been viewed as an important imaging method reserved for microscale objects within the biological sciences. However, during its development in the 1950s and 1960s, it was intended as a technique for

materials science[1]. With the recent advancements in the material sciences related to plasmonic nanoparticles, an increased interest in the characterization of materials with optical microscopy has taken place.

Many optical techniques are certainly available for nanomaterial characterization (e.g., dark field, brightfield, polarized light, fluorescence, etc.). Dark field is widely popular in nanoparticle research, but it relies solely on the collection of scatter and provides limited information about complex samples[2]. Fluorescence can be useful, but only with samples that luminesce or that can be properly stained. DIC microscopy has several traits that make it a valuable tool for the analysis of nanoparticles. The most frequently stated advantages of DIC in comparison to other methods and in regards to plasmonic nanoparticles are: no sample staining required, no halo effects, shallow depth of field, and high lateral resolution[3]. DIC has additional strengths that are valuable to plasmonic nanoparticle research. First of all, two inherent and orthogonal polarization fields are present, and they can be measured simultaneously for spectroscopy purposes[2]. Secondly, the depolarized signal of nanoparticles is not captured in the final image[2], which can be a cause for serious concern in dark field spectroscopy measurements.

The purpose of this article is to provide a clear methodology for utilizing transmitted-light Nomarski DIC microscopy to perform spectroscopy on plasmonic nanoparticles. Although DIC is a powerful technique that can be applied to highly diverse materials, it is also a technique that requires great skill and understanding to operate it properly when imaging nanoparticles. Transmission-based Nomarski DIC microscopy has a complex light path[1] that will only be briefly reviewed here. The optical train of DIC is displayed in **Figure 1**. Light is transmitted through the microscope by first being passed through a polarizer and a beam-splitting Nomarski prism before being focused by the condenser onto the sample plane. After passing through the objective, the light encounters a beam-combining Nomarski prism and an analyzer before exiting to the detector. The two polarizers and Nomarski prisms are critical to formation of the DIC image and are responsible for producing DIC's two orthogonal polarization fields[1]. For the reader interested in knowing more about the working principles and optical path of Nomarski DIC microscopes, or the differences between Nomarski DIC and other styles of DIC, please refer to other well-written accounts on these topics[1,4–7].

It is equally important to understand the basic nature of plasmonic nanoparticles before attempting to perform spectroscopy on them, whether it be with Nomarski DIC, dark field, or any other microscopy technique. In the field of plasmonics, nanoparticles are defined as particulates with dimensions on the scale of 10−100 nm[8,9]. Nanoparticles can take on many shapes (e.g., spheres, rods, stars, dumbbells, etc.), and most of their important properties arise from interactions with light in the ultraviolet-visible-near infrared range of the electromagnetic spectrum. The term "plasmonic" is not restricted to nanoparticles[10]; however, when discussing nanoparticles, it is used in reference to localized surface plasmon resonance (LSPR). LSPR is a phenomenon in which the conduction electrons in a nanoparticle oscillate due to a Coulombic interaction with electromagnetic radiation of a highly specific and relatively narrow frequency band[8]. At these same frequencies, plasmonic nanoparticles exhibit increased absorption and scattering of light, making them observable with optical microscopy. In many cases, it is

preferred to observe the nanoparticles while placing bandpass filters before the condenser[2], to improve imaging contrast and to eliminate light that fails to induce the LSPR effect. Using filters also makes it possible to perform single particle spectroscopy experiments.

LSPR-related optical behavior is highly dependent on the size and shape of the nanoparticles, and it can be investigated with many optical microscopy techniques. However, in order to decipher orientation information of plasmonic nanoparticles with an anisotropic (i.e., non-spherical) shape, it is necessary to utilize polarization of the light field. By carefully rotating the polarization field or the sample substrate at small increments, it is possible to monitor the orientation-dependent spectroscopic properties of individual nanoparticles. Rotation and polarization can also aid in determining whether a spectral feature is due to a dipolar or higher order oscillation of the nanoparticle's surface electrons. However, in the case of isotropic (i.e., spherical) nanoparticles, the spectral profile remains essentially unchanged upon rotating the sample under polarized light.

When viewed through a DIC microscope (**Figure 2**), nanoparticles have an airy disk with a shadow-cast white-and-black appearance against a gray background. Spherical nanoparticles will retain this appearance under rotation and with the changing of bandpass filters; however, the particles will gradually fade from view as the filter's central wavelength becomes further separated from the sphere's only dipolar LSPR wavelength[11]. The appearance of nanorods can change quite dramatically as they are rotated[2]. Nanorods have two LSPR bands with dipolar behavior, the location of which are based on the physical dimensions of the nanorods. When the longitudinal axis of a nanorod is oriented parallel to one of the DIC polarization fields, the airy disc will appear all white or all black if viewed with a bandpass filter associated with that LSPR wavelength. After rotating the sample 90°, it will take on the opposite color. Alternatively, since the transverse axis of a nanorod is perpendicular to the longitudinal axis, the rod will take on the opposite color when switching between filters that match the LSPR wavelengths for the two axes. At other orientations and filter settings, nanorods will appear more like spheres, presenting a variety of shadow-cast airy disc patterns. For nanorods with a transverse axis < 25 nm, it can be difficult to detect signal at that LSPR's wavelength using DIC microscopy.

To perform single particle spectroscopy, it is important to use the correct optical components and to align them properly. An objective capable of DIC microscopy must be used. For single particle experiments, 80x or 100x oil objectives are ideal. Nomarski DIC prisms ordinarily come in three varieties: standard, high contrast, and high resolution. The ideal type highly depends on the purpose of the experiment and the size of the nanoparticles. Standard prisms are fine for many experiments; but when working with smaller nanoparticles (< 50 nm), high contrast prisms can be beneficial, since particle contrast decreases as the particles decrease in size[11]. Adjusting the DIC contrast is achieved either by rotating a polarizer or by translating one of the DIC prisms, depending on the microscope brand or model[6].

After setting Kohler illumination and the polarizer settings, it is critical to not readjust these settings while collecting spectroscopy data. Furthermore, a constant average background signal must be maintained at all times during data collection, even when switching between filters

and angle settings. The actual ideal background value depends on the dynamic range of the scientific camera, but in general, the background should be in the range of 15%–40% of the maximum detection level of the camera. This reduces the likelihood of saturating the camera sensor while enabling optimal particle contrast. For collecting spectroscopy data, it is necessary to work with a scientific camera that captures images in black and white, as opposed to a color camera.

Sample preparation is another critical aspect of imaging plasmonic nanoparticles. It is imperative that operators of DIC microscopy have an understanding of the sample's optical properties and of the sample's substrate. "Pre-cleaned" microscope glass is not sufficiently prepared for imaging nanoparticles, and it must be properly re-cleaned before sample deposition to ensure unobstructed observation of the sample. Many cleaning protocols for microscope slides have been previously documented[12], but it is not a step that is normally reported in experimental studies.

Finally, data analysis methods are the final component to single particle spectroscopy. The maximum and minimum intensities for each nanoparticle must be measured, as well as the local background average. Particles of interest should be located in areas with no background debris, substrate defects, or uneven illumination. One method for determining the spectral profile of a nanoparticle is by calculating particle contrast at each wavelength, using the equation below[11,13–15]:

$$\text{Particle Contrast} = \frac{(\text{Particle Maximum} - \text{Particle Minimum})}{\text{Background Mean}}$$

Alternatively, a single particle's spectrum can be split into its individual maximum and minimum signal components, which represent DIC's two polarization fields, thereby displaying the two simultaneously-collected directionally-dependent spectra, through the two equations:

$$Particle\ Maximum_{adjusted} = \frac{Particle\ Maximum_{measured}}{\text{Background Mean}}$$

$$Particle\ Minimum_{adjusted} = \frac{Particle\ Minimum_{measured}}{\text{Background Mean}}$$

PROTOCOL:

1. Sample preparation with standard glass microscopy slides

1.1. Prepare glass microscope slides for sample deposition.

1.1.1. For best results, purchase glass or quartz microscope slides and cover glass.

1.1.2. Using a scribing pen, place a shallow and short scratch mark onto the center of each glass

cover slip.

1.1.3. Clean all microscope glass, even if it is purchased “pre-cleaned”, to remove glass shards, dust, powder, organic residue, and any other contaminants that affect imaging quality or sample deposition.

NOTE: This cleaning method below works well for the types of samples described here and avoids the use of harsh chemicals. Harsher chemicals can etch the glass and require more care in handling and disposal.

1.1.3.1. Place microscope glass onto storage racks and then into a beaker, or into a staining jar. Do not place microscope glass at the bottom of beakers and other lab glassware without racking, because each piece and surface of microscope glass should be fully exposed to the cleaning agents.

1.1.3.2. Pour ~1 mL of liquid detergent (**Table of Materials**) into the container and top off the container with water. Sonicate for 30 min.

NOTE: Once the cleaning process begins, only handle the glass while wearing gloves, to avoid leaving fingerprint residue on the glass.

