OPTIMIZING MULTIPLEX SINGLE MOLECULE PULL-DOWN (SiMPull) TO QUANTIFY PROTEIN PHOSPHORYLATION PATTERNS

Emanuel Salazar-Cavazos[1], [2]Keith A. Lidke, Diane S. Lidke[1]
[1]Department of Pathology and [2]Department of Physics
University of New Mexico
2325 Camino de Salud
Albuquerque, NM USA 87131
E-mail: dlidke@salud.unm.edu

KEY WORDS: single molecule imaging, single molecule pull-down, SiMPull, Epidermal Growth Factor Receptor, EGFR, TIRF microscopy

Single molecule pull-down (SiMPull) is a powerful technique that allows for interrogation of macromolecular complexes at the individual protein level. Jain *et al.* first demonstrated the ability of this technique to capture macromolecular complexes [1]. SiMPull uses the same sample preparation as IP/Western Blot protocols, but the sample is interrogated using single molecule microscopy. Briefly, cells are lysed and then the protein of interest is captured by antibodies bound to the glass coverslip. If the proteins are fluorescently tagged, either by fluorescent proteins or subsequent antibody labeling, their presence will be quantified by single molecule imaging. In 2016, Kim *et al.* used SiMPull to identify protein post-translational modifications using phosphorylation-specific antibody labeling [2].

We describe important improvements over previous protocols, including 1) reduction of autofluorescence in the green (GFP, Alexa488) channel; 2) a simplified imaging chamber that accommodates higher sample number with lower sample volume, 3) automated data acquisition; 4) the ability to correct for membrane receptor surface expression; and 5) up to 4 color multiplex imaging. We apply our optimized SiMPull assay to the study of Epidermal Growth Factor Receptor (EGFR) phosphorylation kinetics. Using multi-color imaging, we determine the percent of receptors phosphorylated at specific tyrosine sites and the coordination between sites. We find that only a subpopulation of EGFR become phosphorylated under what is considered maximal activation conditions. Furthermore, the coincidence of multiple sites being phosphorylated on the same receptor is low, but not uncommon, suggesting a hierarchy of receptor phosphorylation.

[1] A. Jain, *et al.*, "Single-molecule pull-down for studying protein interactions," *Nat Protoc*, **7**, 445 (2012).
[2] K.L. Kim *et al.* "Pairwise detection of site-specific receptor phosphorylations using single-molecule blotting," *Nat Comm*, 7, 11107 (2016).