Fabrication of Test Samples for Calibration and Testing in 3D Super Resolution Microscopy

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Abstract:

In order to verify the performance of three-dimensional super-resolution microscopy systems, we used nanofabrication techniques in non-traditional ways in order to generate sub-wavelength test samples. Such samples allowed unprecedented control of the arrangement of fluorescent probes, and the resulting images provided valuable metrics regarding super-resolution results.

Introduction:

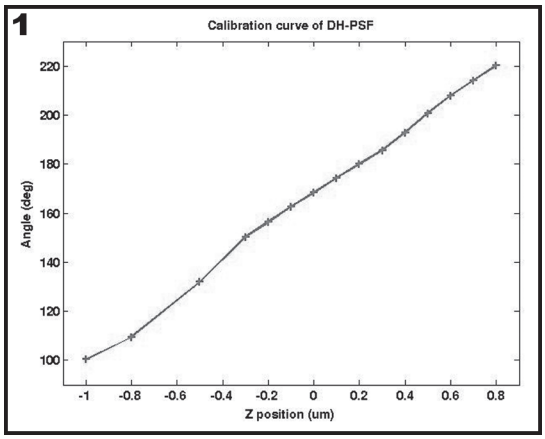
Three-dimensional (3D) super resolution microscopy allows researchers to circumvent Abbe's Diffraction Limit while capturing images of biological specimens with immense detail (one order of magnitude better than the diffraction limit). Due to the increasing resolution and ability to examine nanoscopic features, knowledge of our measurement accuracy and precision is essential. The focus of this project was to create test samples with known dimensions that emulated biological samples in order to calibrate and test 3D super resolution microscopy systems.

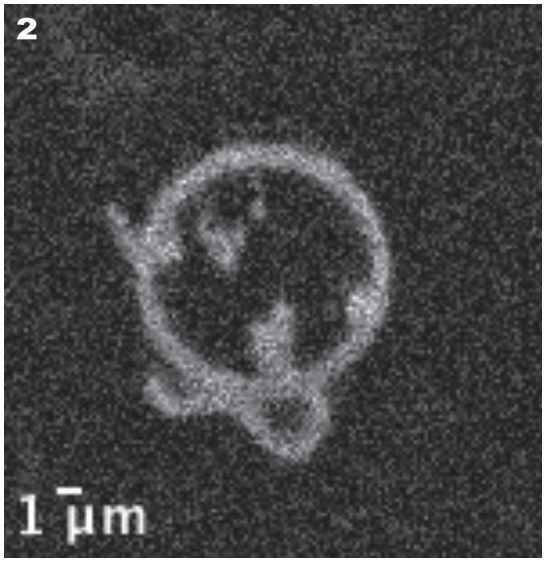
We made two types of samples: apertures in chrome for use in transmission mode, and sub-wavelength arrangements of fluorescent particles. For the transmissive samples, a focused ion beam (FIB) was used to transfer nanoscopic patterns into thin layers of opaque chrome deposited on a glass substrate. These test samples allowed us to evaluate the precision of a given instrument, as well as investigate the extent of optical aberrations across the field of view. Secondly, a new method of nanoscopic fabrication was used to generate a nano-scale patterned sample with a controlled arrangement of fluorescent probes. With such a sample, we were able to demonstrate the proof of concept of this fabrication technique and additionally, test the resolution limit of point sources in close proximity due to the ambiguity caused by overlapping Point Spread Functions.

Experimental Procedure:

Sub-Wavelength Apertures in Chrome. We used AutoCAD (Autodesk, USA) to design sub-wavelength apertures and fabricated the design with a FIB. Before fabrication, 50 nm of chrome was sputtered onto the cover slide (18 × 18 × 0.16 mm) with a reactive DC sputter deposition System. The samples were examined with an Olympus IX83 super-resolution system, which consisted of a 1.45 NA 100× objective, an Andor iXon electron multiplying CCD camera, and white light illumination with a halogen lamp. A double helix-point spread function phase mask was placed in the Fourier plane to collect 3D information from the sample.

