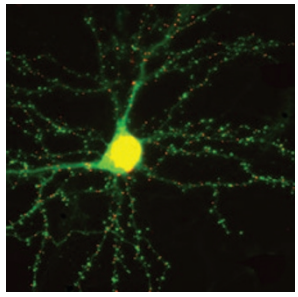
SENSORS AND PROBES

Protein GPS

Antibody-like probes report the localization and amount of endogenous proteins in living cells.

Similar to inhabitants of a city, proteins show all kinds of behaviors when moving about the cell. Following proteins and examining their whereabouts can teach us a lot about who they are and how they do what they do—so methods to track these little citizens of the cell are of great interest.

A global positioning system (GPS) for proteins would ideally label endogenous proteins and not interfere with the proteins' function, localization or expression level. This protein GPS should give us information about the living cell and, optimally, should be specific to targeted cells.



Intrabodies expressed in a live neuron. Image courtesy of G. Gross.

Don Arnold, from the University of Southern California (USC) in Los Angeles, has been studying protein trafficking since the 1990s. Back in those days, he used 'gene guns' to introduce plasmids into neurons in brain slices, and he expressed tagged versions of the proteins he wanted to track inside the cells. "The problem when you do those experiments is that one of two things can happen: the transgenic protein can either go everywhere in the cell, not localizing properly, or it can cause a phenotype," Arnold says. But in the early 2000s he read about small intracellular antibodies, or intrabodies, and decided to try them out.

Intrabodies are small recombinant antibody-like proteins that bind to specific proteins. One can select for intrabodies that bind a protein of interest using phage or mRNA display methods, and the genes encoding the intrabodies can be fused to fluorescent protein genes and introduced into cells so that the endogenous target protein can be followed by microscopy.

BIOPHYSICS

THE PULL OF A CELL

Researchers leash cells to molecular tethers as an easy way to measure single-molecular binding forces.

Taekjip Ha and his postdoc Xuefeng Wang have devised a simple but powerful strategy to measure the tiny forces exerted through single molecules in the cell. The University of Illinois scientists dreamed up a cellular tug-of-war between receptors anchored in the cell membrane and corresponding ligands that they tethered to a surface. To determine the forces, they simply monitored whether the tethers held and the cells adhered.

Cells actively sense and transmit forces, and we know that the stiffness of the environment can determine whether cancer cells become metastatic or whether embryonic stem cells differentiate into other cell types. Force also plays a critical role in processes such as cell adhesion, migration and immune function. Ultimately, events at the cellular scale depend on forces exerted by single molecules.

Ha's work evolved out of an earlier collaboration with Martin Schwartz at the University of Virginia to measure molecular forces using a tension-sensitive fluorescence resonance energy transfer (FRET) sensor. His group calibrated forces corresponding to FRET signals, and the Schwartz group took the biological measurements. "I got kind of unhappy that now the cell biologists are having all the fun," says Ha. He proposed to Wang that he measure FRET at the cell surface, which would be easier than making measurements inside the cell. After a month of applying FRET sensors, Wang came up with the idea of using a tether that could rupture at a known force.

The method works by testing a series of tethers that can tolerate defined tensions before snapping. A receptor must bind its ligand with a force greater than the tolerance of the tether for the cell to snap the tether and detach from the surface. Ha points out that in most single-molecule methods relevant to cell biology, people apply the force, whereas in their system, the cell applies the force. "We are just

"Unfortunately there were many technical problems with intrabodies at first which limited their utility for tracking endogenous proteins, so we set out to make intrabodies that worked better," says Arnold. It took them close to 10 years to optimize the system, but the results made the effort worthwhile. Using intrabodies, Arnold and his team have managed to image the localization and detect the amount of endogenous synaptic proteins in specific neurons of the mouse brain *in vivo*.

To succeed, Arnold teamed up with his next-door USC neighbor Richard Roberts, who had developed the mRNA display method: an *in vitro* method that creates and sifts through trillions of protein sequences for specific functions. Arnold, Roberts and their colleagues also used fibronectin domains as a scaffold for the intrabodies—which are less prone to misfolding and aggregation inside cells than other types of intrabodies, says Arnold.

The last important optimization of the system was to devise a way to match the expression level of the intrabody to that of the target protein inside the cell. The group designed a negative feedback system based on zinc-finger DNA-binding domains and a transcriptional repressor so that when the entire target was bound to the intrabody, the excess intrabody would negatively regulate its own expression. They named the final probes 'FingRs' and made versions targeting the synaptic proteins PSD-95 and Gephyrin.

The group verified that the probes do not interfere with the natural location and function of the proteins and that the neurons—despite bearing brightly colored synapses—were healthy.

Arnold is now collaborating with other groups to apply super-resolution microscopy methods to follow synaptic proteins in the cell in more detail; they plan to study changes in synaptic strength and plasticity. Setting up the system to target other types of proteins will require optimizations, Arnold says, and the transcription control system (as developed) works only for targeting anchored or membrane-bound proteins. But, as he puts it, there are already an awful lot of proteins that fit into this category that are worth following around.

Erika Pastrana

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Gross, G.G. *et al.* Recombinant probes for visualizing endogenous synaptic proteins in living neurons. *Neuron* **78**, 971–985 (2013).

measuring cell behavior, which is much easier to measure than single molecules; and by definition, what we are doing is physiologically relevant," he says.

In creating the tethers, Wang and Ha were inspired by the work of Hermann Gaub and colleagues, which showed that pulling apart two strands of a short DNA fragment from opposite ends requires higher forces than does pulling apart strands from the same end (the former configuration in effect shears the DNA, whereas the latter unzips it). Wang and Ha also designed DNA fragments with seven intermediate attachment points corresponding to a stepwise series of tension tolerances.

The DNA tethers do have some caveats. They eventually fall apart from thermal fluctuations, a factor that limits studies to 5 or 6 hours, and they are only able to measure forces between 10 and 60 picoNewtons, but the researchers are working on new kinds of tethers to push those limits.

Using their tethering method, Wang and Ha found that integrin pulls its ligand in the extracellular matrix with far greater force than is estimated in the literature and that the Notch receptor pulls its ligand relatively weakly. Ha was most surprised by the fact that the same threshold is observed across a number of cell types. "In my talks, I often use the expression 'a quantum of force', and it's as if there is a quantized force value in mechanical signaling," he says. Ligand density does not appear to affect binding thresholds above a level needed for adhesion.

The group is working on measuring forces in a number of contexts, from endocytosis to immune cell activation, and on testing ligands present in different micropatterns. Ha is also interested in studying multiple protein interactions at once and testing cells in clusters. He points out that fluorescently labeled tethers can be imaged to determine which remain intact and which break, and that different ligands can be labeled with unique colors. It should be possible to see how different cells within a cluster sense and respond to tension, as an approximation of what happens in tissues.

Tal Nawy

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Wang, X. & Ha, T. Defining single molecular forces required to activate integrin and Notch signaling. *Science* **340**, 991–994 (2013).