1.1.3.3. Pour out the liquid contents of the cleaning container into a sink. Rinse container several times with ultrapure water to remove all appearance of detergent. Refill the container with ultrapure water. Sonicate the container with microscope glass for another 30 min.

1.1.3.4. Repeat the previous step at least once more. Perform additional rounds of sonication in water until it is obvious that all traces of the detergent have been removed.

1.1.3.5. Pour out the contents of the cleaning container. Rinse the container with ultrapure water. Refill the container with ethanol. Sonicate microscope glass for 30 min.

1.1.3.6. Pour out the contents of the cleaning container into a waste container. Refill with ethanol. Cover the container to prevent loss of ethanol through evaporation. Store the microscope glass in this container until time of experiment. Slides remain clean and usable as long as they remain submerged in ethanol inside a covered container.

NOTE: In some circumstances, it may be more appropriate to store the glass in ultrapure water instead of ethanol. However, storing in water or air makes the glass hydrophobic over time.

1.2. Preparation of nanoparticle solution

1.2.1. Using a micropipette, remove a 100 µL aliquot of 0.05 mg/mL gold nanoparticle solution from its original storage container and eject the solution into a 1.5 mL centrifuge tube.

1.2.2. Centrifuge the sample for 10 min at 6000 x *g*.

1.2.3. Remove the supernatant with a micropipette, in order to remove excess surfactant.

1.2.4. Using a micropipette, place 100 µL of ultrapure water into the centrifuge tube.

NOTE: If not all of the supernatant can be removed on the first attempt, repeat the centrifugation and resuspension steps.

1.2.5. Briefly vortex the sample to resuspend the pellet. Sonicate immediately afterwards for 20 min to fully resuspend and break up nanoparticle aggregates.

NOTE: If the sample is not used immediately, it should be sonicated again for 20 min before depositing solution onto microscope glass.

1.3. Sample deposition

1.3.1. Remove cleaned cover slips and microscope slides from their storage containers. Blow dry the glass with pressurized nitrogen or argon.

1.3.2. Using a micropipette, drop-cast 6 µL of nanoparticle solution from step 1.2.5 onto the cover slip. To spread out the droplet evenly, carefully place a second, larger piece of microscope glass on top of the cover slip, such as a second cover slip or a microscope slide. Avoid getting air bubbles trapped between the two pieces of glass.

1.3.3.1. Turn the sample substrate over, and seal off the edges of the cover slip with a narrow line of nail polish in order to prevent evaporation of the medium solution.

1.3.3.2. Alternatively, to image the sample “dry”, allow the solution to stand for 5–15 min on the cover slip, before removing the unwanted piece of glass. Gently blow the cover slip dry with pressurized nitrogen or argon.

1.3.4. If possible, image samples immediately after preparation. If that is not possible, store samples in a covered container, such as a Petri dish until imaging.

2. DIC imaging

2.1. Align objective and condenser.

2.1.1. After placing the sample onto the microscope, find the focal plane with the sample on it. First locate and focus on the scratch mark created earlier. Then fine tune the focus until nanoparticles come into view.

2.1.2. To determine the accurate placement of the condenser, utilize the Kohler Illumination

method.[5] Kohler Illumination at high magnification (80x, 100x) is more easily achieved by first setting the Kohler Illumination at a lower magnification, such as 20x.

NOTE: Normally, Kohler Illumination does not need to be re-adjusted during imaging of a single sample. However, it is good practice to verify that Kohler Illumination is properly set when switching to a new microscope slide.

2.2. Optimize contrast settings.

2.2.1. Select a region of interest within the sample for imaging. Center the region in the camera's field of view and adjust focus as necessary.

2.2.1.1. If the microscope has the de Senarmont design, start with the polarizer set near to maximum background extinction and gradually rotate the polarizer towards decreasing background extinction. The background intensity will gradually increase.

2.2.1.2. If the microscope does not have a de Senarmont design, start with the optical train set at maximum background extinction. In this case, gradually adjust the objective prism position towards decreasing background extinction.

NOTE: The ideal setting is achieved when the nanoparticles reach their greatest intensity difference (i.e., contrast) from the averaged local background value. For plasmonic nanoparticles, optimal contrast is normally achieved with a relatively dark background, thus at settings near maximum background extinction.

2.3. Image the sample.

2.3.1. Turn off room lighting to prevent stray illumination from interacting with process.

2.3.2. While viewing the nanoparticles with a scientific imaging camera, determine the optimal background level. Using a 10 nm full width at half maximum (FWHM) bandpass filter with its central wavelength co-located with the main LSPR wavelength, view the region of interest. Adjust the lamp intensity or exposure time until the background level is in the range of 15%–40% of the camera's maximum capacity level and no objects within the region of interest exhibit signal intensities that exceed 90% of the camera's maximum intensity level.

NOTE: The goal of step 2.3.2 is to prevent saturating the sensor when switching between filters. The ideal background level will vary between samples and cameras. Once this step is completed, exposure time can be adjusted but not the lamp intensity.

2.3.3. Image the sample with a series of bandpass filters that each has a FWHM of 10 nm and that as a whole enable imaging across the entire wavelength range of interest. Ensure that the background intensity remains consistent from image to image (within ~5% of one another) by adjusting the exposure time. After switching filters, re-focus the sample before image capture.

2.3.4. Save the Images as uncompressed TIFF files and/or in the software's native file format, in order to preserve all information.

2.4. Rotate the sample.

2.4.1. After collecting images of the sample at its original position, the sample can now be rotated and imaged at additional orientations in the light path. Perform rotation at regular intervals (e.g., 10° or 15°) across either a 180° or 360° range.

NOTE: Rotation requires a rotatable sample stage.

2.4.2. As in sections 2.1−2.3, adjust camera settings to provide a consistent background level from image to image.

NOTE: No adjustment to Kohler Illumination should be made.

3. Data analysis using ImageJ

NOTE: The following calculations can be performed in a variety of software packages, and sometimes in the native program used to collect the images. ImageJ is a freely available software from the National Institutes of Health (NIH).

3.1. Calculate particle contrast or intensity.

3.1.1. Open the image with ImageJ.

3.1.2. Select the **Rectangle** tool and draw a rectangle around the main region of interest.

3.1.3. On the Tool bar, select **Image**, then **Zoom**, then **To Selection**. The imaging window will zoom in on the selected area.

3.1.4. On the Tool bar, select **Image**, then **Adjust**, then **Brightness/Contrast**. A new window appears. To enable better viewing of the sample region, adjust the four settings: Minimum, Maximum, Brightness, and Contrast. These adjustments do not alter the scientific data, they merely enable better visibility of the sample region.

NOTE: Steps 3.1.3 and 3.1.4 may be performed multiple times and in reverse order.

3.1.5. Using the rectangle tool again, draw a box around the first nanoparticle to be measured. The box should be only slightly larger than the nanoparticle's airy disc.

3.1.6. On the Tool bar, select **Analyze**, then **Measure**. A new window appears that reports the Minimum, Maximum, and Mean Intensities for the pixels located inside of the selected box.

3.1.7. Drag the box used to measure the nanoparticle to an area immediately adjacent to the particle, where the background contrast is relatively even and no particles or contaminants are present. Retain the original size of the box.

3.1.8. Use the Measure tool to determine the Mean Intensity for the background area.

3.1.9. Measure the remaining particles and an adjacent background area for each.

3.1.10. Repeat the process for each particle in all of the images in the series.

3.1.11. Export the data to a spreadsheet to calculate the contrast or intensity of each particle, across all wavelengths and angles.

3.1.12. Calculate each particle's contrast, using the following equation[13–15]:

$$\text{Particle Contrast} = \frac{(\text{Particle Maximum} - \text{Particle Minimum})}{\text{Background Mean}}$$

NOTE: Using this equation, particle contrast should always be > 0.

3.1.13. Calculate the particle's background-adjusted maximum value by dividing the measured maximum particle intensity by the background mean:

$$Particle\ Maximum_{adjusted} = \frac{Particle\ Maximum_{measured}}{\text{Background Mean}}$$

3.1.14. Likewise, calculate the background-adjusted minimum value by dividing the measured minimum particle intensity by the background mean:

$$Particle\ Minimum_{adjusted} = \frac{Particle\ Minimum_{measured}}{\text{Background Mean}}$$

NOTE: As calculated, the maximum should have a value greater than one, while the minimum will be less than one. It is acceptable to subtract each value by "1", so that the average background is essentially zero, the maximum is represented as a positive value, and the minimum value is assigned a negative value[16]. This latter approach allows the analyst to separately consider what is occurring along each of the polarization fields, which is useful when studying anisotropic particles.

3.1.15. To graph the spectral profile at a given nanoparticle position, plot data with the wavelength along the x-axis and the contrast or intensity along the y-axis.

3.1.16. To graph the rotational profile at a given wavelength, plot the rotation angle along the

x-axis and the contrast or intensity along the y-axis.

