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14. ABSTRACT
This project acquired an instrument module for super-resolution confocal microscopy imaging of reconfigurable colloids. During the project the different methods and instruments available for super-resolution microscopy was evaluated against the needs of colloidal assembly and soft matter science. The techniques and instruments evaluated were stimulated emission depletion microscopy (STED), stimulated illumination microscopy (SIM), and photoactivated localization microscopy (PALM). Our evaluation determined that the SIM method is currently the super-resolution method most compatible with our group's needs in soft matter assembly. Furthermore

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Report Title

Final Report: DURIP: Super-resolution module for confocal microscopy of reconfigurable colloidal matter

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Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

Received Paper

TOTAL:

Number of Papers published in peer-reviewed journals:

(b) Papers published in non-peer-reviewed journals (N/A for none)

Received Paper

TOTAL:

Number of Papers published in non peer-reviewed journals:

(c) Presentations

No presentations to report.

Number of Presentations: 0.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received Paper

TOTAL:

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received Paper

TOTAL:

Number of Peer-Reviewed Conference Proceeding publications (other than abstracts):

(d) Manuscripts

Received Paper

TOTAL:

Number of Manuscripts:

Books

Received Book

TOTAL:

Received	Book Chapter

TOTAL:

Patents Submitted

No Patents Submitted

Patents Awarded

No Patents Awarded

Awards

No Honors and Awards to Report

Graduate Students

NAME	PERCENT_SUPPORTED	Discipline
Lilian Hsiao	0.00	
Laura Colon-Melendez	0.00	
FTE Equivalent:	**0.00**	
Total Number:	**2**	

Names of Post Doctorates

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Names of Faculty Supported

NAME	PERCENT_SUPPORTED	National Academy Member
Michael J. Solomon	0.00	
Joerg Lahann	0.00	
Ronald Larson	0.00	Yes
Sharon Glotzer	0.00	Yes
Nick Kotov	0.00	
FTE Equivalent:	**0.00**	
Total Number:	**5**	

Names of Under Graduate students supported

NAME	PERCENT_SUPPORTED	Discipline
Cathleen Chong	0.00	Chemical Engineering
FTE Equivalent:	**0.00**	
Total Number:	**1**	

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period: 0.00

The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:..... 0.00

The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:..... 0.00

Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):..... 0.00

Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:..... 0.00

The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense 0.00

The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields: 0.00

Names of Personnel receiving masters degrees

NAME
Total Number:

Names of personnel receiving PHDs

NAME
Lilian Hsiao
Total Number: 1

Names of other research staff

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Sub Contractors (DD882)

Inventions (DD882)

Scientific Progress

Please see attached PDF file for Scientific Progress and Accomplishments Report.

Technology Transfer

No specific Technology Transfer activities to report; interactions with industry during this project were with the commercial instrument vendors whose super-resolution microscopy systems were evaluated for application in colloidal self-assembly.

Final Technical Report. DURIP: Super-resolution module for confocal microscopy of reconfigurable matter

Principal Investigator. Michael J. Solomon, University of Michigan Ann Arbor MI 48109 (mjsolo@umich.edu)

Final Report Summary

This project acquired an instrument module for super-resolution confocal microscopy imaging of reconfigurable colloids. During the project the different methods and instruments available for super-resolution microscopy was evaluated against the needs of colloidal assembly and soft matter science. The techniques and instruments evaluated were stimulated emission depletion microscopy (STED), stimulated illumination microscopy (SIM), and photoactivated localization microscopy (PALM). Our evaluation determined that the SIM method is currently the super-resolution method most compatible with our group's needs in soft-matter assembly. Furthermore, demonstration testing established that the performance of commercially available SIM modules were significantly better than the currently best available resolution of confocal microscopy. After competitive bidding, the Zeiss ELYRA SIM system was acquired, installed, and made available for use to DoD supported researchers. During the no cost extension period of the grant, the instrument was used to generate new understanding about the behavior of nanoscale colloidal assemblies of particle sizes smaller than can currently be imaged by confocal microscopy.

