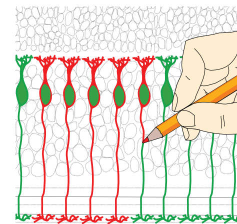
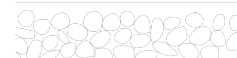
MOLECULAR BIOLOGY

A tool belt for GFP

Nanobodies turn any GFP-expressing cells into targets for *in vivo* functional manipulation.





Nanobodies deliver functional capabilities to GFP-expressing cells. Image courtesy of J. Tang and T. Cherry.

GFP is no longer just a label. It can now be used to activate a virtually limitless set of functional tools in the cell types that it marks.

The transformation of GFP from reporter to reagent was driven by the desire of Connie Cepko at Harvard University and her colleagues to understand the complex mammalian retina. In the thicket of cell populations in the central nervous system, "you're always looking for ways to functionally assay an individual cell type," says Cepko.

As a young graduate student in the Cepko lab, Jonathan Tang identified transgenic mice in which interesting subtypes of retinal interneurons were labeled with GFP. The victory soon resembled a dead end as he questioned whether GFP had uses beyond imaging. Digging in the GFP literature, Tang discovered nanobodies.

Nanobodies are derived from a single-chain protein found in camelid species and have excellent binding specificity in a much smaller, more stable and less aggregation-prone form than those of typical antibody reagents. "Obviously camelids—these are alpacas, llamas, camels—aren't your garden-variety lab creature," says Cepko, but a number of nanobodies that bind GFP have been developed to brighten or dim fluorescence or to target reporter fusion proteins for degradation. Tang's insight was to use GFP as a scaffold that brings pairs of GFP-binding nanobody fusion proteins together to reconstitute a functional biological complex, enabling a host of new activities in the cell.

He attached one nanobody to a DNA-binding domain and another to a transcriptional activation domain and then screened a large number of nanobody pairs for the ability to

MOLECULAR ENGINEERING

ANTIBODIES, MADE TO ORDER

Informed by existing molecular recognition motifs, researchers design effective scaffolds for directed evolution of post-translational modification–specific antibodies.

In many cases, it is reasonably straightforward to obtain or generate antibodies to detect, purify and even determine binding partners for your favorite protein. However, many of the most interesting functional details emerge only when a target is profiled on the basis of the presence or absence of specific post-translational modifications (PTMs). For example, it is estimated that up to 30% of all eukaryotic proteins undergo phosphorylation, a process that can switch proteins 'on' or 'off' or radically alter localization or binding properties. And here, antibodies generally fall short.

"Various academic labs have really dived down into how successful the hybridoma strategy is for making anti-PTM antibodies, and the numbers are shockingly low," explains James Koerber, a postdoc in the lab of James Wells at the University of California at San Francisco. Wells and Koerber have now devised a strategy that draws on the strengths of rational design and directed evolution to dramatically accelerate the generation of phospho-specific antibodies. Their team had been striving to generate PTM-targeting reagents for the better part of a year, but failure with both conventional immunization and phage display–based screens forced them to become creative. "We reasoned that since we're doing this all *in vitro*, perhaps we could design a scaffold with a region in it that's perfectly designed to bind a phosphorylated residue and then use mutagenesis to diversify other regions of the antibody," says Koerber.

As a template, they started with a structural element common to phosphate-binding proteins, known as a 'nest' motif, and then combed through antibody structural data to find candidates with nest-like structures. They identified a mouse antigen-binding fragment (Fab) domain with a loop motif that normally mediates binding to aspartate residues, which are negatively charged like a phosphate group, and preliminary

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simultaneously bind GFP and transcribe a luciferase reporter in human cells. The best pairs gave several-hundred-fold higher expression than background.

The researchers delivered the GFP-dependent transcription system into mouse retinal tissue by electroporating a mix of three plasmids: two bearing the nanobodies and one carrying a responsive promoter fused to a red fluorescent protein or Cre recombinase. They observed sensitive red fluorescence or Cre readouts in GFP-expressing cells, even when GFP was below levels that could be visualized directly. Little to no target gene expression was detected in cells lacking GFP, with occasional leakiness attributed to the common promoters used.

A plethora of mouse lines exist with genetic elements flanked by recombination sites, so this method should allow a variety of genes to be turned on or off by Cre in GFP-positive cells. The researchers also showed GFP-dependent expression of a light-controlled ion channel in sensory neurons, from which they could record electrical activity. "Pairing GFP specificity with optogenetics power was really exciting," says Cepko.

Cepko notes that electroporation works robustly with multiple plasmids, though uptake is not uniform across a tissue. The lab is also developing adeno-associated virus delivery vectors and hopes to generate a series of transgenic mice that will express nanobody reagents ubiquitously.

The system is very flexible. The researchers successfully tested the Gal4, LexA and chemically inducible rTetR DNA-binding domains and also tuned the degree of transcriptional activity using variants of VP16 or an alternative activation domain. They proved portability by using the system in zebrafish to direct several types of GFP-expressing embryonic cells to express a red fluorescent protein.

GFP has long been a powerful imaging tool for biologists. As a scaffold it expands the repertoire of countless existing GFP transgenic lines into the functional realm.

Tal Nawy

RESEARCH PAPERS
Tang, J.C.Y. *et al.* A nanobody-based system using fluorescent proteins as scaffolds for cell-specific gene manipulation. *Cell* **154**, 928–939 (2013).

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experiments indicated that this Fab also bound peptides with phosphorylated serine or threonine residues—albeit very weakly. Koerber decided to try improving on this selectivity via phage-display screens with 'humanized' versions of this Fab containing randomized variants on this loop structure. "It was just that whiff of an inkling of binding that then led him to invest a significant amount of effort," recalls Wells.

The gamble paid off: four rounds of selection yielded scaffolds with dramatically improved affinity and specificity for phosphoserine and phosphothreonine peptides. Structural analysis revealed that the features contributing to these interactions resided within a single complementarity-determining region of the Fab. The researchers therefore created new phage-display libraries in which selected structural residues outside this region were randomized, enabling them to conduct screens for molecules that can distinguish these PTMs in specific sequence contexts. Using this approach, they obtained a set of over 50 antibodies that bound to seven out of their ten candidate peptides with high target- and phospho-specificity, even in the absence of counterselection to weed out antibodies that also bind the unphosphorylated peptides. Several of these molecules achieved affinities comparable or superior to conventionally derived antibodies.

The researchers also obtained a phosphotyrosine-specific scaffold, although its somewhat unusual binding mechanism suggests that this scaffold may lack the target-discrimination capacity of its phosphoserine and phosphothreonine counterparts. However, Wells believes that the structural insights into antibody target recognition that can be obtained through this approach will guide the design of superior antibodies for this particular modification and a broad spectrum of other PTMs. His group is now focused on automating this workflow as part of a larger reagent-development consortium, the Recombinant Antibody Network, with the ultimate goal of assembling a comprehensive 'open-source' toolbox of anti-PTM Fab sequences that can be readily shared and synthesized. "Our priority is to move towards renewable reagents, so that once these things are made they're there forever," says Wells.

Michael Eisenstein

RESEARCH PAPERS
Koerber, J.T. *et al.* Nature-inspired design of motif-specific antibody scaffolds. *Nat. Biotechnol.* doi:10.1038/nbt.2672 (18 August 2013).