Supporting Information

Microretroreflector-Sedimentation Immunoassays for Pathogen Detection

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S1. Fabrication of assay tubes.

Materials and tools:

- qPCR tubes
- Bunsen burner
- 18 gauge Luer-Lok™ needles (for interface ports)
- Dremmel tool
- 90 second epoxy (Araldite 2043 or similar)
- Tygon microbore tubing (OD = 0.070”, ID= 0.040″)

Tubes were obtained and separated into individual pieces using scissors or a precision knife. Each tube was then cut approximately 1 mm from the conical end to create the inlet opening. An 18 gauge needle was then heated using a Bunsen burner and used to carefully create the outlet hole in the side of each tube. Holes were made to the approximate diameter of the Tygon tubing. The tubing was cut to length and, prior to assembly, all materials were cleaned by bath sonication in anhydrous ethanol. Upon assembly, epoxy adhesive was applied to affix the tubing to the qPCR tubes and allowed to dry overnight. The Luer-Lok™ interface ports were created by removing the tips of 18 gauge needles using a Dremmel tool with the appropriate metal cut-off wheel attachment. Once the interface ports were cleaned and the epoxy on the assembled qPCR tubes dried, the interfaces were inserted into the Tygon tubing.

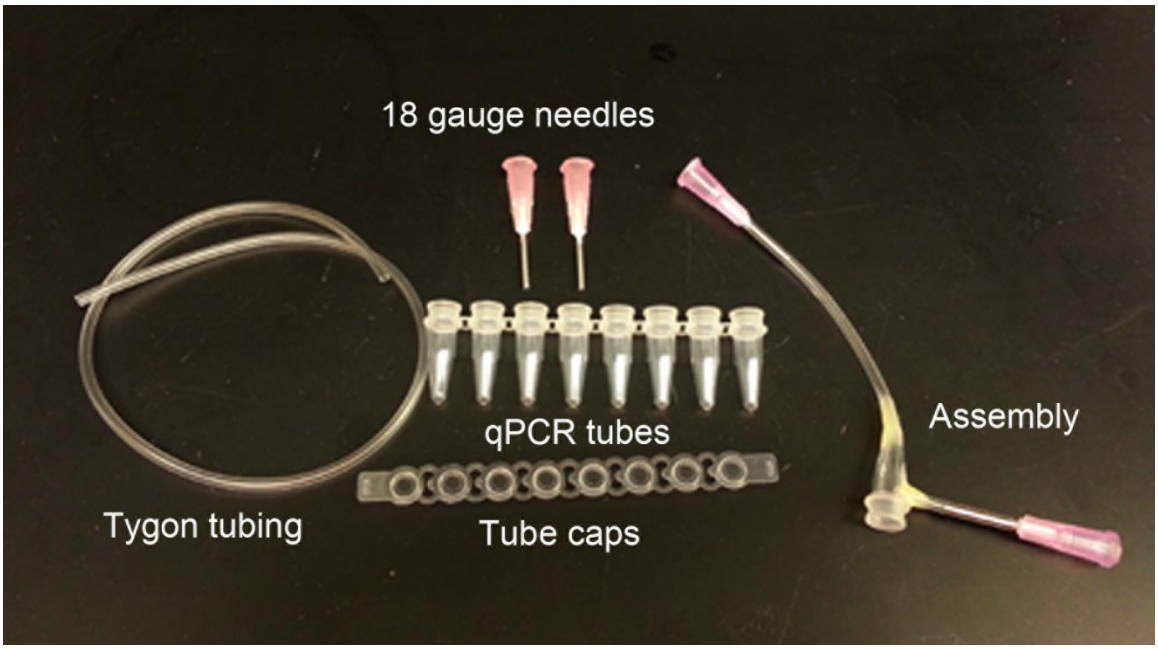

Figure S1. Components of assay tube system.

S2. Fabrication and functionalization of retroreflector cubes.

A 400 nm copper layer is deposited by electron beam evaporation onto a silicon wafer followed by a 5 μm SU-8 5 photoresist layer (Microchem, Inc., Newton, MA) deposited by spin-coating at 2000 rpm for 1 minute. The SU-8 is baked on a hot plate at 95 ˚C for 3 minutes and exposed to a 254 nm wavelength UV light source (UVP CL-1000, Upton, CA) and baked, once again, at 95 ˚C for 5 minutes to cure the epoxy-based photoresist. A second 200 nm copper layer is electron beam evaporated onto the substrate. A 70 nm polystyrene layer is deposited by spin-coating 2.5% polystyrene in toluene (w/v) at 2300 rpm for 1 minute. The cube pattern is then printed using an in-house helium ion beam proximity lithography tool,[1] through a gold-coated, silicon nitride stencil mask containing a 1 cm^2 area of 5μm by 5μm square openings with 10 μm pitch. Following the patterning step the resist is developed in a toluene bath for 40 seconds with slight agitation. The underlying copper layer is then etched with copper etchant TF-49 reagent (Transene Company, Danvers MA). The square pattern is then transferred into the SU-8 by oxygen reactive ion etching, under 1mTorr O_2 feed, and 13.56 MHz RF signal at 50 W in the presence of a 50 Gauss axial magnetic field, leaving cubic structures. The bottom copper layer is then dissolved using copper etchant, leaving behind an undercut underneath the cube structures. The undercut is critical for providing an opening for copper etchant to dissolve the remaining copper during the final step for cube release. Titanium is then evaporated, top-down, onto the substrate to form a 10 nm adhesion layer and a 100 nm of gold is deposited, at an angle relative to the surface normal by physical vapor deposition, coating only three of the optically transparent SU-8 surfaces. The cubes are later released by dissolving the remaining copper.[2]

