Using Pre-Cleaned Coverslips to Optimize Coverslip Preparation for Single-Molecule Fluorescence Microscopy

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Abstract:

Single-molecule fluorescence microscopy has become a popular tool for exploring structural changes and dynamics of biological systems. In our laboratory, we use single-molecule techniques to track conformational changes of immobilized nitric oxide synthase (NOS) and determine their relationship to catalytic activity. Properly immobilizing biomolecules like NOS on a glass surface requires careful attention to coverslip cleaning and preparation. There are many protocols available for cleaning glass coverslips, but these protocols are time-consuming and often use harsh conditions. Alternatively, commercially cleaned and passivated coverslips are available but are quite expensive. In this poster, we examine the possibility of using purchased pre-cleaned coverslips (Schott Nexterion) that come ready to be prepared for single-molecule measurements. We present figures and measurements of merit comparing the pre-cleaned coverslips with ozone cleaned coverslips; demonstrating the effectiveness of pre-cleaned coverslips for single-molecule fluorescence microscopy.

Introduction:

Single-molecule fluorescence microscopy (SMFM) has become an indispensable tool in the biosciences by giving insight into the kinetics and dynamics of biological molecules while leaving samples minimally perturbed (1). SMFM utilizes fluorescence, a process that occurs when a photon of light is absorbed by a conjugated fluorophore, which re-emits the photon at a higher wavelength. The first detection of a single molecule using SMFM was reported by Tomas Hirshfield in 1976 when he observed the presence of fluorescently labeled globulin proteins flowing past a detector (1). His success led to the continued application of SMFM to many other structures such as enzymes, DNA, and other cellular structures.

For SMFM to work properly, the coverslips used must be exceptionally clean because nonspecific molecular binding can interfere with the quantification of background noise (2,3). To prevent these interactions, surface passivation can be used. This process facilitates the specific binding of desired molecules to the surface. PEG passivation is very effective, and it has become a standard for SMFM, but the cleaning procedure can be very tedious, complicated, and involve harsh conditions (2). One option is for labs to buy coverslips that have already been cleaned and passivated, but these are very expensive. A second, less expensive option is buying coverslips that have been cleaned but not passivated. However, some may question the robustness, longevity, and consistency of this commercial cleaning. A third method is buying coverslips that have not been pre-cleaned and subjecting them to a quicker and easier cleaning procedure, such as ozone cleaning. Ozone cleaning involves using high powered UV light to generate ozone. The ozone reacts with contaminants on the coverslip surface, forming volatile compounds that evaporate from the surface of the coverslip. This method can take as little time as 10-15 minutes, and then passivation can proceed. Here we explore the cleanliness and coverage quality of pre-cleaned coverslips and ozone cleaned coverslips for SMFM applications.

References and Acknowledgements

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Results:

Comparing the different coverslip types that were untreated, both the Platinum coverslips and Batch 2 Schott coverslips had the lowest means and standard deviations (Figure 1,2). For the ozone treatment, only the means of the Batch 1 Schott and Platinum coverslips were significantly different from their untreated counterparts (Figure 1,2). Comparing how surface cleanliness improves surface coverage, ozone cleaning of platinum coverslips significantly reduced the coefficient of variation compared to its untreated counterpart (Figure 3,4). For the remaining coverslips, there was no significant difference in the coefficient of variation between ozone cleaned and untreated coverslips (Figure 3,4). Ozone cleaning significantly increased the mean counts of Batch 1 and 2 Schott slides (Figure 3,4).

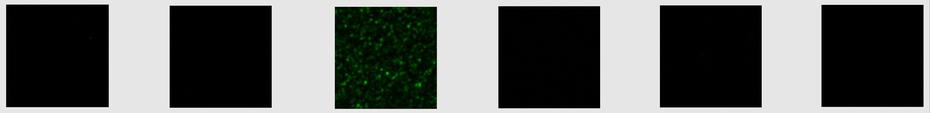

Figure 1: 15x15 μm area of a variety of coverslip types that are ozone cleaned or untreated. From left to right: Platinum untreated. Platinum ozone cleaned. Batch 1 Schott coverslip untreated. Batch 1 Schott coverslip ozone cleaned. Batch 2 Schott coverslip untreated. Batch 2 Scott coverslip ozone cleaned. These images are representative images from each category and are standardized to the same count maximum.

