Bifunctional Nanoarrays for Probing the Immune Response at the Single-Molecule Level

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In the immune recognition process, surface proteins on T-cells along with their corresponding ligands on antigen-presenting cells (APCs) become organized into a spatially patterned microscale motif, known as the immunological synapse (IS). Since the IS displays a clear pattern of adhesion, costimulatory, and signaling molecules, T-cells should be extremely sensitive to the arrangement of these molecules on the nanoscale. In fact, artificial APC surfaces of supported bilayer membranes with nanoscale barriers were shown to modulate IS patterns in living T-cells, revealing prolonged signaling from T-cell receptor (TCR) microclusters that had been mechanically trapped by the nanostructures in the peripheral region [1].

To probe the geometric factors that affect T-cell response, we employ biomimetic arrays of individual protein binding sites created by molecular-scale nanolithography and site-selective biochemical assembly [2]. Nanoarrays are fabricated using nanoimprint lithography (NIL) and self-aligned pattern transfer to form metallic nanodots with diameters $\lesssim$ 5 nm [3] (Fig. 1). Single-molecule T-cell studies, however, require *bifunctional nanoarrays*, in which two co-stimulatory molecules are presented simultaneously. In the IS, the central supramolecular activation cluster (c-SMAC) of TCRs is surrounded by a ring of peripheral supramolecular activation cluster (p-SMAC), which is formed by adhesion molecules: leukocyte function associated antigen-1 (LFA-1) on T-cells and the corresponding intercellular adhesion molecule-1 (ICAM-1) on APCs. In our bifunctional arrays, which are formed on glass coverslips, AuPd nanodots are functionalized with a biotinylated UCHT1 Fab antibody (which binds to TCR) anchored to the nanodots via a streptavidin linker. The surrounding surface is functionalized with a his-tag ICAM-1 linked to a PEG-silane via a nickel-NTA bridge to facilitate binding of LFA-1, which enhances cell adhesion (Fig. 2).

Cell assays were performed on nanodots arrayed in clusters of various size, spacing and density. Fresh human naive CD4+ cells were plated on the bifunctional surfaces and fixed after 5 min. Phosphorylated tyrosine (pY), an indicator of immune response signaling strength, was stained for fluorescence microscopy. We found, for example, on extended hexagonal arrays with inter-dot spacing from 60 nm to 300 nm, the average pY intensity decreased with increasing spacing to a threshold ~ 100 -150 nm spacing (corresponding to a density of ~ 50 -120 TCR binding sites per μm^2), at which point the signal dropped to nearly that of cells on the background (outside the pattern area, a negative control) (Fig. 3).

We have also begun addressing a key question relevant to all single-molecule experiments involving nanoparticles, namely, how many molecules are bound to an individual nanoparticle? Photobleaching analysis of the functionalized nanodot arrays was applied to estimate the molecular occupancy of the dots in each cluster, either by counting the steps in a bleaching curve or by simply dividing the total bleaching intensity by the average measured step size. The relationship between the molecular occupancy and factors such as nanodot size, concentration and incubation time are studied, so that this nanoscale platform can be optimized to ensure true single-molecule control.

[1] Altered TCR Signaling from Geometrically Repatterned Immunological Synapses, K. D. Mossman et al, 2005, Science 310 (5751): 1191-1193.
[2] Nanolithographic control of the spatial organization of cellular adhesion receptors at the single-molecule level, M. Schvartzman et al, 2011, Nano Letters 11 (3): 1306-1312.
[3] Robust Pattern Transfer of Nanoimprinted Features for Sub-5-nm Fabrication, M. Schvartzman et al, 2009, Nano Letters 9 (10): 3629-3634.