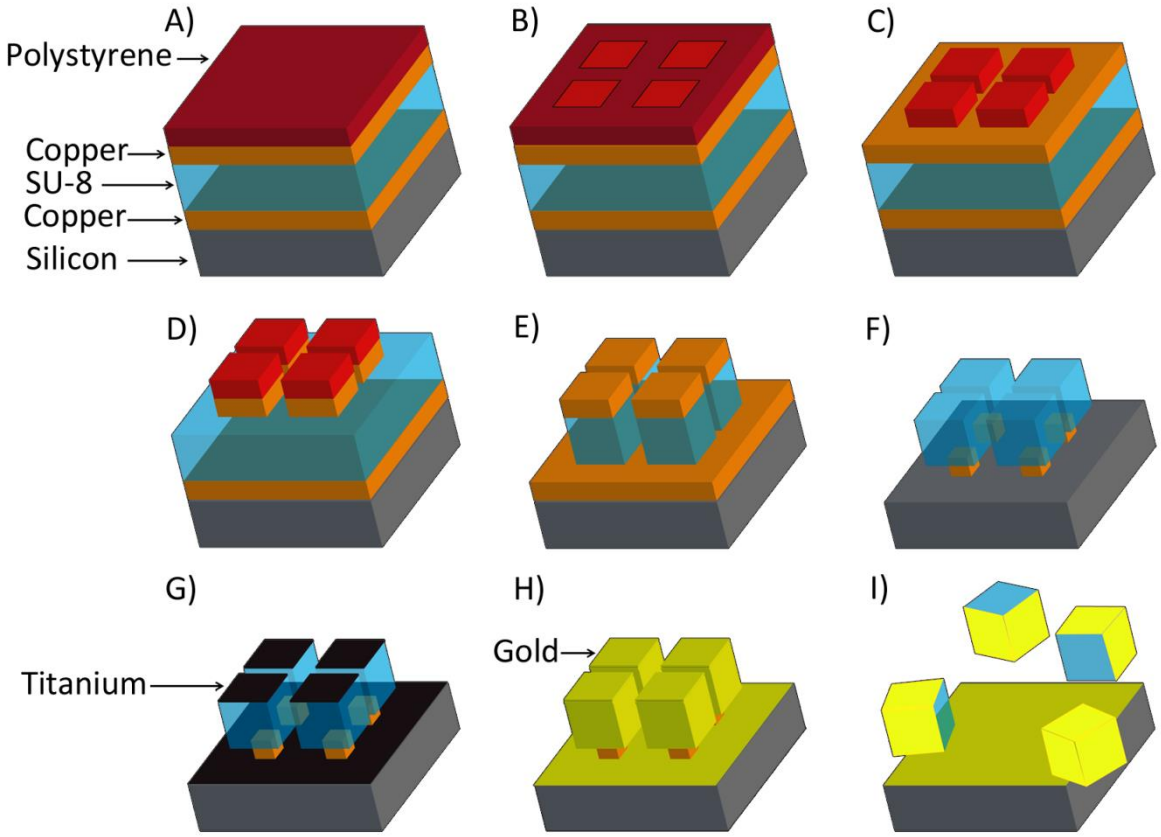

Figure S2. (A) Material layers are deposited. (B) Cube pattern is printed by ion beam proximity lithography. (C)Resist is developed in a toluene. (D) Underlying copper layer is etched with copper etchant. (E) Pattern is transferred into the SU-8, by reactive ion etching. (F) Last copper layer is dissolved and cube structures are undercut. (G) Titanium is evaporated, top-down, onto the substrate. (H) Gold is evaporated at an angle relative to the surface normal. (I) Cubes are released by dissolving the remaining copper.

To optimize the antibody functionalization of the cubes, individual batches of ECD/NHS activated cubes were pelleted and resuspended in 400 µL of 25 µg/mL, 125 µg/mL and 250 µg/mL of antibody solution, respectively, for overnight incubation at 4 °C with constant mixing

by inversion. The conjugated cubes were then pelleted and resuspended in 2% BSA, 100 mM hydroxylamine for passivation at room temperature for 3 h. The cubes were then washed with PBST, and resuspended in 1 mL PBS. Functionalized cubes were then spotted on glass slides (~5 µL per sample) and observed by optical microscopy, as shown in Figure S3. An antibody concentration of 250 µg/mL was chosen to minimize aggregation of the cubes.