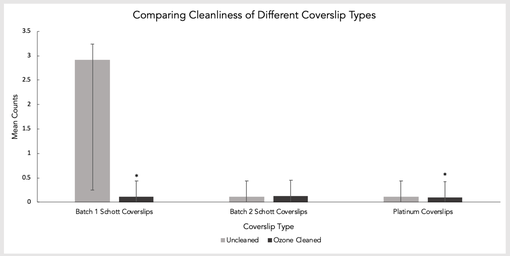

Figure 2: Comparing fluorescence intensity from various coverslip types with and without ozone cleaning. The fluorescence intensity is measured by focusing on the surface of the slide. The mean counts, shown here, reflect the cleanliness of slide, with lower counts corresponding to a cleaner slide. * Indicates a significant difference from its counterpart in the same coverslip type ($p<0.05$). For each subcategory n=15 images from 3 slides.

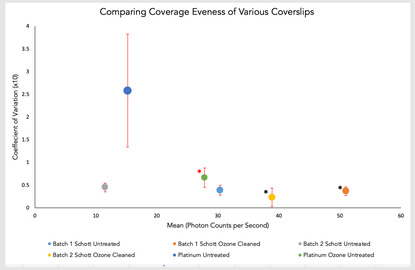

Figure 3: Comparing the evenness of surface coverage between different coverslip types. Surfaces were covered with streptavidin conjugated with AF594 following surface passivation with PEG-PEG Biotin. The mean refers to the brightness of an image area and the coefficient of variation corresponds to the differences in counts within an image area. Greater coefficient of variation indicates unevenness of coverage. * Indicates a significant difference between the means from the same coverslip type ($p<0.05$). * Indicates a significant difference between the coefficients of variation from the same coverslip type ($p<0.05$). Red error bars represent standard deviation of the coefficient of variation. For each subcategory n=15 images from 3 slides.

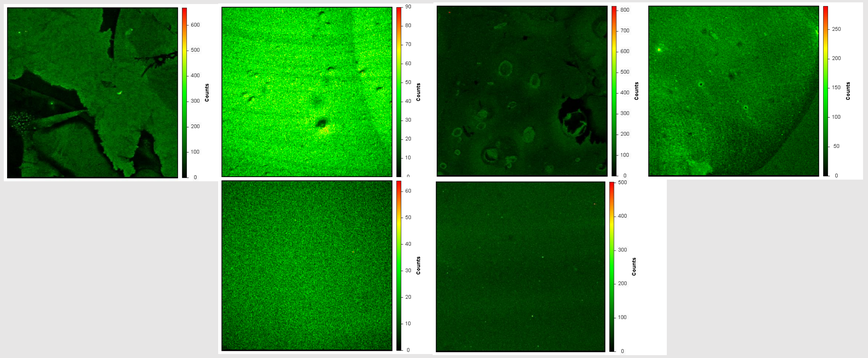

Figure 4: 600x600 μm area of PEG treated coverslips that are incubated with streptavidin AF594 that are either untreated or ozone cleaned. From left to right and top to bottom, the images correspond to areas from Platinum coverslips untreated and ozone treated. Batch 1 Schott coverslips untreated and ozone treated. Batch 2 Schott coverslips untreated and ozone treated. These images are representative images from each category.