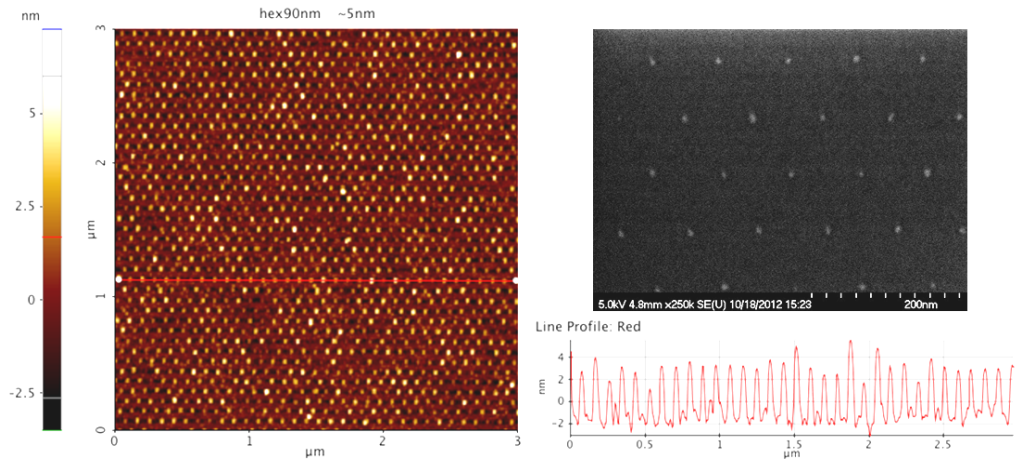

Figure 1. AFM and SEM images of the AuPd nanodot arrays (90 nm spacing hexagonal array as an example).

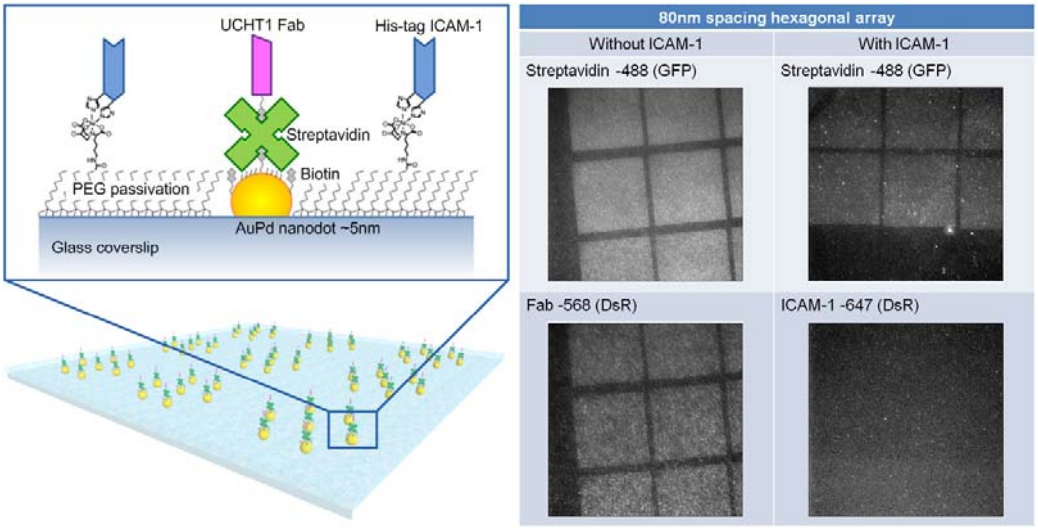

Figure 2. Schematic diagram and fluorescence image of the bifunctional nanoarray.

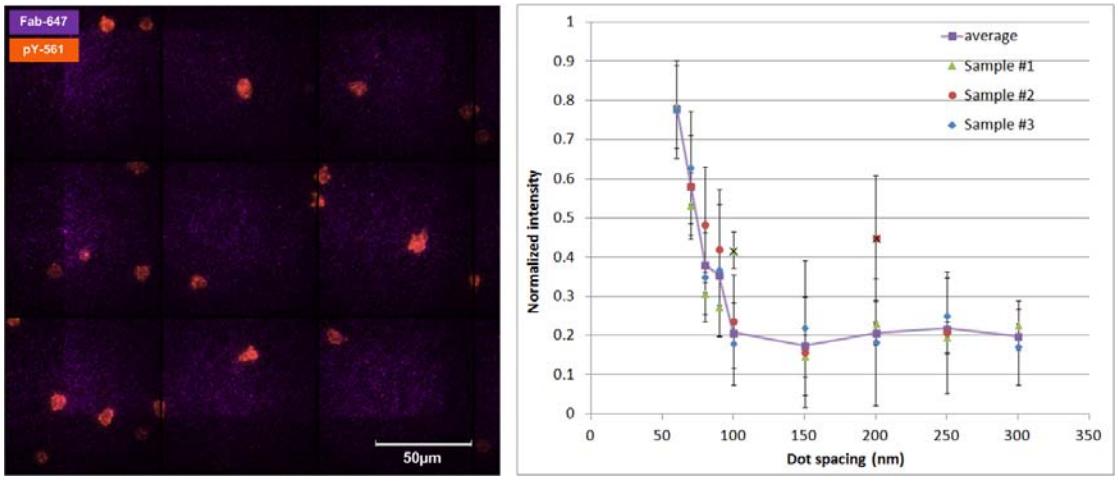

Figure 3. Cell assay on extended hexagonal arrays. Left: Fluorescence image on a 200µm x 200µm array (90 nm spacing). Right: pY intensity as a function of nanodot spacing.