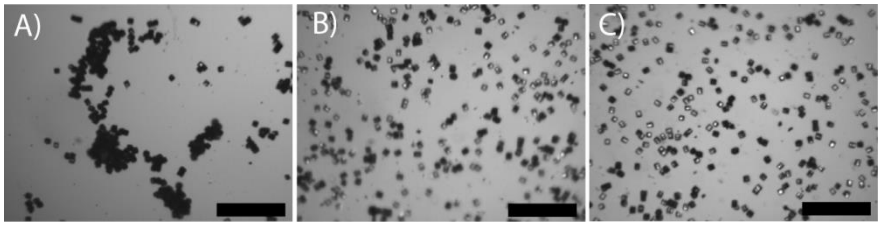

Figure S3. Optical microscopy images of cubes functionalized with: (A) 25 µg/mL of antibody, (B) 125 µg/mL of antibody and (C) 250 µg/mL of antibody. Scale bars are 100 µm.

S3. Image capture and counting of retroreflecting cubes.

Images of three regions within the observation window are taken and within each of those regions, three images are taken (process schematic shown in Figure S4 and example images shown in Figure S5-S9). The camera, with in-line light source, is positioned at a 35˚ angle (to reduce the effect of specular background reflection) and has a 50 μm depth of field; as a result, approximately one-third of the region is in focus in each recorded image. The height of the camera is therefore adjusted to capture three focal planes over the region. The focused regions within each focal plane image are cropped and stitched together to create a single image. A binary threshold is applied to this single image then counting is automated using NIH ImageJ 1.46r.[3]

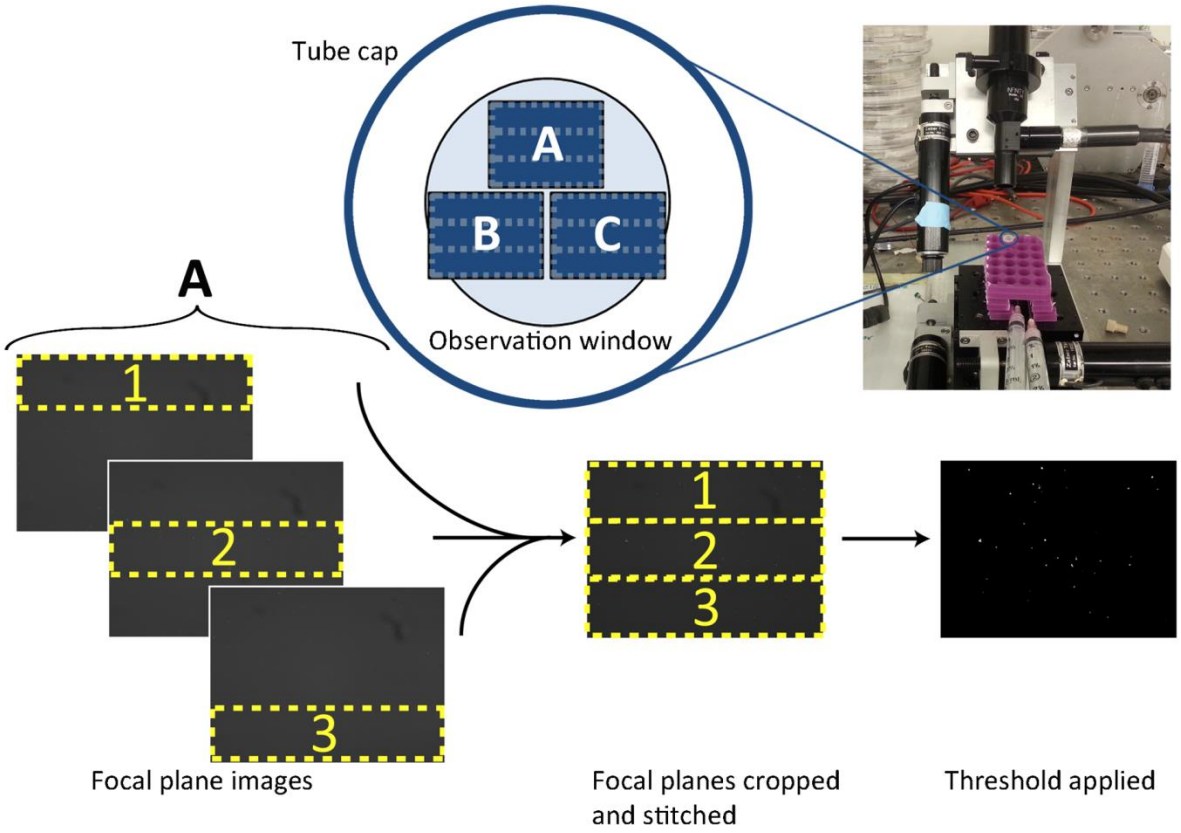

Figure S4. Schematic of image capture and processing steps. Letters A, B and C represent three regions within the observation window to be imaged. Numbers 1, 2 and 3 represent focal planes within each image.