REPRESENTATIVE RESULTS:

When working with samples that are large enough to be seen with the naked eye, placing landmarks on the glass substrate is not normally required. However, when working with nanomaterials or when rotation of the sample is required, landmarks can provide an easy method for locating, distinguishing, and tracking the orientation of the sample. Although more sophisticated techniques can be utilized for leaving landmarks on glass substrates[17], scratching the glass with a scribing pen is an economical and simple method that works in many situations. It is important to avoid examining sample regions that are immediately adjacent to these landmarks, since scratch marks create a complex background with the potential of impacting data (**Figure 3**). However, at the tips of the scratch marks, "spider webs" often extend outward from the scratch. These lines are quite valuable as landmarks, but again, nanoparticle should not be collected for data if they overlap with these defects.

In order to achieve optimal imaging with DIC microscopy, it is of vital importance to determine the proper focal plane. Objects that are slightly out of focus will appear fuzzy, have blurred edges, and have decreased contrast. **Figure 4** displays gold nanoparticles that are out of focus to varying degrees. Nanoparticles in the bottom right corner are in focus, while nanoparticles become farther out of focus as they approach the upper left corner of this image. Because DIC has a shallow depth of field, it is not uncommon for some nanoparticles to be in focus while others are out of focus when imaging them on a glass substrate. As a result, it is critical to consistently focus on the same exact particles when making adjustments to the microscope during an experiment.

Figure 5 provides an example of the effect of adjusting the polarizer settings while imaging gold nanospheres. Five nanoparticles are in focus, while one is slightly out of focus. A 540 nm bandpass filter with 10 nm FWHM was also in the optical path. In this series of images, the background brightness was adjusted with ImageJ after image acquisition in order to make the five particles more apparent against the background. When the polarizer is set at 0° in a de Senarmont designed Nomarski DIC microscope, it is orthogonal to the analyzer (**Figure 5A**). At 0°, the particles appear mostly white, with a dark stripe running across their mid-section. This is indicative of cross-polarization for nanosphere samples. When the polarizer is rotated to different angles (**Figure 5B-E**), the particles appear to be casting dark shadows towards the southwest. The black and white components to the signal arise as a result of DIC's two polarization fields and provide information about the orientation of plasmonic nanoparticles when working with bandpass filters. As the polarizer is rotated towards higher angles, the shadow pattern remains similar. However, the particle contrast values change dramatically. This is best demonstrated by measuring the contrast values for the individual particles, using the equation provided above. The particle highlighted with the yellow box has contrast values of 0.65 (crossed polarizers), 0.84 (polarizer shift of 5°), 1.10 (10°), 0.44 (20°), and 0.23 (45°). Therefore, for this sample, the optimal imaging setting is with a polarizer shift of 10°. Plasmonic nanoparticles often require a polarizer setting in the range of 5°–15°, and smaller increments than these should normally be used to identify the ideal setting. For further information on the

imaging and analysis of spherical gold nanoparticles, readers are referred to the prior work by Sun et al.[11].

Anisotropic-shaped nanoparticles produce patterns of higher complexity than nanospheres. Gold nanorods were imaged (**Figure 6**) at their longitudinal LSPR wavelength, 650 nm. In the initial image (**Figure 6A**), five bright nanorods and several dimmer particles are apparent. Instead of having a shadow-cast appearance, three of the rods have predominately black airy discs while two are mostly white. The polarizer was set at 10° to the left of the crossed polarization setting. In **Figure 6B**, crossed polarization was used; only three of the particles appear, as fully white airy discs. The others have disappeared or appear to be slightly out of focus. With the polarizer set at 10° to the right of crossed polarization (**Figure 6C**), the patterns are now reversed of what was observed in **Figure 6A**. The polarizer was next turned to 45° right of crossed polarization (**Figure 6D**), the maximum setting, to demonstrate that particles retain their colors at this setting, but contrast has declined significantly. In the remaining figure panels, the collection of nanorods was incrementally rotated a full 90° clockwise while the polarizer was set at 10° to the right of crossed polarization. The pattern gradually changes for each nanorod, and after a full 90° rotation, the particles have reversed their colors from the initial setting. In brief, if one of the axes of a plasmonic nanorod is lined up with one of the two polarization fields, and if the nanorod is imaged at that axis' LSPR wavelength, the nanorod will appear to be mostly white or mostly black, depending on which polarization field it is aligned with (**Figure 6A,C**)[2]. If the nanoparticle is rotated a full 90° (**Figure 6H**), it will now be lined up with the opposite polarization field and take on the opposite color. If instead the nanoparticle was rotated only 45° (**Figure 6F**), then it will be in a position where the particle will exhibit its greatest shadow-cast appearance, showing striking similarity to what is observed with the plasmonic nanospheres. As a result of this optical behavior, plasmonic nanoparticles with an anisotropic shape often look flat instead of having the three-dimensional shadow-cast appearance of nanospheres. The result of this difference in optical behavior is that it can be exploited in order to distinguish between plasmonic nanoparticles that are spherical and anisotropic in shape, as has been previously discussed in multiple research studies[2,3,6,7,11,13,16].

Finally, **Figure 7** displays representative single particle spectroscopy data, as contrast of a gold nanosphere (**Figure 7A**)[11], intensity of a single gold nanorod with its longitudinal axis oriented parallel to one of the polarization fields (**Figure 7B**)[6], and intensity profile of a single gold nanorod at its LSPR wavelength and during rotation of the stage (**Figure 7C**)[6]. Either method of presentation reveals the width and location of the LSPR effect. For plasmonic nanoparticles with an anisotropic shape, the intensity and rotation data reveal the directionality of the effect, and hence, the orientation of the particle on the sample substrate, which has been previously proven through correlative studies on such particles using DIC and transmission electron microscopy[2,16,18].

FIGURE LEGENDS:

Figure 1: The light path in transmitted-light Nomarski-based DIC microscopy. After leaving the light source (S), light passes through a polarizer (P), a beam-shearing Nomarski prism (NP), the

condenser (C), the focal plane (FP), the objective (O), a beam-combining Nomarski prism (NP), the analyzer (A), and finally the detector (D).

Figure 2: Examples of plasmonic nanoparticles imaged at their LSPR wavelengths with 10 nm bandpass filters, using a DIC microscope. Both images are collected at 100x. (**A**) Silver nanospheres with 40 nm diameter imaged at 480 nm with a bandpass filter having 10 nm FWHM. (**B**) Rod-like gold nanoparticles imaged at 700 nm using a bandpass filter with 10 nm FWHM.

Figure 3: Image of a scratch made into a glass cover slip with a scribing pen. Near the end of the actual indentation, a series of narrow and shallow "spider web" lines branch out from the scratch itself, resulting in a pattern that can be utilized as an imaging landmark. This image was collected using 100x magnification and broadband white light.

Figure 4: Gold nanospheres imaged with broadband white light at 100x. Particles in the lower right are in focus but particles drift farther from the focal plane towards the upper left corner. Object in middle of image is debris.

Figure 5: Gold nanospheres imaged under a series of different polarizer settings at a wavelength of 540 nm and a magnification of 100x. Background brightness was adjusted post-imaging with ImageJ to make particles more apparent. Polarizer setting of (**A**) 0° (polarizer orthogonal to analyzer), (**B**) 5°, (**C**) 10° (the best contrast of this series of images), (**D**) 20°, and (**E**) 45°. Measured contrast of particle in yellow box is (**A**) 0.65, (**B**) 0.84, (**C**) 1.10, (**D**) 0.44, and (**E**) 0.23.

Figure 6: Example of imaging anisotropic plasmonic nanoparticles: gold nanorods at their longitudinal LSPR wavelength of 650 nm and a magnification of 100x. Particles of main interest are enclosed in yellow box. Polarizer settings are: (**A**) left 10°, (**B**) 0°, (**C**) right 10°, (**D**) right 45°. With the polarizer set to right 10°, the stage was rotated clockwise by (**E**) 20°, (**F**) 45°, (**G**) 70°, and (**H**) 90°.

Figure 7: Representative results of single particle spectroscopy data. (**A**) Gold nanosphere spectroscopy displayed in terms of DIC Contrast. Each data point represents an average of 20 nanospheres for each particle diameter, and data capture relied on 10nm FWHM bandpass filters. (**B**) A single gold nanorod displayed as DIC Intensity data, using two different polarizer settings (2° on either side of crossed polarization). (**C**) DIC Intensity data for a single gold nanorod at the LSPR wavelength of 680 nm, while it was rotated 180° and the polarizer was held at 2° off the crossed polarization position. Figure 7A is adapted with permission from Sun et al., *Analytical Chemistry*. 81 (22), 9203-9208 (2009), and Figure 7B,C from Stender et al., *Analytical Chemistry*. 84 (12), 5210-5215 (2012). Copyright American Chemical Society.

DISCUSSION:

When imaging with DIC microscopy, it is critical to optimize the optical components before collecting data. Even minor adjustments to the polarizer in the middle of an experiment can

result in significant impacts to the final data[6]. Moreover, different materials require different polarizer settings. Although large step sizes were utilized here to demonstrate the effect of polarization angle, in an actual experiment, it is imperative to optimize the polarizer setting within 1°–2° of the optimal contrast setting. The polarizer setting should also be recorded for future reference. It is also recommended to always work on the same side of the crossed polarizer (0°) point. Switching back and forth does not provide any advantages, but it can lead to confusion, due to the reversal in signal.

