Multiplexed Super-Resolution Imaging of Signaling Microclusters in T cells

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***Abstract*—Engagement of the T cell receptor (TCR) leads to the formation of microclusters containing the TCR and many different signaling molecules. These microclusters are known to be the sites where a number of key downstream signaling pathways are triggered, leading to T cell activation. The nanostructure and relative molecular distributions within the microclusters are not well characterized due to limitations in conventional light microscopy. While the resolution limit of microscopy has been recently surpassed by techniques known collectively as super-resolution microscopy, there exists a spectral limit to light microscopy. Multiplexed visualization of different fluorescent molecules is restricted to a maximum of six colors due to the limited availability of non-overlapping wavelength profiles for different fluorescent probes. Moreover, in super-resolution microscopy, only a few fluorescent probes have the properties required for precise single molecule localization, limiting most super-resolution studies to two colors.**

To overcome these spectral limits, alternative multiplexing schemes have been devised (Schubert, et al. 2014, Jungmann et al. 2014, Tam et al., 2014). These strategies utilize cycling of pre-labeled fluorescent probes that are bound to the cell, imaged, and then either photo-bleached or chemically quenched. Such multiplexing strategies can indeed bypass the spectral limit of microscopy, but the eventual accumulation of fluorescent probes will likely lead to steric blocking of additional binding sites in the cell, preventing further multiplexing. Furthermore, as fluorescence bleaching is known to be a toxic process, prolonged photo- or chemical- bleaching will likely cause unwanted effects such as reverse cross-linking and denaturation of cellular proteins. We have developed a novel imaging technique called multiplexed antibody size-limited direct stochastic optical reconstruction microscopy (madSTORM) that allows potentially unlimited multiplexing and minimizes the steric blocking and toxicity issues of previous methods. In addition, we have overcome limitations of current super-resolution imaging software in registering samples for drift correction and alignment, achieving >2 fold improvement in precision over previous dSTORM studies. Using our precise multiplexed dSTORM imaging technique we have successfully visualized 11 different markers in the same T cell with an average localization precision of 2.5 nm and alignment precision of 3.5 nm. In future studies we will simultaneously probe all molecular components of the TCR microcluster in human Jurkat T cells and primary mouse T cells.