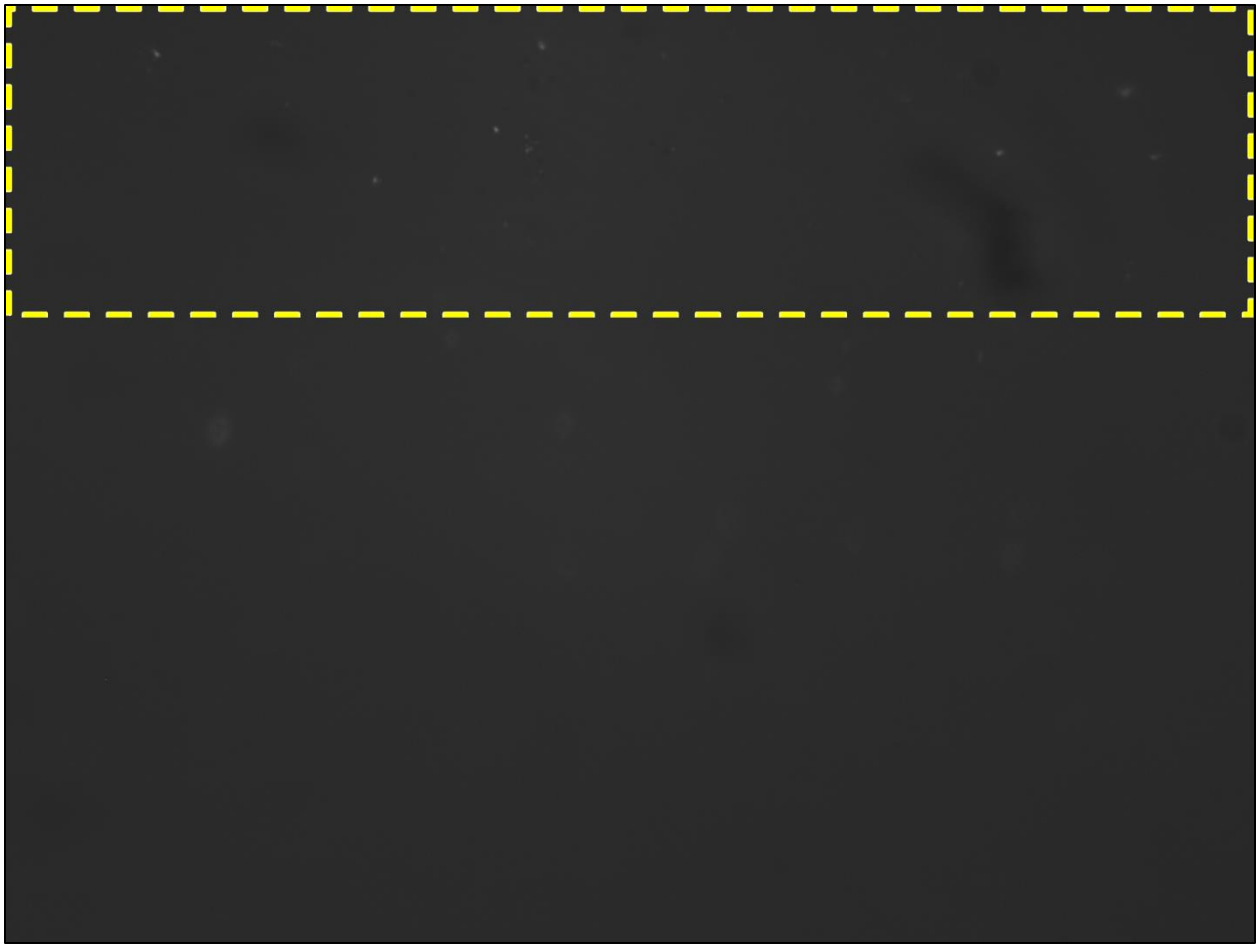

Figure S5. Image of Region A: focal plane 1 (depicted in Fig. S3 above), showing only top part of the image in focus.

Figure S6. Image of Region A: focal plane 2 (depicted in Fig. S3 above), showing middle part of the image in focus.