Next in importance, it is critical to monitor the background intensity when planning to perform spectroscopy. This is best accomplished by adjusting the camera exposure time, or by adding neutral density filters to the light path. Adjusting apertures or lamp intensities can impact the Kohler Illumination and alter contrast values. The background needs to be relatively even across the sample, so that the selection of a background region does not alter the contrast calculation. Sample specimens that are not adjacent to a clean background space should be avoided. Moreover, the background intensity cannot be initially set too high or too low. If the background intensity is set too high, there is an increased risk that some signals will exceed the maximum range of the camera, making it impossible to calculate the contrast in those regions. If the background intensity is set too low, it will be extremely difficult to achieve good contrast between the dark component of the DIC signal and the background signal. Understanding the typical or expected behavior of a sample can aid in selecting the proper background intensity.

Finding the proper focal plane is also essential. One of Nomarski DIC's advantages is that it has a shallow depth of field. However, this makes it more challenging to focus on thin samples, such as nanoparticles. With thicker samples, the challenge is in finding the actual focal plane of greatest interest. Many focal planes may be interesting and have nanoparticles on them, so it is important to determine early on the nanoparticles of greatest interest.

In the case of nanoparticles, it is important for the microscopist to recognize that they are viewing an airy disc or "point spread function" of the object[2]. In general, the airy disc is useful in determining whether a plasmonic nanoparticle has a shape that is isotropic or anisotropic, but nanoparticle imaging is in fact much more complex than what is discussed here. Complex nanoparticle aggregates can sometimes resemble isotropic particles, and as a result, electron microscopy methods are then necessary to characterize the nanoparticle patterns[2,16,18,19]. To image plasmonic nanoparticles with a DIC microscope, it is crucial to use filtered imaging and to image the particles at one of their highly-absorbing plasmonic wavelengths[6]. Imaging at an improper wavelength or without filters can result in the capture of shadow-cast patterns that are difficult to decipher.

When imaging nanoparticles alongside objects that are larger than the diffraction limit of light, it is important to remember that the microscope's objective "sees" a relatively flat focal plane. A common misconception of DIC is that it enables viewing of an object in actual 3D relief. This is caused by the shadow-cast patterning, which indeed makes many objects appear to be three-dimensional. However, to collect vertical information on multiple focal planes, it would be necessary to raise or lower the stage and collect a sequence of images. This can be very difficult

to perform and to interpret, especially for thicker samples, such as cells. Thus, the microscopist needs a deep understanding of all materials involved when performing such experiments and must record the positions of the individual focal planes that were utilized.

Finally, data analysis step is as critical as data collection. When measuring the contrast or intensity values of the sample, several factors should be kept in mind. Typically, the analyst is primarily interested in the minimum and maximum values for the particle of interest. When the contrast to noise ratio for the sample is sufficiently high, and if the background area is clean and evenly illuminated, then a simple geometric shape can be drawn around the sample region without concern of signal being introduced by contaminants. Furthermore, if the background is clean and evenly illuminated, a background measurement can be made in any area immediately adjacent to the sample. However, if there are contaminants or if the background is uneven, then the analyst must make a critical review of the sample's environs, and the analyst needs to assess whether it is even possible to make a reasonable background measurement. It is also critical to measure the sample and background areas with the same-sized and shaped tool, in order to avoid the introduction of bias into the calculation. In general, smaller-sized measurement areas have a lower likelihood of detecting outliers (e.g., contaminants, bad pixels, etc.) but larger sampling areas often provide a more reliable measurement of the background's mean value.

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DISCLOSURES:

The author has nothing to disclose.