In this final report, we first present a summary of the technical comparison of the STED, SIM and PALM super resolution microscopy methods that was performed. This technical comparison established SIM as the technique that is currently most adaptable to the imaging needs of soft matter assembly at the University of Michigan. Second, we report on the demonstration testing that evaluated the relative performance of commercially available SIM systems. Finally, we report first results from a scientific study that, by virtue of the acquired device, evaluates new features of self-assembly at scales that could not previously have been resolved by optical microscopy.

Results of Super Resolution Technique Evaluation

Commercially available superresolution imaging methods are STED microscopy (stimulated emission depletion microscopy), PALM (photoactivated localization microscopy) and SIM (structured illumination microscopy).

STED improves on the diffraction limits of microscopy by using a second laser to block fluorescence emission from the diffuse front of the diffraction layer. This second laser profile has been shaped as a doughnut. This beam depletes the fluorescence of the specimen's emission in the region of the doughnut. Thus only the central portion of the image region, at the center of the doughnut, is free to undergo fluorescence and contribute to the image. Strengths of the method are that it can track colloidal dynamics. Weaknesses of the method are that axial resolution is not significantly improved by this method, because the doughnut shaped depletion is principally configured in the objective plane. More crucially, only a very limited series of dyes performs well with

STED, and, after extensive evaluation and demonstration of possibilities with an instrument vendor, we found that none of these dyes is yet capable of being reliably incorporated into colloidal and soft matter.

PALM improves on the diffraction limits of microscopy by sequentially exciting and bleaching single photoactive molecules. By stochastically exciting a set of individual molecules over a long period of time, molecules that are indistinguishable in space because of the diffraction limit of microscopy because resolvable because they are distinguishable in time due to the stochastic nature of the excitations. Strengths of the method are that it is inexpensive. Weaknesses of the method are that is fundamentally a measurement that can only be deployed for fixed samples. Because superresolution is obtained by distinguishing between fluorphores in time, the image must be fixed and the duration to acquire one image is long. If photoactivation cycles are required for the fluorphore, then image acquisition times can be an hour or more. The recent method of PALM with independent running acquisition can reduce the acquisition time to a few minutes, albeit for the case of a particular photochromic compound.

SIM improves on the diffraction limits of microscopy by producing a Moiré pattern between the incident illumination and the subwavelength features in the specimen. The intensity of illumination is modulated (in space) by means of a diffraction grating. Interference of this pattern with the specimen results in long wavelength intensity changes that are resolved by the microscope objective. The spatial position of the subwavelength features can be deconvoluted from the Moiré pattern. Strengths of the method are that the improvement in resolution is both in the objective plane and in along the axial direction. The method works at a variety of wavelengths (although resolution is most improved at the lowest visible wavelengths). The improvement is resolution is about a factor to two – from ~ 250 nm down to about 125 nm. A weakness of the method is that the time to acquire one image is slowed by the need to product the Moiré pattern along a number of axes. Thus image acquisition is slower than STED but faster than PALM – image acquisition time is about ~ 1 frame/s for SIM. This time scale suggests that kinetics of self-assembly might ultimately be observable by this method and this possibility is important to our aims for reconfigurable colloidal assembly.

Thus, comparison of the three commercially available superresolution methods yields the following conclusions: (i) the limited range of available dyes for STED limits its current applicability to colloidal assembly; (ii) the long image acquisition times for PALM limits its current applicability to colloidal assembly; (iii) SIM, with its moderate image acquisition times, good spatial resolution in both lateral and axial directions, and compatibility with a range of dyes represents a good first choice for extending superresolution imaging into the area of colloidal self-assembly. Specifically, the ~ 125 nm resolution indicates that the method might be able to resolve the structure of colloidal crystals comprised of colloids of size 150 – 300 nm, which can currently not be studied by due to the diffraction limits of confocal microscopy. Colloids in this size range yield blue iridescence, and therefore are important for science and technology that requires structural color across the full spectrum. Furthermore, the image acquisition rate of SIM suggests that the kinetics of self-assembly might be resolvable.