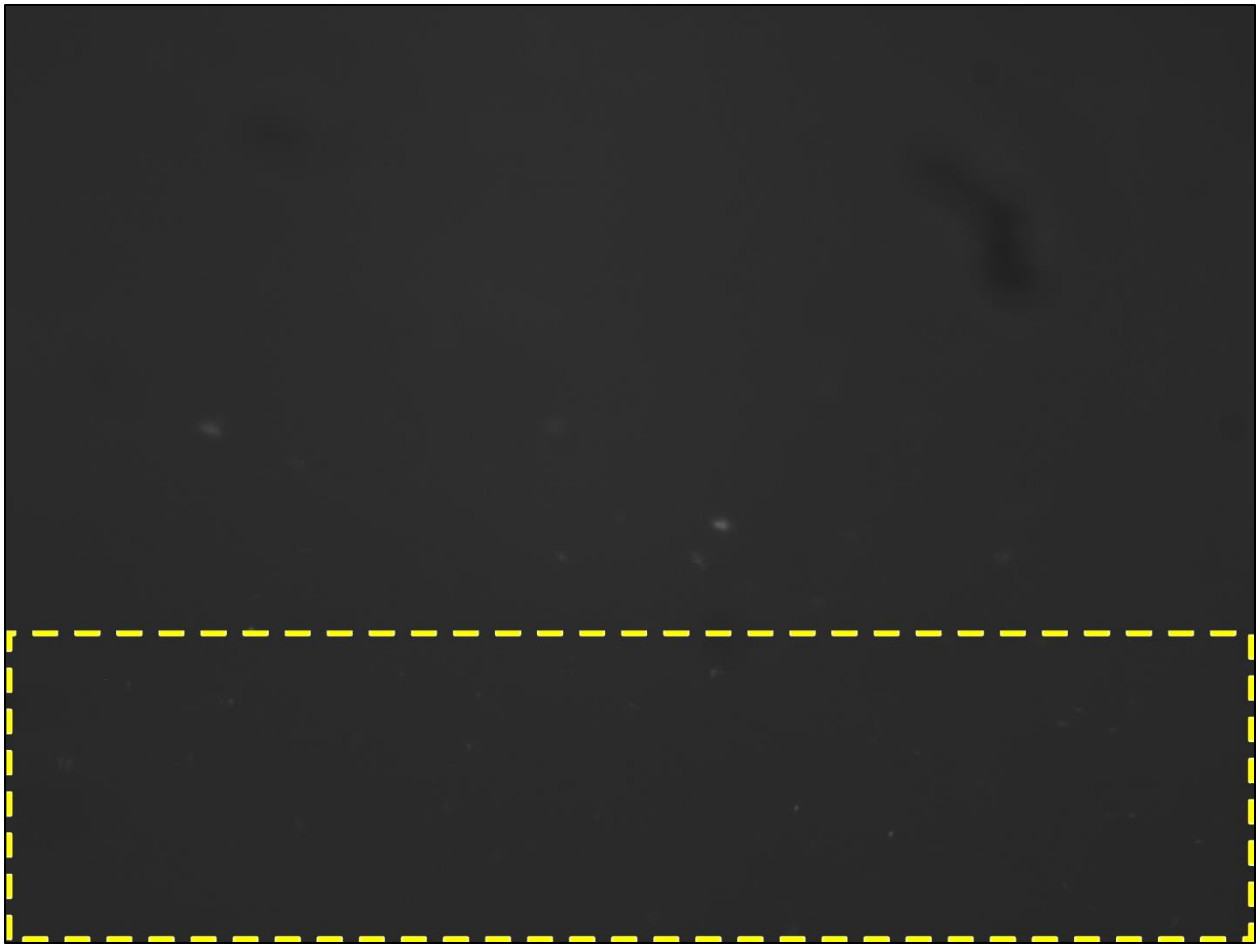

Figure S7. Image of Region A: focal plane 3 (depicted in Fig. S3 above), showing bottom part of the image in focus.