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Figure 1

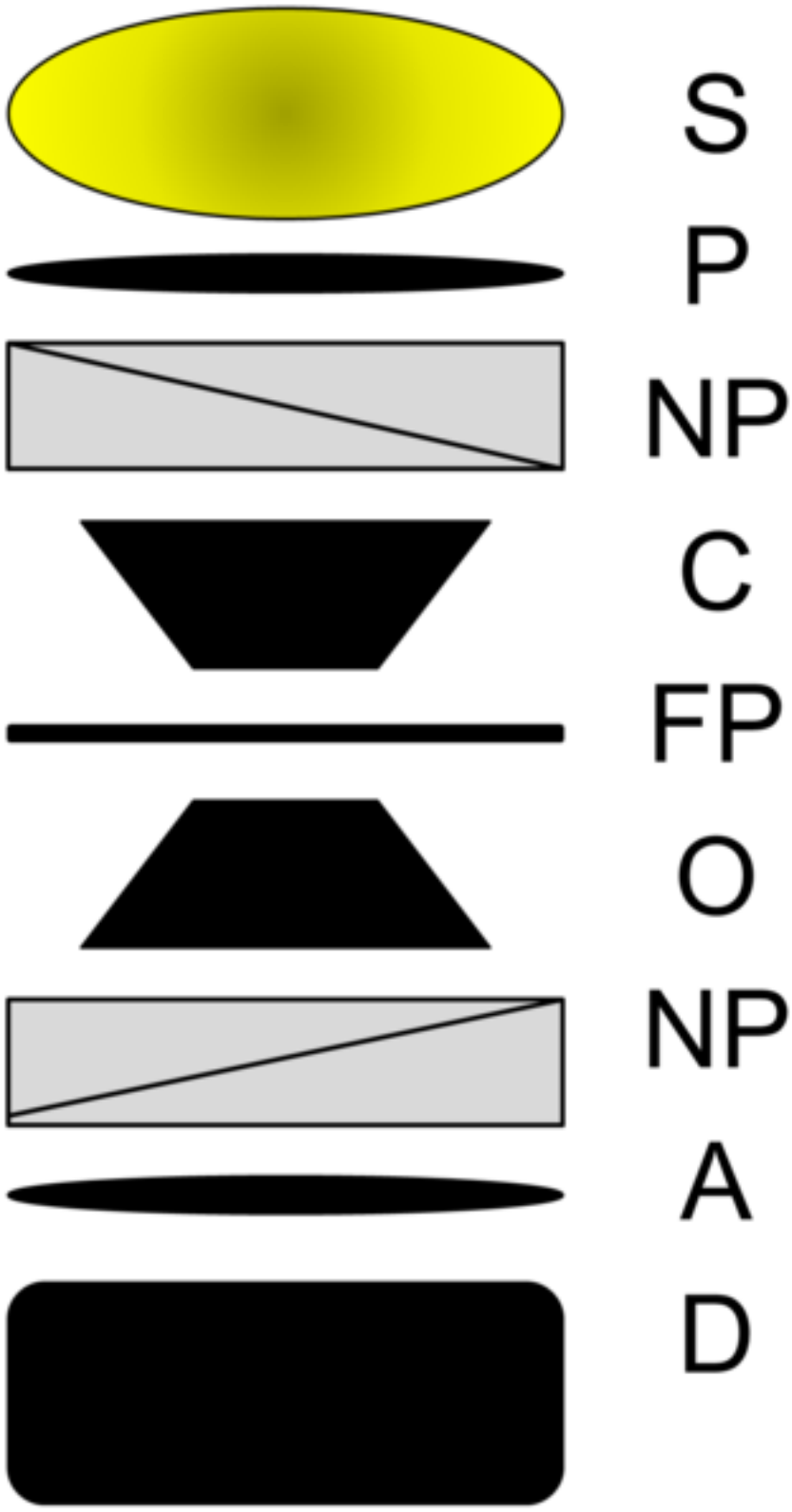

Figure 2

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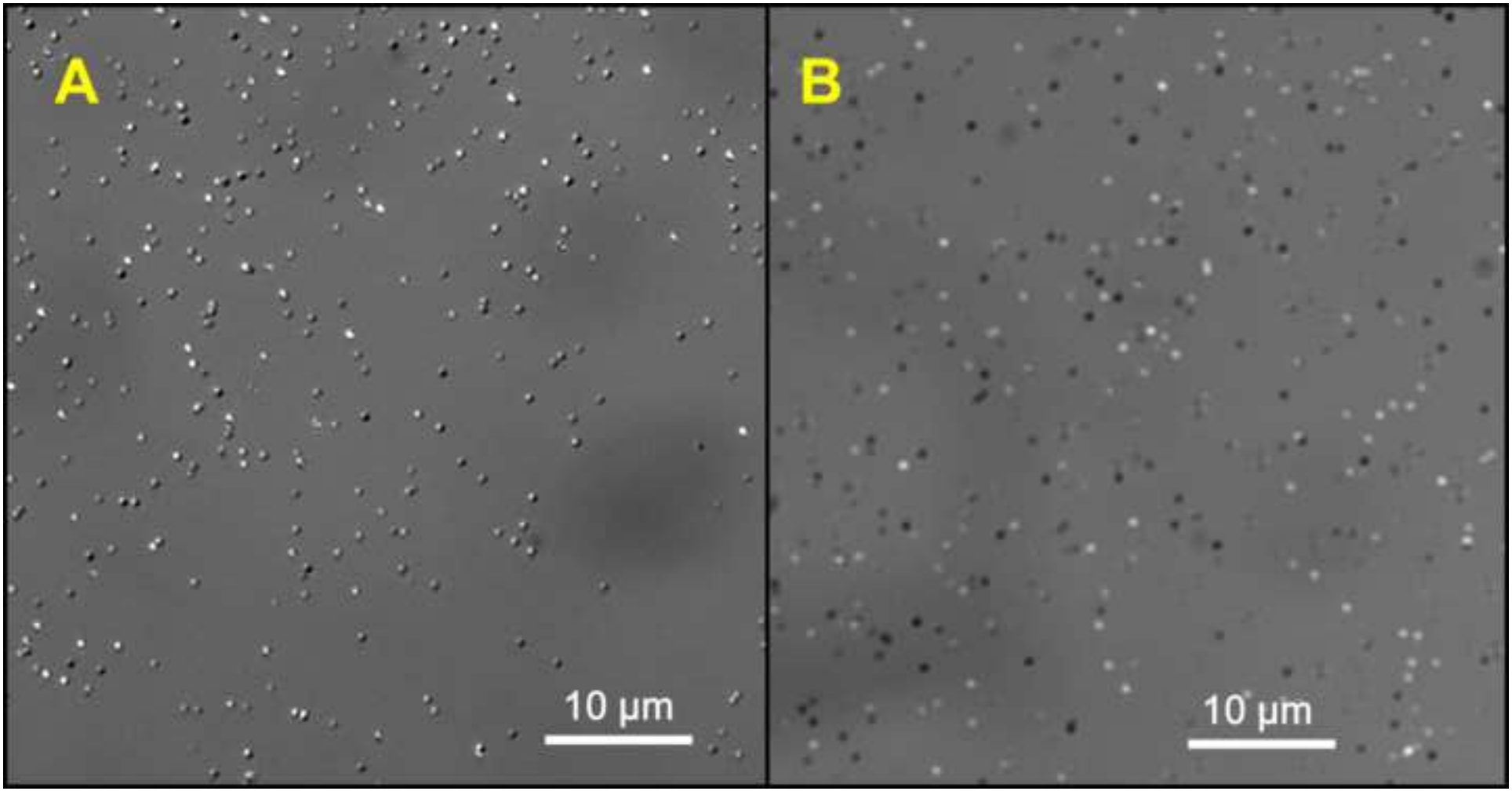

Figure 3

Click here to access/download;Figure;Stender-Fig3-v2.tif

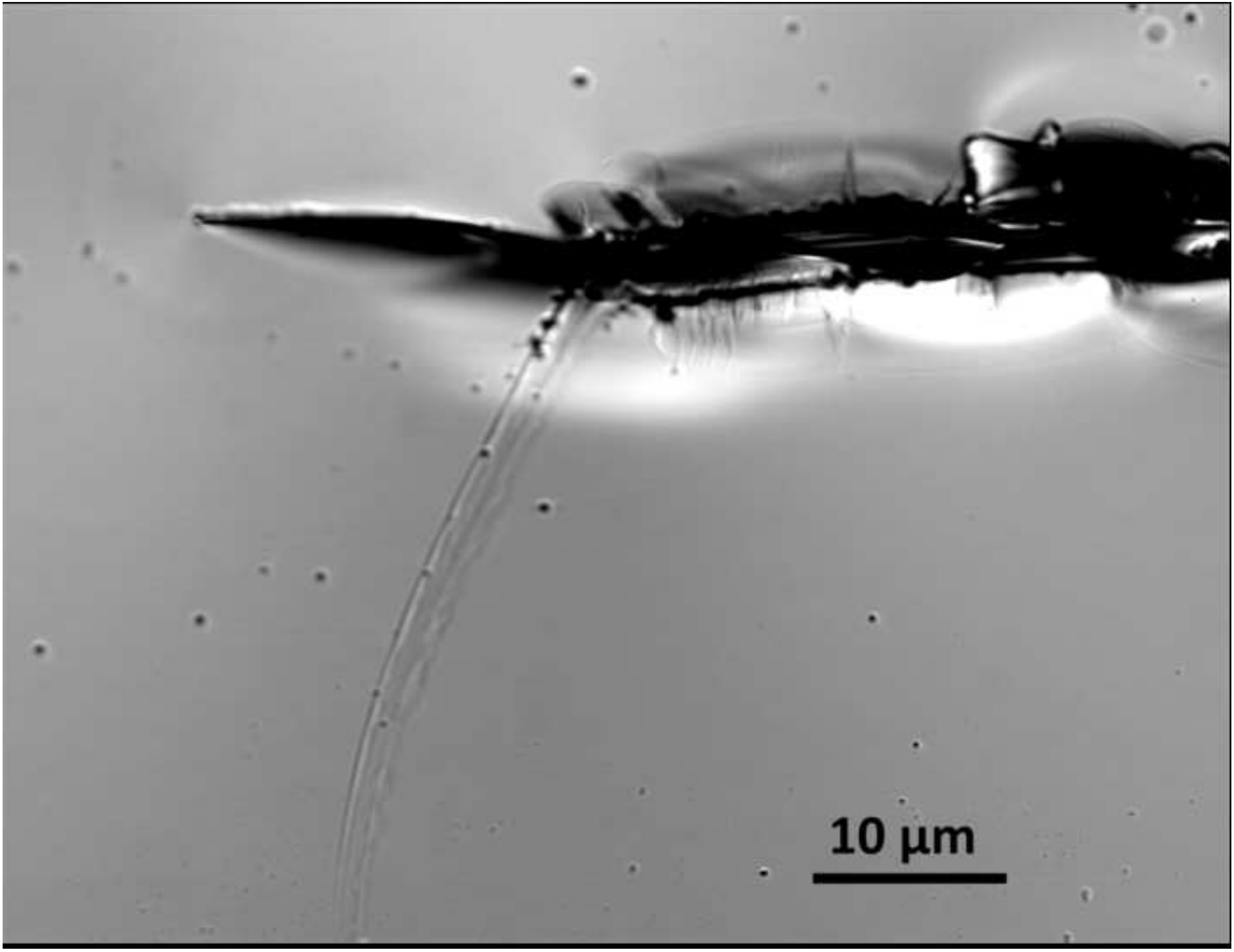

Figure 4

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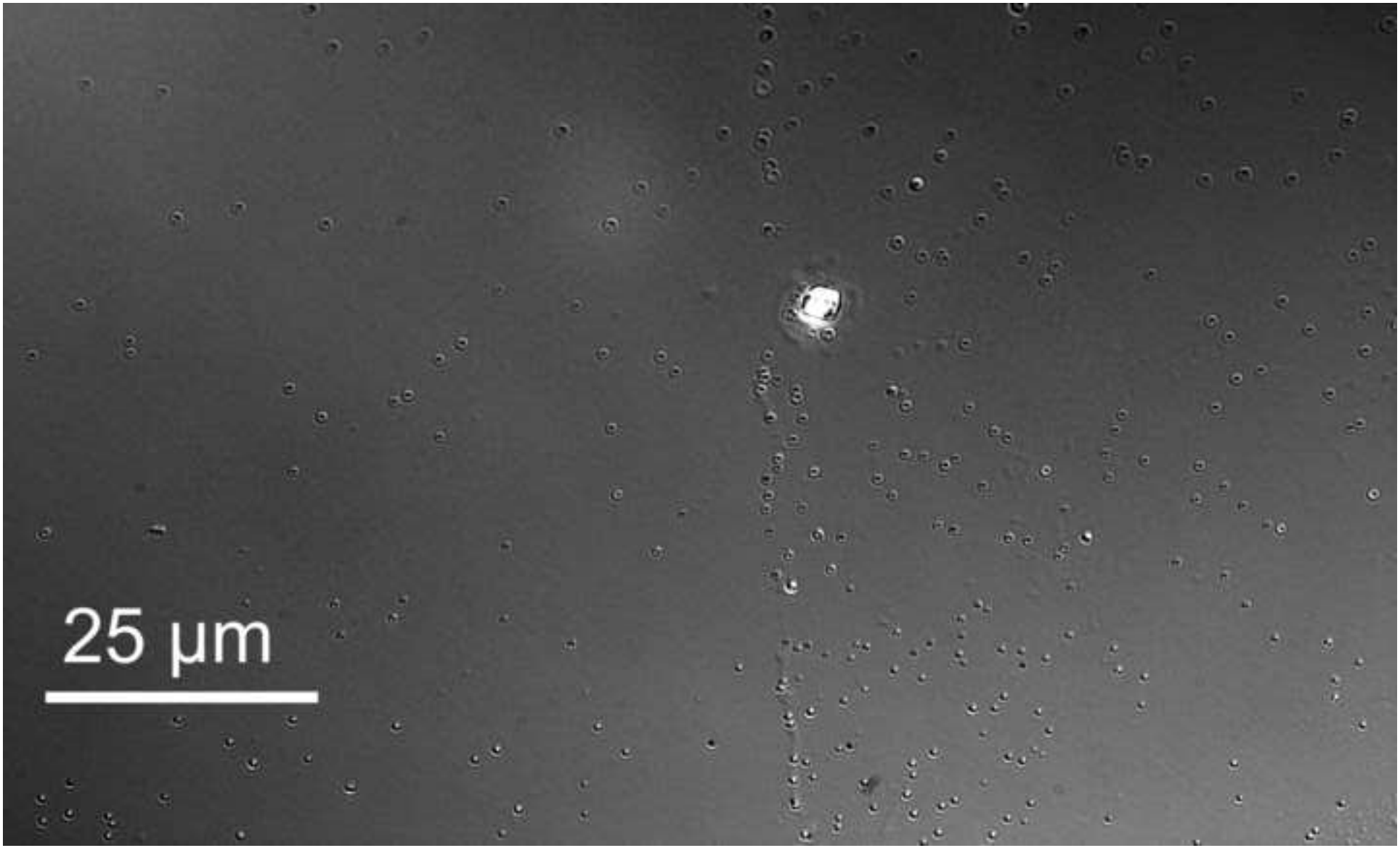