Thus, instrument demonstrations were undertaken for SIM systems from commercial vendors.

Results of Super-resolution Instrument Evaluation

We demonstrated two systems with the potential to meet the following specifications: (i) minimum objective plane resolution of at least 150 nm and axial resolution of 300 nm using an excitation wavelength that includes 405 nm, 488 nm, and 561 nm for specimens up to 20 microns in thickness; (ii) configured for both 2D and 3D SIM; (iii) multi-channel imaging of up to 4 different dye wavelengths with methods such as sequential imaging. One device was the Nikon N-SIM system; the other was the Zeiss Elyra system. Colloidal systems produced from particles of a range of different sizes were tested on both systems, and also compared to best available confocal microscopy (CLSM) imaging, so as to ascertain relative performance of the systems as well as benchmark SIM relative to CLSM.

Figure 1, 2, 3, compare the performance of CLSM, Nikon SIM, and ELYRA SIM imaging for the case of ~ 450 nm, 200 nm, and 100 nm particles. While quality of CLSM and SIM images of assemblies of 450 nm particles are comparable, the relative image quality progressively favors SIM as the particle size is decreased from 200 nm to 100 nm. Figures 4 and 5 further evaluate the scope for specialty uses of the instrument. In Figure 4 the scope for deducing particle coordination number at high resolution was evaluated – this is a method important to evaluating the quality of self-assembly. In Figure 5, the scope for imaging Janus functionality in two colors was evaluated. Both these specialty applications show significant scope for application of SIM; the instrument demonstrations indicated in our judgment that image resolution is significantly better than diffraction limited confocal microscopy imaging. Both SIM platforms performed similarly and both improved upon CLSM imaging for studies of soft matter assembly.

Based on competitive bidding, the Zeiss ELYRA system was acquired; all DURIP funds were committed to the acquisition, with significant University of Michigan funds added to the DURIP amount, in addition to the committed cost sharing, to fund the full cost of the instrument acquisition. The device has the following capabilities: The Zeiss ELYRA S.1 Imaging System performs super-resolution optical microscopy by the method of structure illumination microscopy (SIM). The SIM method uses gratings to resolve distances as small as 100 nm when imaging colloidal structures. This microscope is equipped with 405 nm, 488 nm and 561 nm lasers, and specially designed gratings that optimize resolution at each of these wavelengths. The SIM method generates Moiré fringe patterns are used to reconstruct an image with fine-scale features through software analysis. Multiple channel imaging is available. The microscope has an installed z-piezoelectric drive that allows it to take 3-dimensional stacks that can be super-resolved.

Initial Results with Instrument

Scope and Motivation. In an early use of the instrument, we are quantitatively evaluating the minimum resolution of the instrument, how this minimum resolution

varies with physical properties of the colloidal specimen studied, and how this minimum resolution compares, quantitatively, to the best available alternative characterization method, which is confocal microscopy.

As a first step in this work, a comparative study of the resolving power of confocal microscopy (Nikon A1R) and structured illumination microscopy (Zeiss) was conducted by examining assemblies of spin-coated colloidal particles. Samples of different diameter sizes – 200, 310, and 450 nm – were imaged. The image quality and resolution was determined. The resolving power of standard optical microscopy methods is limited by the Abbe diffraction limit. The structured illumination microscopy (SIM) method that was introduced through the DURIP acquisition can improve upon the standard resolution limits of microscopy. SIM is able to resolve sub-diffraction limit features by illuminating a grid-patterned laser light onto a sample. The images thereby produced include a Moiré pattern from which a high resolution image can be deconvolved. Resolution of features as small as 100 nm can be achieved. The ability to resolve sub-diffraction limit features can find application in the analysis of the structure of photonic bandgap crystals made of nano-sized isotropic or anisotropic particles, which explains our interest in this microscopy method.