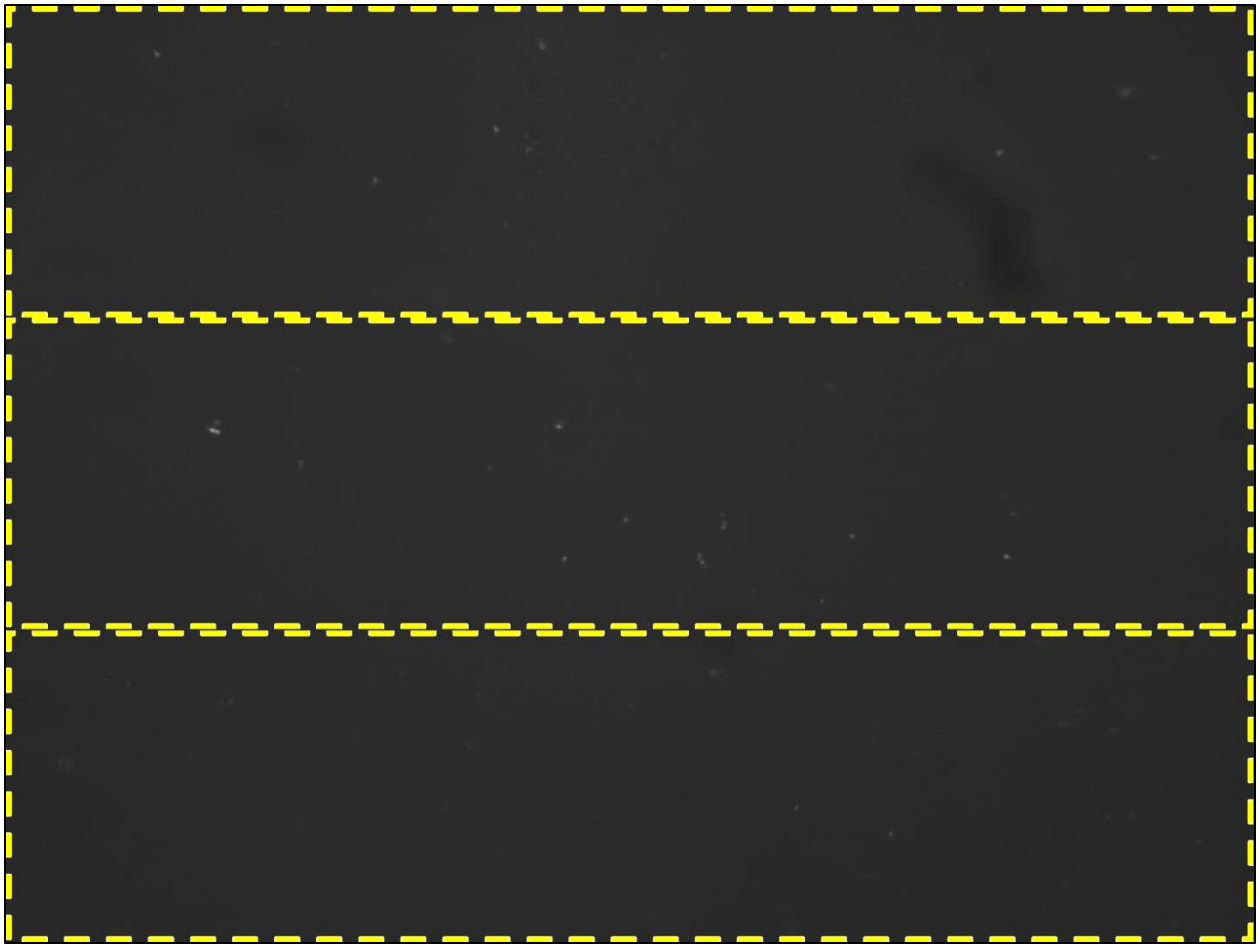

Figure S8. Focal planes cropped and stitched together to create a single image showing the entire Region A in focus.

Figure S9. Binary threshold applied to stitched image of Region A.

S4. Monitoring of polypropylene observation window modification by attenuated total internal reflection fourier transform infrared spectroscopy and X-ray photoelectron spectroscopy.

After each major modification step, the samples were analyzed by ATR-FTIR and XPS, Figure S10 and S11.