Figure 5

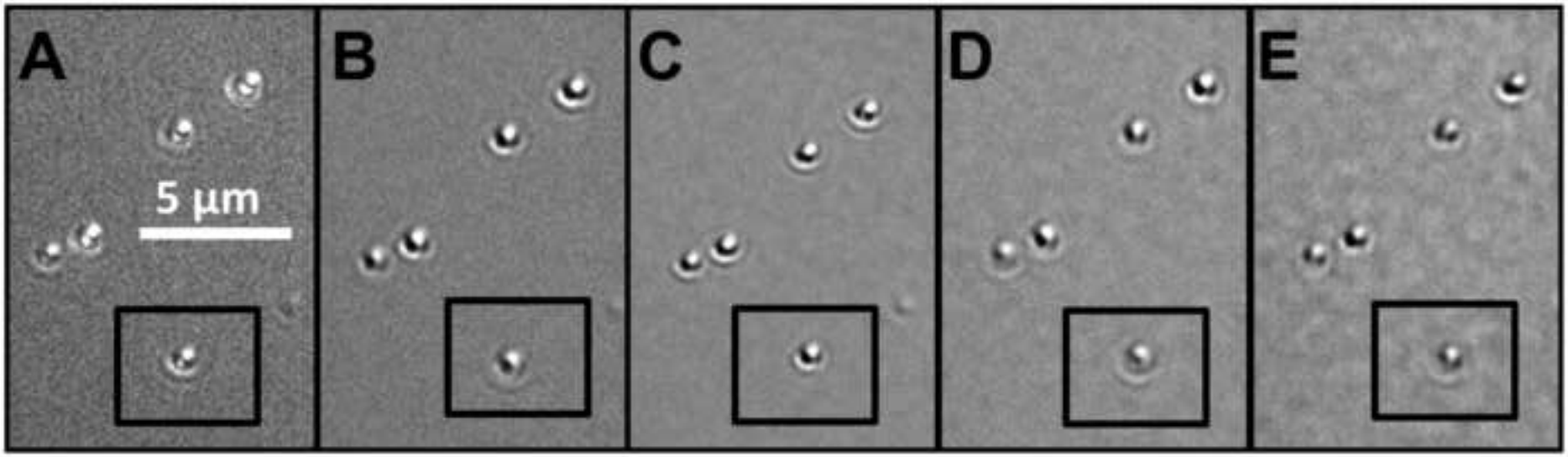


Figure 6

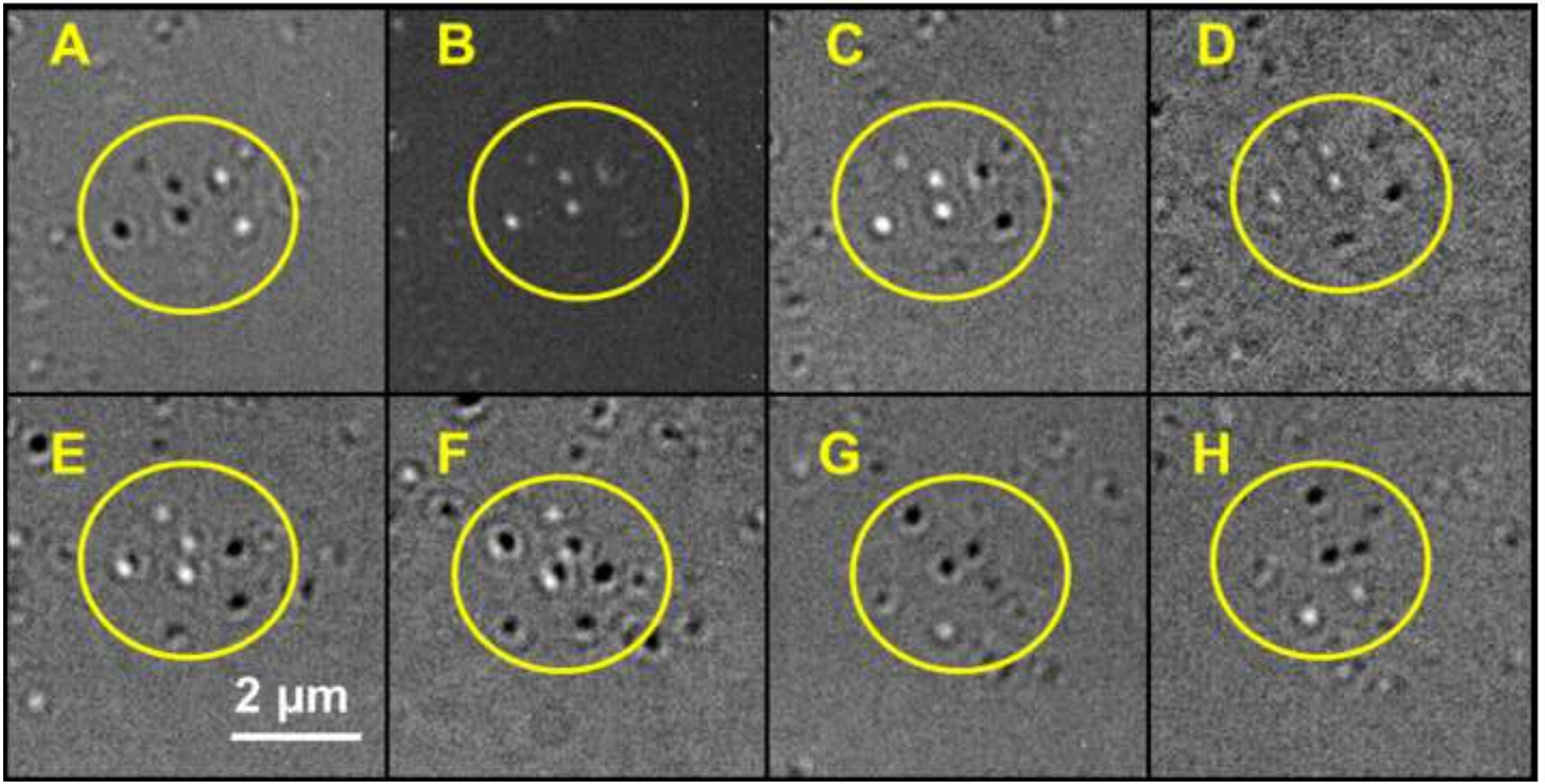
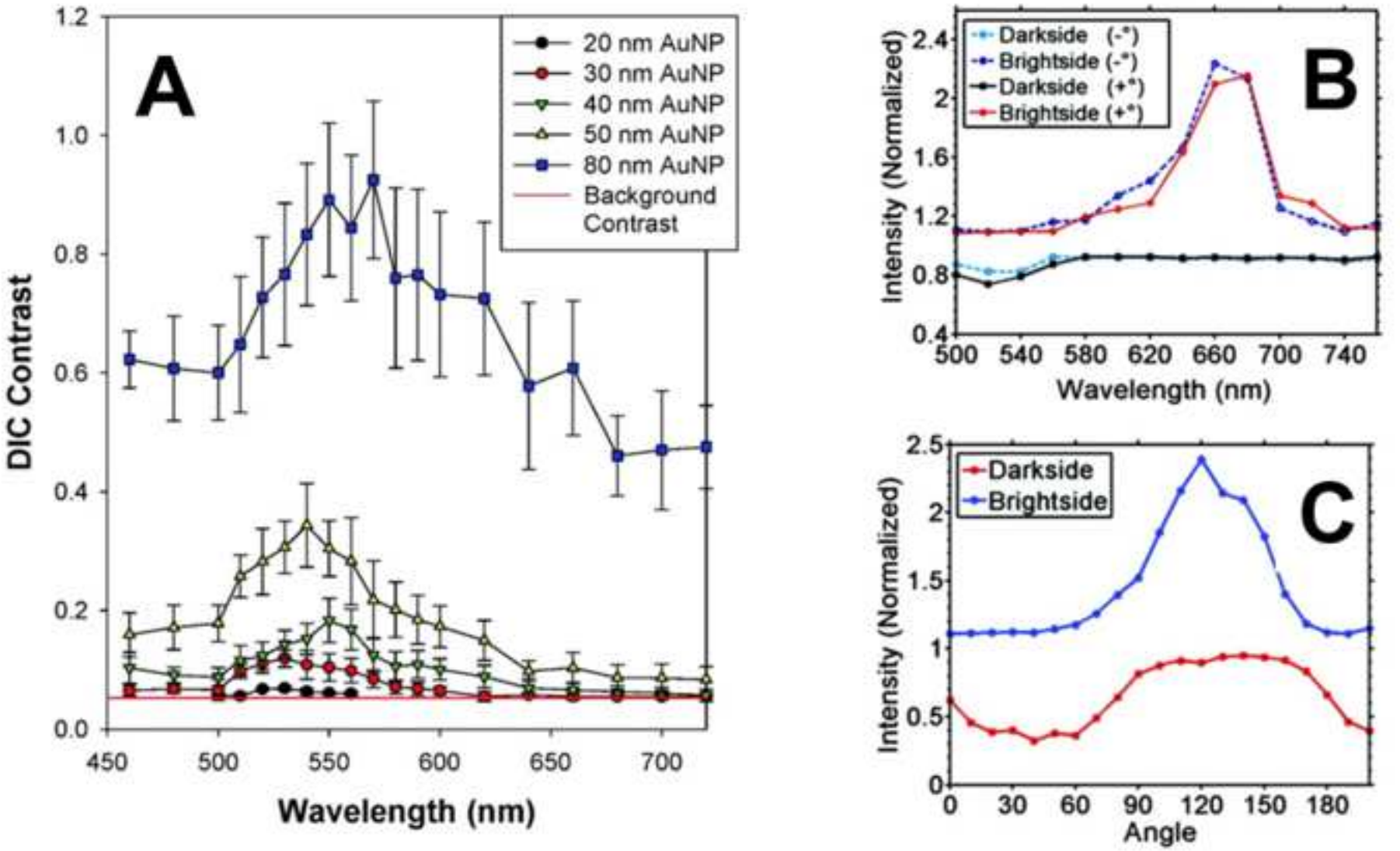