Materials and Methods:

Pre-cleaned coverslips were purchased from Schott Nexterion (Louisville, KY). These coverslips were placed individually in sterile 50mL falcon tubes and vacuum-sealed to preserve cleanliness. The lids on the falcon tubes were turned very loosely, which allowed the air to be removed from the falcon tube. Two separate batches of these pre-cleaned coverslips were used in this study. Also, a box of Platinum Line coverslips from ThermoFisher (Waltham, MA) was procured, and these are not pre-cleaned. For ozone cleaning, a UV Ozone Cleaner from Ossila (Sheffield, UK) was used. Three coverslips from each box were ozone cleaned prior to passivation, and another three coverslips from each box were solely passivated.

The protocol for PEG passivation of glass coverslips was adapted from Gidi et al. (2). In a hood cleaned with acetone, a silicone mold form Grace Bio-Labs (Bend, OR) was placed onto a glass coverslip. The desired wells were washed with 110 μl of dry-acetone and emptied after each wash. Then the wells were airdried to remove any residual acetone. The coverslip was placed in a desiccator apparatus and heated in an oven at 90ºC for 5 minutes. To the desired wells, 99:1 25%v/v PEG-Silane and Biotin-PEG-Silane was added and then heated for 15 minutes at 90ºC to facilitate an alcohol condensation between the silanol of the glass and the ethoxide of silane component of PEG. The desired wells were washed three times with Ambion water (Austin, TX).

In this study, a confocal microscopy and avalanche photodiodes from ISS (Champaign, IL) were utilized, and the experiments were carried out over a period of two months. To assess coverslip cleanliness, we compared untreated coverslips (coverslips right outside of the box) to their ozone cleaned counterparts from the two batches of Schott Nexterion coverslips (pre-cleaned) and Platinum Line coverslips. The wells on the coverslips were washed with water three times and then filled with water and imaged at five different locations using the water objective. The area of the images was 600 μm by 600 μm. The wavelength was set to 594 nm and the intensity was adjusted to achieve a power of around 630 nW when measured immediately before the laser entered the microscope. Utilizing the same settings and coverslip categories, we compared how coverslip cleanliness related to coverage quality. The coverslips were passivated according to the aforementioned protocol. The wells were then washed three times with fluorescence buffer, which is composed of 10 mL of 10X fluorescence buffer stock, 1 mL of 10 mg/mL BSA, and 89 mL of Ambion water mixed and run through a sterile 0.2 μm polyethersulfone membrane filter. The 10X fluorescence buffer stock is made of 1 M KCL and 0.5 M HEPES at a pH of 7.4. Then 110 mL of 100 nM solution of streptavidin-conjugated-Alexa Flour 594 from ThermoFisher was added, which binds biotin tagged molecules. This was allowed to incubate for fifteen minutes. Then the wells were washed three more times with fluorescence buffer and filled with fluorescence buffer once more. Next, the coverslips were once again imaged at five different locations using the air objective. This was to determine the quality of the surface that had been created. A patchy uneven spread of Alexa Flour 594 indicates a poor surface, while even coverage suggests higher quality surface passivation.

Discussion

To assess the cleanliness of the coverslips, we compared the mean counts of untreated coverslips to their ozone cleaned counterparts. We concluded that ozone cleaning improved the cleanliness of Batch 1 Schott and Platinum coverslips (Figure 1,2). However, cleanliness of coverslips did not necessarily correspond to the evenness of surface coverage when the coverslips was treated with PEG and incubated with streptavidin AF594. We found that ozone cleaning did not impact the surface coverage of the Batch 1 and 2 coverslips but enhanced the surface coverage of Platinum coverslips (Figure 3,4). Therefore, we conclude that Schott coverslips are adequate to use right of the box because their background counts are low and their surface coverage is even. However, commercially uncleaned coverslips such as the Platinum coverslips can be ozonated to improve the surface quality for single-molecule fluorescence microscopy, noting that surface coverage was varied (Figure 3,4). Further directions would be to examine different coverslip cleaning procedures that are cheaper, as ozone cleaning is expensive. Additionally, we could analyze how different surface passivation protocols, such as with BSA, might affect the evenness of the coverage on a coverslip.