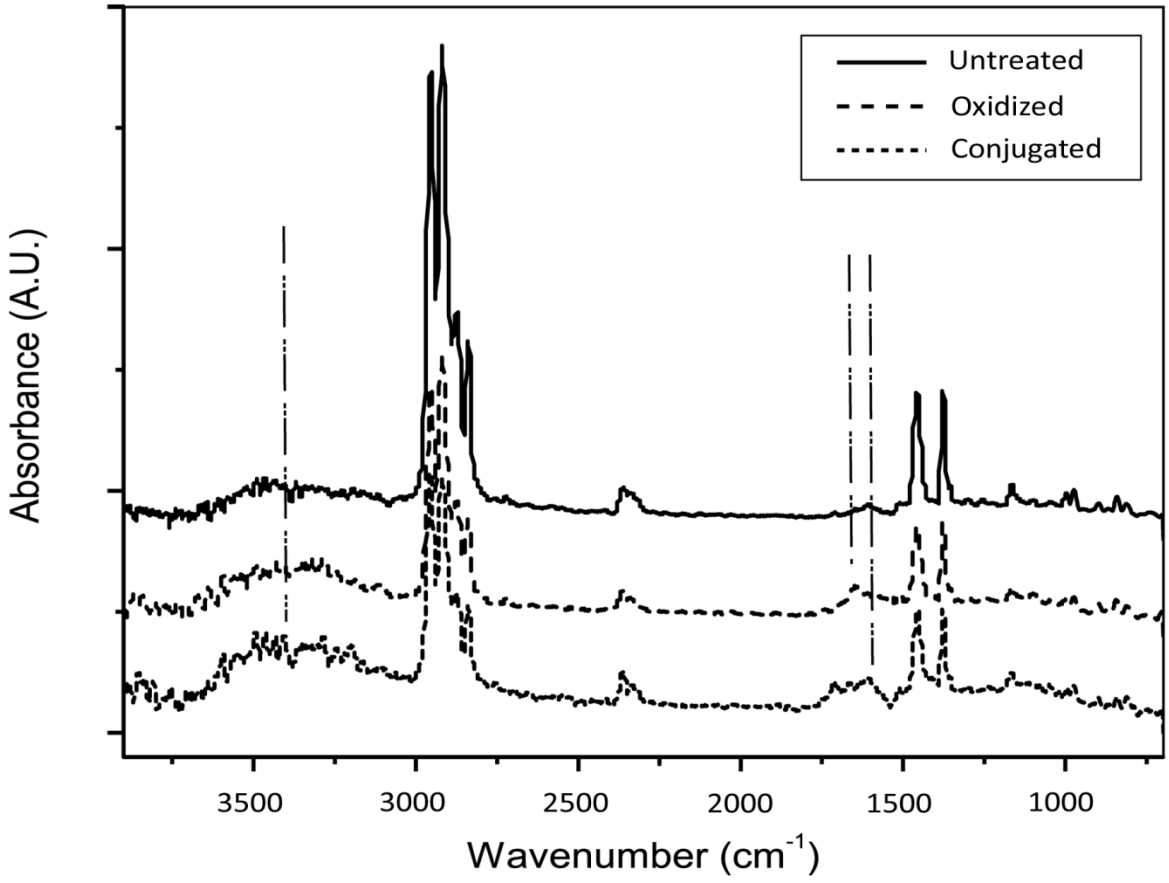

Figure S10. FTIR spectra of polypropylene observation window at different stages of functionalization.

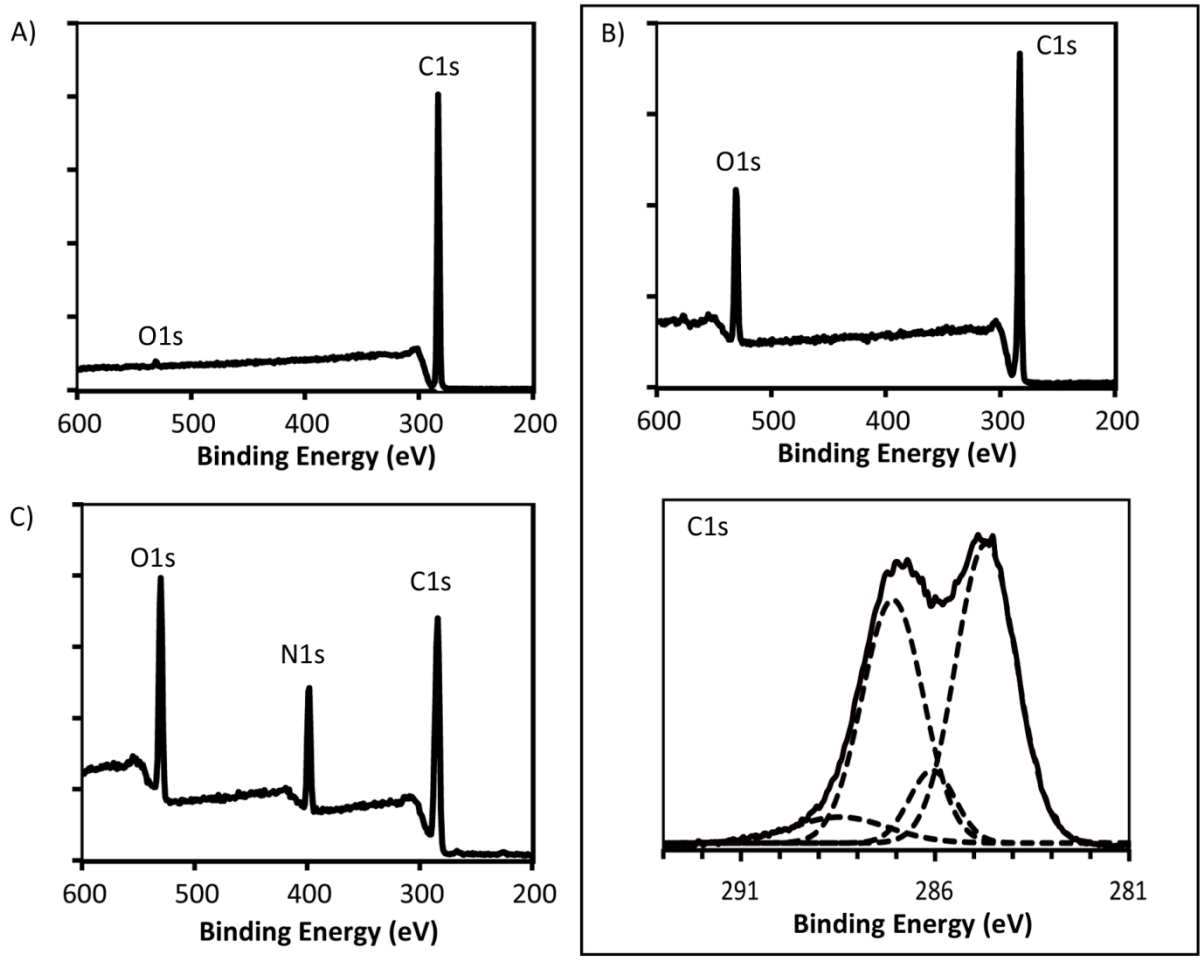

Figure S11. XPS survey spectra of A) untreated polypropylene observation window, B) oxidized polypropylene with high resolution C1s scan and C) antibody-conjugated polypropylene.