Figure 7



Name of Material/ Equipment	Company	Catalog Number
Contrad 70	Decon Labs, Inc.	1002
Ethanol	Fisher Scientific	A962-4
Glass microscope cover slips	Ted Pella	260148
Glass microscope slides	Ted Pella	26007
Gold nanorods	Nanopartz	DIAM-SPR-25-650
Gold nanospheres (80 nm)	Sigma Aldrich	742023-25ML
ImageJ	NIH	N/A
Nail polish	Electron Microscopy Sciences	72180
Nikon Ti-E microscope	Nikon	N/A
Nitrogen gas	Airgas	N/A
ORCA Flash 4.0 V2+ digital sCMOS camera	Hamamatsu	77054098
Scribing pen	Amazon	N/A
Ultrapure water		

	Comments/Description
	For cleaning microscope glass, Available through many chemical suppliers
	For cleaning and storing microscope glass
	Free Software availabe for data analysis from NIJ
	Many options available online for under $10. Not necessary to buy an expensive version.
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22 January 2019

Re: Rebuttal Documentation for JOVE Manuscript

Dear Phillip Steindel, Ph.D.

As per our email exchange earlier in January, I have decided to make major revisions in my original manuscript, beyond those suggested by yourself and the two Reviewers. After reading the original reviews, I felt that it would be wise to narrow the scope of my manuscript to focus on spectroscopy of plasmonic nanoparticles with Differential Interference Contrast (DIC) Microscopy. This is my main area of expertise and should solve many of the major concerns that arose during the first round of reviews. I have also changed the title of the paper and made significant changes to the figures. It is now ready for another round of reviews. Thank you for your consideration and for your feedback earlier this month.

As requested, I have addressed all of the editorial and reviewer feedback, which you can find below.

Regards,

Dr. Anthony S. Stender

Editorial comments:

Author's responses are provided in maroon text.

Changes to be made by the author(s) regarding the manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
2. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
3. Please revise the protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.
4. In the JoVE Protocol format, "Notes" should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section.

Response: I have made these requested changes in this latest document.

5. Please provide specific values to be used in the protocol. We cannot film a generalized protocol; we need specific settings of a specific experiment. See below for some examples.

Response: I have re-written the manuscript to follow a very specific protocol instead of a more generalized approach that the first manuscript entailed.

6. 1.2.2: Please specify the composition of sample solution used in this step.
7. 2.3.1: Please specify the color filters that are used.
8. 2.3.2: Please specify the lamp intensity and camera exposure time.
9. 3.1.1, 3.1.2: Software steps must be more explicitly explained ('click', 'select', etc.). Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc.).
10. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Notes cannot usually be filmed and should be excluded from the highlighting.

Response: These steps have been provided in highly specific detail now. The number of "Notes" comments have been reduced significantly. In many cases, I was able to move that content into either the Discussion or the Introduction sections, where it seems more appropriate.

11. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Response: Since this is my first submission to JOVE, I was somewhat unfamiliar with these requirements, but hopefully these concerns have been sufficiently addressed.

12. Discussion: Please discuss any limitations of the technique and future applications of the technique.

Response: Limitations are now discussed, both in the Introduction and the Discussion sections. Since sample prep and optical alignment are the key concerns, the problems associated with poor sample prep and improper optical alignment are discussed. Furthermore, since this document focused on single particle spectroscopy, mention is made to problems that are encountered when particles aggregate, which is not truly single particle spectroscopy.

13. Figure 2B: Please describe what are the left, top right, bottom right images are. Please specify the distances.
14. Figures 3 and 4: Please add a yellow box in other panels as well (A, B, D, and E).
15. Figure 4: Please provide measured contrast of particle in yellow box for panel D.
16. Figure 8: Please label different panels. Please ensure that the panels are of the same dimensions if possible.

Response: Many of the figures have been replaced or shuffled around. I used consistent labeling schemes this time, and I believe I don't have any issues leftover of the type I had during the first round of reviews.

17. Table of Materials: Please sort the items in alphabetical order according to the name of material/equipment.

Response: This has been resolved.

18. References: Please do not abbreviate journal titles.

Response: I think I originally abbreviated based on the "Author Instructions" sheet I downloaded from JOVE, and perhaps I misinterpreted what was being asked of during the first submission. Hopefully the reference list is properly presented now. I used EndNote and downloaded JOVE's EndNote style.

Reviewers' comments:

Reviewer #1:
Some questions and comments are the following:

Abstract: "Several important steps must be followed carefully in order to acquire repeatable and quantifiable data".
Comment: DIC is not quantitative and it does not provide quantifiable data. The contrast depends on the phase gradient magnitude and orientation, beam shear amount, and bias. The author has to clarify what he has in mind under "quantification".

Response: Because of the change in the paper's emphasis, I have changed this sentence to read "Several important steps must be followed carefully in order to perform repeatable spectroscopy experiments." I have also taken great care throughout the paper to remove the words "quantify" and "Quantification" to avoid confusion.

Abstract: "Rotation of the sample may be necessary, particularly when the material exhibits orientation-dependent optical properties (i.e. anisotropy)".
Comment: This is not correct. Standard DIC is not suitable for specimen with anisotropy (birefringence). It is possible to use a PlasDIC (Wehner, E. (2003) PlasDIC, an innovative relief contrast for routine observation in cell biology. Imag. Microsc. 4, 23)

Response: PlasDIC has two primary selling points: 1) it enables imaging of samples supported on plastic substrates (petri dishes, microscope slides, etc.) – hence the "Plas" in PlasDIC; and 2) it provides focused imaging across a deeper depth of field than Nomarski DIC. In many situations, the latter point is actually a disadvantage when imaging nanoparticles, especially if the position of the focal plane is important (e.g. monitoring a cell surface, monitoring an interface of any kind, etc.) The article suggested by the Reviewer does not specifically mention birefringence. The classic paper by Mehta and Sheppard (Optics Express, 2008, 16, 19462) actually gets to the point that I believe this Reviewer was trying to make (see page 19467 of Mehta article). Plastic substrates can induce "spurious birefringence", thereby interfering with the phase gradients produced by the sample and dampening the sample's signal. PlasDIC fixes this problem. However, since in my experiments I am working with glass substrates, it is reasonable to assume that "spurious birefringence" is not a factor, in following with Mehta's paper (pages 19470 and following). For this assumption to hold true, it is certainly true that imaging should not be attempted

too near to any locations of stress or strain birefringence within the material (such as the scratch marks that are etched into the glass in my experiments), which is discussed in the manuscript. The Wehner article cited by Reviewer 1 verifies that Nomarski DIC is an approved method for imaging samples on glass.

Abstract: "Because DIC microscopy has two orthogonal polarization fields that contribute to the final image, the coloration of the imaged signal reveals the orientation of anisotropic, nanoscale objects".
Comment: The coloration of the imaged signal does not reveal the orientation of anisotropic, nanoscale objects. The coloration of the imaged signal reveals the optical thickness of isotropic, nanoscale objects (Shribak, M. (2012) Differential interference contrast microscopy. Biomedical Optical Phase Microscopy and Nanoscopy (ed. by N. T. Shaked, Z. Zalevsky & L. L. Satterwhite), Elsevier, Amsterdam, Boston, Heidelberg, London.)