Materials and Methods. 200 and 310 nm diameter polystyrene (PS) beads were dyed following existing procedures (L. Jung-Hyun, J. G. Ismael, B. S. Valerie, C. M. J., *Journal of Colloid and Interface Science* **363**, 137 (2011)). The dye used was Nile Red dispersed in tetrahydrofuran (THF). The resulting suspensions were centrifuged and resuspended in DI water four times to remove excess dye and THF. The suspension was solvent transferred to a solution of 1:1 water/ethanol, yielding a 2 wt.% PS particle suspension. A 2 wt.% solution of 450 nm Nile Red-labeled poly(hydroxystearic acid) stabilized poly(methyl methacrylate) (PMMA-PHSA) particles was also prepared in hexane. 100 µL of each suspension were then spin-coated **(**WS-400B Lite, Laurell Technologies) onto 1.5 thickness glass coverslips (Fisher Scientific). The suspensions were pipetted onto the spinning coverslips and spun for 60 seconds at 1750 rpm and 100 rpm/s to allow solvent evaporation.

Solutions of photopolymer (CD501 Sartomer) and photoinitiator (Irgacure 2100) were prepared in both dimethyl sulfoxide (DMSO) and dioctyl phthalate (DOP). Immediately after spincoating the PMMA sample, 25 µL of the DOP-photopolymer-photoinitiator solution were pipetted onto it. The sample was then mounted on a glass slide and placed under a UV light to immobilize the particles. The same procedure was repeated for the PS samples using the DMSO-photopolymer-photoinitiator mixture. Images were acquired under confocal (Nikon A1R) and SIM (Zeiss Elyra S1) microscopes at .032 microns/pixel; confocal images were acquired using a 100x NA = 1.45 objective, and SIM images with a 63x NA = 1.4 objective. Imaging software (ImageJ) was used to acquire pixel intensity distributions of a sample region.

Results and Outlook. Figure 6 is visual comparison of the confocal and SIM microscopy results for the 200 nm, 310 nm, and 450 nm particles. While the spacings between the particles are apparent for the 450 nm particles in both confocal and SIM images (Fig. 1 e, f), the 310 nm and 200 nm particles are harder to distinguish in the confocal (Fig. 6a, c) than in the SIM images (Fig. 6 b,d). Figure 7 illustrates the resolving performance of

the SIM and confocal microscope. Regions of the SIM and confocal pictures of the 310 nm PS beads were analyzed using the freely available image processing tool, ImageJ. The SIM plot (Fig. 7b) clearly distinguishes between the various peaks, which represent particle density, while the comparable confocal microscopy plot (Fig. 7a) of the pixel intensities has less definition due to the microscope's resolution limitations.

As a next step, an optimized, automatic method for analyzing the SIM images is necessary to ensure that information is not lost in the analysis of the peaks and to therefore realize image quality. A more thorough analysis of the peaks in the pixel intensity distributions can be conducted by measuring the full width at half maximum. The width of the peaks can be compared against nominal particle sizes and sizes obtained from scanning electron microscopy imaging. Additionally, imaging of binary suspensions of particles with different dyes can be done to compare image resolution quality. Finally, the scope for measuring kinetics of soft-matter assembly by SIM will be investigated.