Nano-Scale Patterning of Fluorescent Probes. We used AutoCAD (Autodesk, USA) to design nanoscopic features and fabricated the design with the FIB. After gold deposition of 4 nm to reduce charging, the FIB milled 50 nm into the silicon dioxide cover slide (18 × 18 × 0.16 mm). F8789 fluorophores (ThermoFisher Scientific) were sonicated for 15 minutes. A 10^3 dilution of F8789 fluorophores and HPLC Water was made and sonicated for five minutes. The glass cover slide was placed in the March Jupiter III reactive ion etcher for five minutes (standard procedure) to remove the 4 nm of gold. Next, 5 µl of 10^3 F8789 fluorophore, water solution was pipetted over the milled area. After 30 minutes, the solution evaporated and the sample rinsed with HPLC Water, 3 × 500 µl. The remaining liquid was allowed to evaporate at room temperature in a dark covered container that blocked light. After evaporation was complete, the sample was examined with a Nikon stochastic optical reconstruction microscopy (STORM) system, with a similar objective and camera as above. The illumination involved a 647 nm 500 nW laser, and a dichroic mirror to separate the excitation and emission (fluorescence) light.





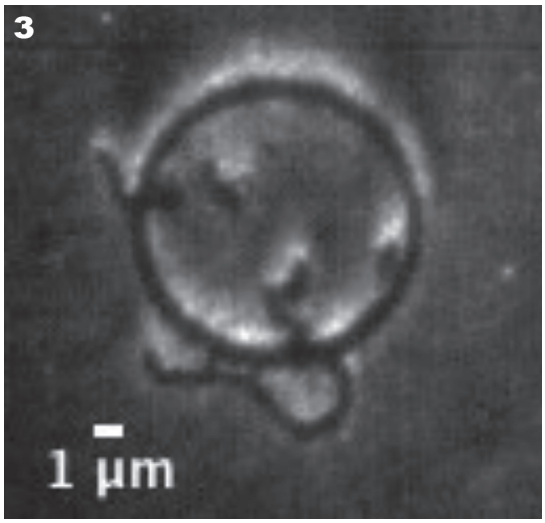


Figure 1, top: *Calibration Curve: Calibration curve of the rotation of the Double Helix point spread function through a Z stack, analyzing Angle vs. Z position.*

Figure 2, middle: *Fluorescent Probes patterned in the shape of a Cell: A fluorescent image of mock bacterial cell designed in CAD.*

Figure 3, bottom: *Fluorescent Cell post STORM: A fluorescent image of a designed bacterial cell after photobleaching.*

Results and Conclusions:

The sub-wavelength apertures in chrome provided an aberration sample. We successfully tested the aberration sample and found that 50 nm of chrome was too transmissive and allowed for light transmission not localized in the apertures. With the data collected, a calibration curve for the double helix-point spread function phase mask was created as seen in Figure 1. The nano-scale fluorescent probe sample was excited with a 647 nm laser, showing fluorophore localization in the patterned areas (Figure 2). Due to the diffraction limit, the feature size increased, but this demonstrated resolution limit of point sources in close proximity.

During photobleaching, images were taken of a mock bacterial cell. With photobleaching, fluorophores not localized within the patterned area fluoresced as seen in Figure 3. From the images collected, we were able to prove the nano-scale patterning of fluorescent probes concept.

Future Work:

The aberration slides for three-dimensional super resolution microcopy systems are being further developed. To improve the results and quality of the apertures in chrome experiment, the same design will be replicated on a cover slip with 100 nm of chrome, with holes varying in sizes of 50, 100, 150, and 200 nm. From this new sample, the aberrations from the center of the field of view will be compared to the aberrations at the edges of the field of view. Next, new methods will be designed and tested for nano-scale patterning of fluorescent probes to create a standard calibration slide for a variety of three-dimensional resolution microscopy systems.

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