Response: Because the word "coloration" is ambiguous and can have many meanings, and because I am now focusing on spectroscopy of plasmonic nanoparticles, which can exhibit orientation-dependent DIC signals, I edited this sentence. The new sentence is, as follows:
"Because DIC microscopy has two inherent orthogonal polarization fields, the contrast of the imaged signal reveals the orientation of rod-shaped plasmonic nanoparticles."

I have also removed all use of the word "coloration" from the document to avoid further confusion.

Line 39: "Rotation of the sample may be necessary, particularly when the material exhibits orientation-dependent optical properties (i.e. anisotropy)".
Comment: Not correct. This is a little help when the specimen is birefringent.

Response: Again, because of the changed emphasis of the paper, and because I am emphasizing Nomarski-DIC, I have edited this sentence as follows:
"Using a standard Nomarski style DIC microscope, rotation of the sample may be necessary, particularly when the plasmonic material exhibits orientation-dependent optical properties."

Line 40: "Because DIC microscopy has two orthogonal polarization fields that contribute to the final image, the coloration of the imaged signal reveals the orientation of anisotropic, nanoscale objects".
Comment: Not correct. See above.

Response: I already addressed this comment – see above.

Line 64: "DIC has additional strengths that are valuable to materials research: introducing a waveplate to the light path enables optical staining, thickness and refractive index measurements are possible, two inherent and orthogonal polarization fields are present and can be measured separately, and depolarized signal is not captured in the final image".
Comment: Not correct, depolarized signal strongly affects the image contrast. See, for example, Inoué, S., and K.R.Spring.1997. Videomicroscopy: The Fundamentals. Second edition, Plenum Press, New York, NY.

Response: As previously stated, this comment is referring to a specific instance (when plastic substrates are used with Nomarski DIC). Several of the citations listed in the manuscript have demonstrated that when using glass substrates, no “spurious birefringence” and hence, no depolarized signal from nanoparticles will appear in the captured data. However, the capture of depolarized signal with dark field microscopy is a serious limitation to that technique in regards to using it for spectroscopy of plasmonic nanoparticles.

Line 76: "If these components are misaligned, or if Kohler Illumination is not properly achieved, the quantitative aspects of the data are unreliable".
Question: Which quantitative aspect has the author in mind?

Response: I have deleted this sentence, due to the new format of the paper.

Line 94: "Isotropic objects retain their appearance (and contrast) when rotated in the optical path, while anisotropic objects undergo important and significant changes as they are rotated".
Comment: The author should use another term for this in order doesn't confuse the reader. For example, one of kind of optical anisotropy is birefringence.

Response: Unfortunately, it is not possible to use different adjectives to describe plasmonic nanoparticles, because isotropic and anisotropic have been accepted by the nanoparticle community as the preferred terminology in describing the shapes of nanoscale particles. I have re-written the text to make it clear that these terms are used to describe shape properties.

Line 96: "Isotropic objects retain their appearance (and contrast) when rotated in the optical path, while anisotropic objects undergo important and significant changes as they are rotated".
Comment: The reader could be interested that currently is available the orientation-independent DIC, which does not require rotation of the specimen under investigation (see, for example, J.E. Malamy, M. Shribak, "High resolution imaging of epithelial cell migration and wound healing in a Cnidarian model using an orientation-independent DIC microscope" Journal of Microscopy, vol. 270, No. 3, p. 290-301 (2018)).

Response: For this paper, I am focusing on the use of traditional commercially available Nomarski DIC., since many users are not in the position to modify their microscopes (or user facility microscopes) in such a manner. Modifications to the light path are intriguing and are currently being employed by several groups, and as such, would make for a good review article. However, that goes beyond the scope of this paper and could not be discussed adequately within the space constraints for this paper.

Line 120: "Note: Many cleaning protocols for microscope slides have been previously documented, but it is not a step that is typically reported in the literature".
Comment: The reader could be interesting to see the following reference: Inoué, S., and K.R.Spring.1997. Videomicroscopy: The Fundamentals. Second edition, Plenum Press, New York, NY.

Response: I am aware that many cleaning protocols are published in the literature, but this was a poorly thought-out sentence on my part. Originally, I was expressing my opinion that this “is not a step that is

typically reported in ”peer-reviewed journal articles”. In short, this was meant as a complaint about peer-reviewed journal articles, and I realize now that this is not the forum to complain about this. Several of my peers in other departments who work with cell imaging and microscopy have told me that they never clean their microscope slides, because they are pre-cleaned. This is not acceptable when working with nanomaterials. However, many researchers in nanoscience fail to report in journal articles what cleaning methods they actually use. That being the case, I discussed a very basic and effective method but did not claim it to be the only one available. The other traditional methods employed in nanoscience are discussed in detail in the Ligler article cited in the manuscript, but they rely on harsher chemicals than what is used here.

Line 187: "2.1 Align Objective and Condenser".
Comment: Major microscope manufacturers, such as Nikon, Olympus, etc., offer three types of DIC prisms: high-resolution, high contrast and standard. The reader would be interesting which type to choose and why.
Major microscope manufacturers offer three types of objective lenses: standard, DIC quality and polarization quality. The reader would be interesting which type to choose and why.

Response: I have updated the article to include a brief discussion on this topic.

Line 211: "If the microscope has the de Senarmont design, start with the polarizer set near to maximum background extinction and gradually rotate the polarizer towards decreasing background extinction. The background intensity will gradually increase".
Comment: The author may wish to add a simple description how bias depends on the polarizer rotation: Bias (in nm)= Wavelength (in nm) X Angle (in deg.)/ 180. Then he can use a two-beam interference mathematical model to illustrate a change of the background intensity (see, for example, Shribak, M. (2012) Differential interference contrast microscopy. Biomedical Optical Phase Microscopy and Nanoscopy (ed. by N. T. Shaked, Z. Zalevsky & L. L. Satterwhite), Elsevier, Amsterdam, Boston, Heidelberg, London).

Response: Discussing the calculation of bias is beyond the scope of this article and this application, and it would not be appropriate in the Protocol section. It is discussed in one of my earlier papers that is cited by this manuscript (Ref #2). This discussion would be more appropriate for a full technical review paper of DIC microscopy than a JOVE protocol paper of this type.

Line 225: "Color filters may be placed into the light path to limit the wavelength range reaching the detector; without using filters, the detector will be capable of detecting a broad spectrum of wavelengths."
Comment: It is necessary to mention that color filter reduces the chromatic aberration of objective and improves the image contrast. What is the optimal bandwidth of color filter?

Response: The specifications for the filters are included in the updated manuscript. Because of this specific application, the materials, and the bandwidth of the color filters, I did not specifically discuss “chromatic aberrations”. I felt that would lead to a lengthy tangential discussion. However, I did state that when using filters, the contrast is affected (as Reviewer 1 commented) and that the sample must be re-focused when switching between filters.

Line 237: "Rotate the Sample."
Comment: See comment above.

Response: This content has been updated.

Lines 263, 281, 286: formulas
Comment: This is not a rigor mathematical analysis. The author has to eliminate influence of the microscope parameters, such bias, wavelength, etc. and take into account the properties of the specimen only.

Response: This is not intended to be, nor is it necessary to be, a rigorous mathematical analysis. Instead, this is the simple formula for calculating contrast that is widely accepted in microscopy and optics, and it can be found in many standard microscopy and spectroscopy textbooks, including Randy Wayne's "Light and Video Microscopy" and Murphy and Davidson's "Fundamentals of Light Microscopy and Electronic Imaging", which are now cited as sources for that equation in the manuscript.

Reviewer #2:

This manuscript described a method for preparing and inspecting microscale and nanoscale objects with DIC microscopy. To achieve repeatable and quantifiable data, the author detailed protocols for several important steps such as etching landmarks into the sample substrate, substrate cleaning, optical path aligning with respect to specific material, focal plane positioning, illumination intensity determination, anisotropic sample rotation and tracking, data acquisition and data analyses. The methodology presented in this paper would help researchers or microscopists in the field of biological or material characterization on optical method, to achieve reliable results. The manuscript is well-written and the procedure described is instructive and clear. Therefore, I recommend the publication of this manucrsript as it is.

Response: Many updates have been made to narrow the scope and to refine the procedural aspects of the manuscript. The emphasis now is aimed only towards an audience that wishes to use DIC microscopy for plasmonic nanoparticles.

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Title: Wavelength-Dependent Differential Interference Contrast Microscopy: Selectively Imaging Nanoparticle Probes in Live Cells

Author: Wei Sun, Gufeng Wang, Ning Fang, et al

Publication: Analytical Chemistry

Publisher: American Chemical Society

Date: Nov 1, 2009

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Title: Influence of Polarization Setting on Gold Nanorod Signal at Nonplasmonic Wavelengths Under Differential Interference Contrast Microscopy

Author: Anthony S. Stender, Ashley E. Augspurger, Gufeng Wang, et al

Publication: Analytical Chemistry

Publisher: American Chemical Society

Date: Jun 1, 2012

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