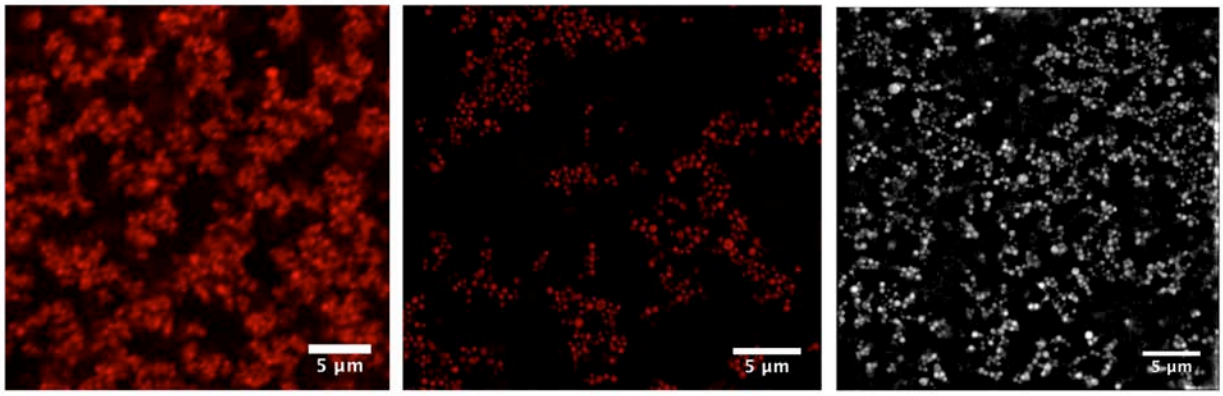

Figure 1. From left to right: CLSM, Zeiss SIM, and Nikon SIM images of ~ 450 nm particles assembled into disordered structures.

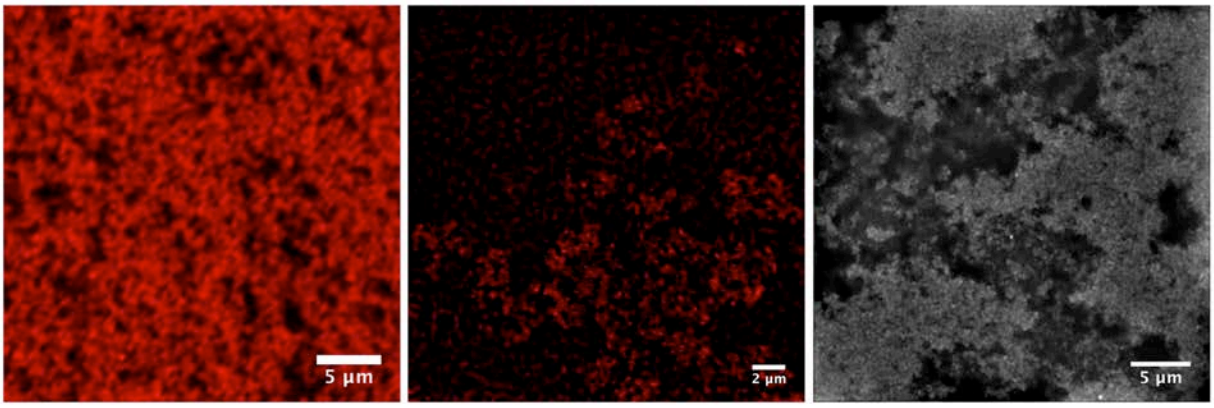

Figure 2. From left to right: CLSM, Zeiss SIM, and Nikon SIM images of ~ 200 nm particles assembled into disordered structures.

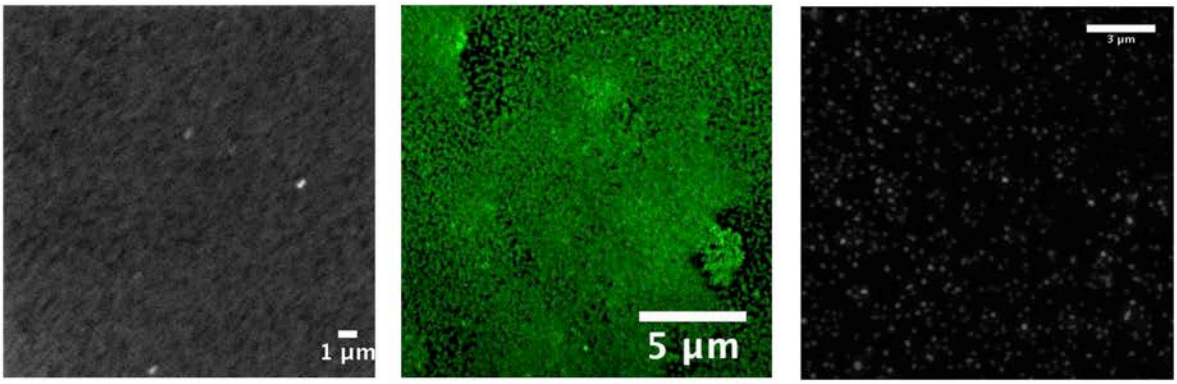

Figure 3. From left to right: CLSM, Zeiss SIM, and Nikon SIM images of ~ 150 nm particles assembled into disordered structures.

