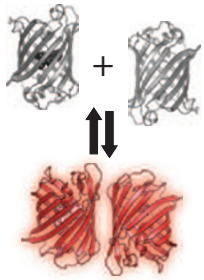
SENSORS AND PROBES

Together we shine

A dimerization-dependent red fluorescent protein provides a new strategy for biosensor design.

Robert Campbell and members of his laboratory at the University of Alberta, Edmonton, focus on engineering fluorescent protein–based indicators for imaging biological processes such as the detection of protein-protein interactions, enzyme activities or changes in small-molecule concentrations.



Schema of ddRFP's mode of action. Image courtesy of S.C. Alford.

Typically, the design of biosensors for these types of applications relies on one of three principles: modulation of fluorescence resonance energy transfer between two fluorescent proteins, changes in fluorescence intensity of a single fluorescent protein or its reconstruction from two split halves. All these strategies have been most commonly applied to build green or yellow fluorescent sensors; their red counterparts have proven much harder to engineer or to get to work.

Making sensors shine in the blood-colored side of the spectrum could offer advantages for *in vivo* imaging and would enable combinatorial experiments using different hue-emitting sensors.

One thing that might explain the difficult 'behavior' of red fluorescent proteins is their tendency to exist as multiunit proteins. This gregarious property is in many cases undesirable from the engineering standpoint and many efforts have aimed at converting them into monomeric or dimeric variants.

To Campbell, however, it became apparent that the oligomeric nature of red fluorescent proteins was precisely what made them bright. So instead of engineering this property out, he figured out a way of exploiting it. "The dimerization-dependent brightness [that

STEM CELLS

THE PLANARIAN PROMETHEUS, QUANTIFIED

Tools to study stem cell function in planarians continue to accumulate. Researchers now image growing stem cell clones in these animals and make quantitative phenotypic measurements *in vivo*.

The ability of some animals to regenerate body parts has been a source of fascination since at least the eighteenth century. In recent years, the power of molecular genetics has been brought to bear on the question of how this is actually achieved. Among the invertebrates, the planarian *Schmidtea mediterranea* is emerging as perhaps the most robust model for this purpose.

Planaria are flatworms that can rapidly regenerate any body part, which requires the function of a special type of radiation-sensitive stem cell called the neoblast. These cells are distributed throughout the body and are required for regeneration both after injury and during normal homeostasis. Peter Reddien and his colleagues at the Massachusetts Institute of Technology, in work published about a year ago, showed that a single neoblast can give rise, upon transplant, to the entire flatworm. The cells can thus stringently be defined as pluripotent.

In the course of these studies, graduate student Dan Wagner found that sublethal doses of irradiation would yield a few surviving neoblasts throughout the body of the flatworm. Subsequent expansion of these cells yields *in vivo* clonal colonies. When the researchers imaged these growing clones, they observed a linear relationship between the number of dividing, pluripotent cells and of postmitotic, differentiated cells. Furthermore, the relationship appeared to be autonomous to the neoblasts: it holds true irrespective of location within the body. "This observation presented the opportunity to use clones as a method to compare expansion and differentiation from colony to colony, animal to animal and in multiple conditions," explains Reddien.

The radiation dose used to kill off most of the planarian neoblasts turned out to be key. Too low a dose gives colonies that are crowded together and are difficult to reliably

red fluorescent proteins exhibit] suggested to us a new strategy to make [red fluorescent protein]-based biosensors," he explains, one based on two dimly fluorescent—or ideally nonfluorescent—monomeric proteins that give rise to a bright dimeric protein when they come together.

To achieve this, graduate student Spencer Alford performed many iterative screens in bacteria searching for the right red fluorescent protein pair that would exclusively form heterodimers, shine brightly when bound together and remain dim or nonfluorescent when alone. Another critical factor was that the affinity of these two proteins for each other would need to be relatively low, so as to not favor their complete association when expressed in cells.

"The hardest part of all of this was deciding on the right compromise," says Campbell. After some years of optimization and endless mutational tweaking, the group settled on a red fluorescent protein pair, dimerization-dependent (dd)RFP, that exhibited a tenfold increase in fluorescence upon dimerization and had a dissociation constant (K_d) of 33 micromolar. Although a satisfactory compromise for the time being, increasing ddRFP's K_d will be one of the group's major goals in the future.

Alford, Campbell and their colleagues used ddRFP to make red-emitting versions of a couple of existing protein-protein interaction sensors such as one that responds to rapamycin and a split cameleon-based calcium biosensor. The group also built a caspase sensor in which the activity of the enzyme made the two fluorescent partners dissociate.

It turns out that ddRFP is made from one fluorescent protein monomer that has a preassembled chromophore buried in its core and a second protein that has lost its chromophore but whose main job is to stabilize and increase the brightness of the first one.

This type of empirical process often involved in protein engineering continues to surprise and excite Campbell: "My lab is 100% protein engineering, and we rely on collaborators to then use these tools for biological applications."

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RESEARCH PAPERS
Alford, S.C. *et al.* A fluorogenic red fluorescent protein heterodimer. *Chem Biol.* **19**, 353–360 (2012).

image; too high a dose kills the flatworm or yields too few cells to analyze statistically. The researchers typically use irradiated flatworms with three or fewer starting neoblasts.

Defining three simple phenotypes—colony loss, failed proliferation and failed differentiation—they initially examined genes with already reported functions in regeneration in the flatworm. They irradiated the flatworms, allowed the few remaining neoblasts to form colonies either in the presence or absence of short interfering RNA for genes of interest, then imaged colony phenotypes 1 or 2 weeks later and quantified the numbers of proliferating and differentiating cells. They observed phenotypes consistent with the known functions of the tested genes (in neoblast self-renewal and differentiation).

Encouraged, they went on to use the *in vivo* colony assay to study 28 irradiation-sensitive genes they had previously identified by expression profiling, which are candidates for involvement in some aspect of neoblast function. Sixteen of these genes, including a transcription factor (a class of gene that has not been implicated in neoblast function before), showed perturbation of colony growth when knocked down. Several of these genes they also confirmed as functioning in normal homeostasis.

Although it is a relatively time-intensive method and therefore more suited for screening a list of already defined candidates, *in vivo* measurement of clonal neoblast outgrowth is likely to be a powerful way to study gene function in planarians. In particular, Reddien emphasizes the rapidity with which colonies grow as well as the synchronicity of the assayed cells. "Often when you study stem-cell phenotypes, things are unfolding in a complex way, it's easy to miss the primary defect by studying the whole system at some late time point after perturbing gene function. With the colonies, we've got the process stripped down to a short time window; every gene can be assessed in the same time course, and we know roughly what happened from start to finish."

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RESEARCH PAPERS
Wagner, D.E. *et al.* Genetic regulators of a pluripotent adult stem cell system in planarians identified by RNAi and clonal analysis. *Cell Stem Cell* **10**, 299–311 (2012).