SINGLE MOLECULE

Single molecules made simple

A single-molecule pull-down method provides a simple way for biologists to examine their favorite protein at the single-molecule level.

Intracellular signaling depends on specific dynamic physical interactions between proteins. But detailed analysis of these interactions is challenging, and single-molecule experiments are typically limited to defined systems using purified proteins. This presents several hurdles. Purification protocols must be developed for the proteins of interest, and because interaction partners are generally lost during purification, it is necessary to purify each constituent protein and reconstitute the system. If identities of all constituents are in question, this presents a quandary for the researcher. Furthermore, recombinant proteins expressed in bacteria may lack important post-translational modifications.

Taekjip Ha at the University of Illinois at Urbana-Champaign has been conducting single-molecule fluorescence experiments on purified proteins for years and decided there must be an easier way. He says, "Protein-protein interactions are such a fundamental thing, and so many people are using pull-downs and western blots that if you can somehow couple that into a single-molecule imaging platform, that could have a wide appeal." His recently reported single-molecule pull-down method (SiMPull) promises to be just such a method.

A 'pull-down', or immunoprecipitation assay, relies on an antibody to bind the target, or bait, protein, in a cell extract and beads coated with a secondary antibody to bind and isolate the antibody-bound bait protein along with any interacting proteins. The identities of the interacting proteins can be determined by western blot or mass spectrometry.

This sounds simple, but as with many experimental methods there is always a catch. Here it is the beads, which are quite sticky and bind not only the bait protein but other cellular proteins as well. Careful washing can remove nonspecifically bound protein while retaining the real interactors, but striking the right balance can be hard. Proteins that bind to the bait directly or indirectly with low affinity are easily washed away before nonspecific binders.

Ha says, "We thought maybe you could bypass all that and just pull down proteins directly from the cell extract and analyze the complexes at the single-molecule level." SiMPull does just that. Ha and colleagues constructed a passivated flow cell in which they coated the imaging surface with antibodies to the bait protein. Flowing the extract through the flow cell captures the bait and any interacting proteins. A single gentle wash step removes unbound protein. "Over the last 10 years we have really optimized the polymer-coated surface preparation. Essentially nothing sticks to the surface except the antibodies that we have. That greatly reduces nonspecific interactions," says Ha.

Captured proteins are detected via intrinsic labeling with a fluorescent protein or immunolabeling with a fluorophore-labeled antibody followed by total internal reflection fluorescence microscopy. Complexes formed by two or more interacting proteins can be detected using a combination of labeled antibodies or fluorescent tags and stoichiometry can be determined by monitoring sequential photobleaching steps. The assay is also very sensitive. "We can now use a single cell as opposed to 5,000 cells for a regular western blot," says Ha. "And this can be done in 2 minutes."

Ha and colleagues demonstrated the performance of SiMPull by pulling down a variety of exogenously expressed protein complexes including the protein kinase A tetramer, G-protein receptor homo- and heterodimers, and mTOR and its binding partner Raptor. Finally, they showed they could detect endogenous PKA-AKAP protein complexes from mouse brain and heart tissue extract, displaying a sensitivity 20-fold higher than in a western blot.

Will SiMPull replace conventional pull-downs and western blots? It lacks the sizing ability of western blots and is more similar to dot blots, so this will limit applications. Good antibodies and stable interactions are still requirements, but it should simplify detection of low-affinity interactions.

The real potential is in the new applications it will allow and its accessibility to biologists. Ha is optimistic and says, "I'm building a device to loan to a cell biologist collaborator. I want it to be easy for people to use."

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RESEARCH PAPERS

Jain, A. *et al.* Probing cellular protein complexes using single-molecule pull-down. *Nature* **473**, 484–488 (2011).