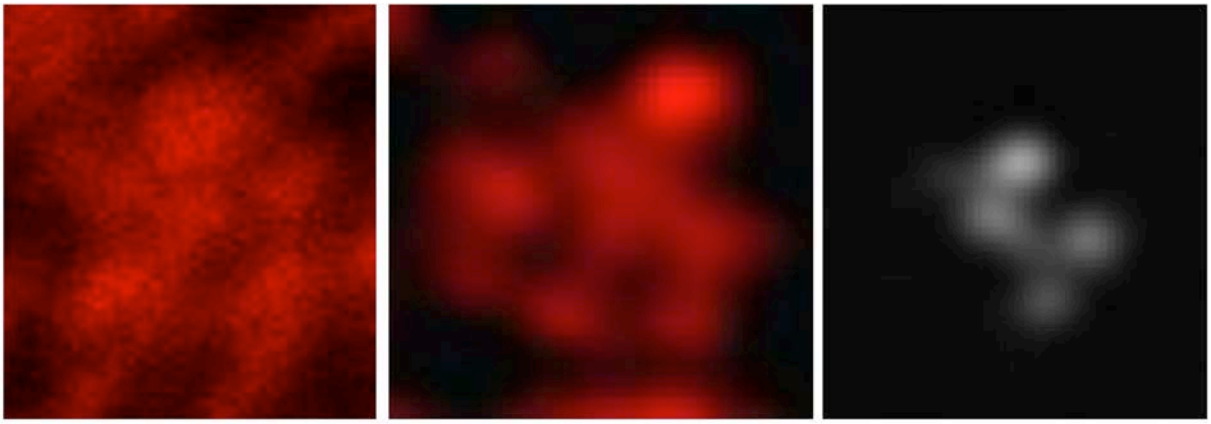

Figure 4. From left to right: CLSM, Zeiss SIM, and Nikon SIM images that compare the scope for determination of the coordination structure of ~ 200 nm colloids in assemblies.

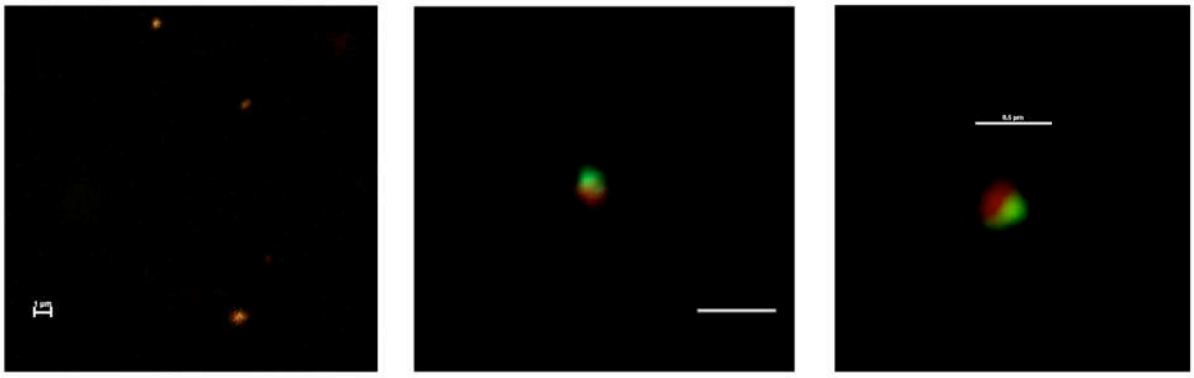

Figure 5. From left to right: CLSM, Zeiss SIM, and Nikon SIM images of Janus particles by two-color imaging.