S5. Specific capture of antibody functionalized cubes.

Anti-rabbit IgG antibodies, anti-mouse IgG antibodies and D1.3 anti-lysozyme antibodies were conjugated to thermally evaporated gold spots on silicon via DSP thiol coupling chemistry.[4] Rabbit antibody-functionalized anti-*E. coli* and anti-MS2 cubes added were spotted on the antibody coated gold areas and then washed with PBST. The resulting substrates were then dried and observed via SEM.

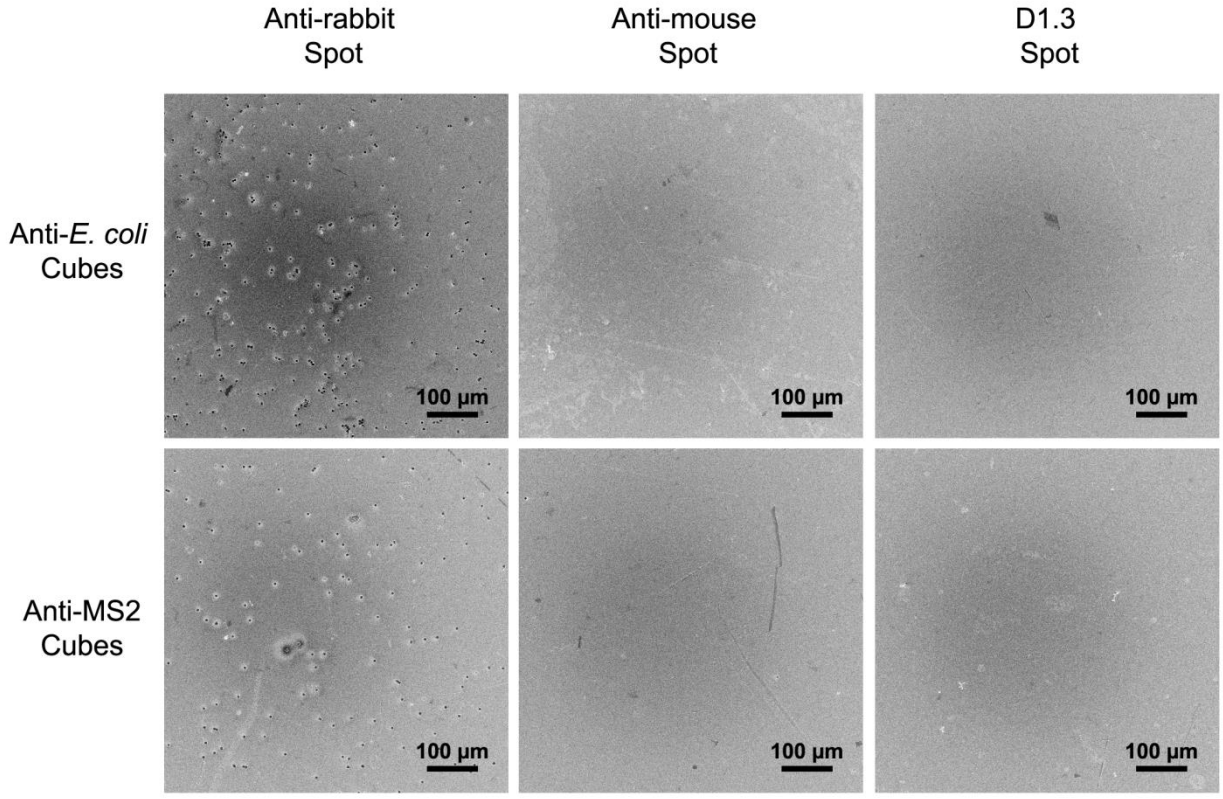

Figure S12. Specific capture of antibody functionalized cubes.

References.

(1) Nasrullah, A.; Smith, D.; Sherlock, T.; Ruchhoeft, P.; Litvinov, D. *J. Vac. Sci. Technol. B*, **2009**, *27*, 2674.

(2) Sherlock, T.; Nasrullah, A.; Litvinov, J.; Cacao, E.; Knoop, J.; Kemper, S.; Kourentzi, K.; Kar, A.; Ruchhoeft, P.; Willson, R. *J. Vac. Sci. Technol. B*, **2011**, *29*, 06FA01-06FA01.

(3) Noursadeghi, M.; Tsang, J.; Haustein, T.; Miller, R. F.; Chain, B. M.; Katz, D. R. *J. Immunol. Methods* **2008**, *329*, 194.

(4) Hermanson, G. T. *Bioconjugate Techniques*; Academic Press: Amsterdam, 2008.