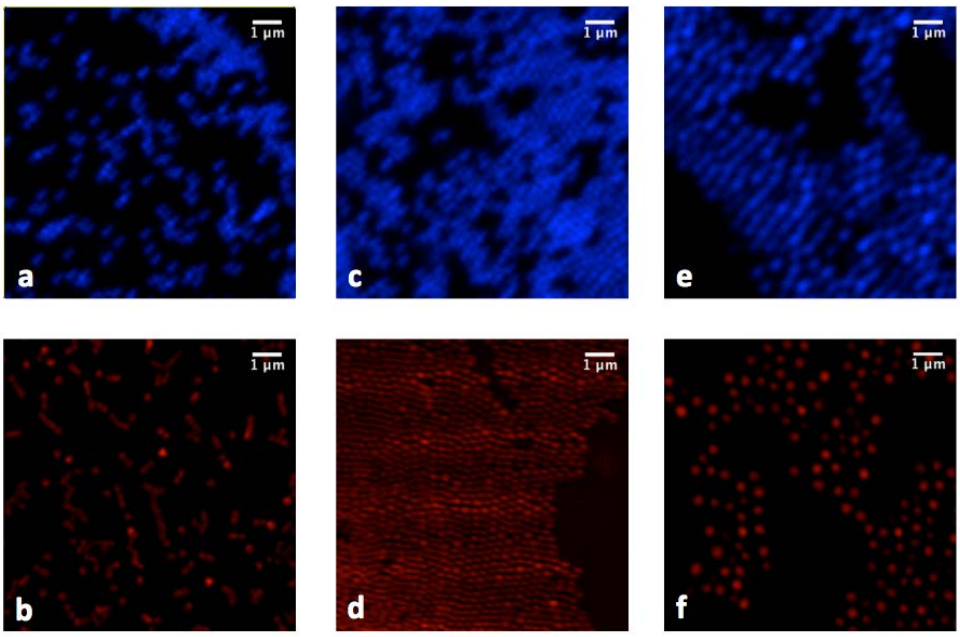

Figure 6. The blue images were taken using confocal microscopy, the red images were taken using SIM. (panels a, b): 200 nm PMMA-PHSA particles, (panels c, d): 310 nm PS particles, (panels e, f): 450 nm PS particles. Credit: Cathleen Chong & Laura Colon-Melendez.

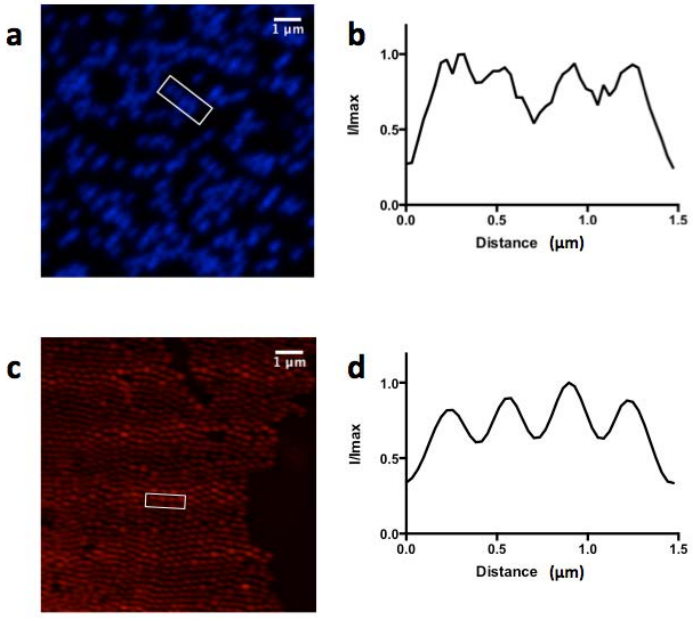

Figure 7. Analysis of 310 nm particles under the confocal (blue, panel a) and SIM (red, panel b) image. The boxes on the image represent the location of the peak intensity

analysis. The corresponding plots on the right demonstrate the results of the particles of each image. Credit: Cathleen Chong & Laura Colon-Melendez.