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MUTAGENESIS

A better way to pick a plum

Working with naturally mutation-prone cell lines could spare researchers a lot of the bother involved in directed evolution studies; Roger Tsien and colleagues demonstrate how letting cells do all the grunt work of sequence modification allowed the group to develop mPlum, a robust and substantially red-shifted red fluorescent protein (RFP) variant.

'Directed evolution' is among the hottest buzzwords in molecular biology, referring to the increasingly popular strategy of performing iterative cycles of introducing variations into a molecular library, then applying some sort of selective pressure to identify changes that maximize a specific property of the molecule of interest. With such strategies, it becomes relatively simple to develop catalytic RNAs, highly specialized proteins or optimized DNA sequences with a speed and efficiency that would be simply unrealistic with a conventional trial-and-error approach.

Roger Tsien of the University of California San Diego is widely recognized as a leader in the study of fluorescent proteins for cell biology research. Directed evolution techniques are well suited to the development of such reagents, as Tsien's group recently demonstrated in an article announcing the production of more than a half-dozen new monomeric orange, red and yellow fluorescent proteins (Shaner *et al.*, 2004). However, when conducted *in vitro*—as such experiments typically are—the evolution process can be a laborious one, requiring several stages of DNA manipulation, transfection and screening. In another recent study from Tsien's group (Wang *et al.*, 2004), he presents a new alternative that promises to accelerate and simplify this powerful method.

The technique is based on a naturally occurring process called somatic hypermutation (SHM). The foundation of the mammalian immune system is the ability to generate recognition molecules with strong specificity for a broad variety of potential antigens. The first stage of this process is V(D)J recombination, in which gene segments are shuffled in different combinations to produce a wide array of T-cell receptor and immunoglobulin molecules. The affinity-selection process is further refined for antibodies via SHM, wherein an endogenous B lymphocyte nuclease introduces double-strand breaks at various locations in the genome—typically at sites of highly active transcription—that are then repaired by an error-prone polymerase. This process results in the widespread introduction of sequence changes; cells producing higher-affinity antibodies proliferate, ensuring selection for alterations that optimize binding.

To test this system as an experimental approach, Tsien's group attempted to develop a red-shifted variant of RFP. A monomeric RFP (mRFP1.2) was cloned into an inducible retroviral expression vector and transduced into a B-cell lymphoma line known to undergo constitutive hypermutation in culture. Cells were subjected to several rounds of selection, consisting of induction of RFP expression followed by fluorescence-assisted cell sorting (FACS) to separate cells expressing variants with a high intensity of fluorescence at a red-shifted wavelength. After 23 rounds of selection, the Tsien group isolated one variant, which they called mPlum, that showed a peak emission wavelength of 649 nm, 37 nm longer than mRFP1.2 (**Fig. 1**) and 12 nm longer than the furthest-red emitter identified so far. Although its quantum yield was slightly diminished relative to mRFP1.2, mPlum proved to be robust in terms of elevated resistance to photobleaching, suggesting that the selection process had also favored highly photostable variants.

Although SHM is largely localized to variable immunoglobulin genes (IgV) in B cells, Tsien's team observed RFP mutation even in cell lines where integration took place outside these loci. Nonetheless, mutagenesis was most effective when the retrovirus was integrated into an IgV locus, and by the 23rd round of selection this was the only type of integration event detected. As such, the authors indicate that a more targeted approach for delivering target genes to these sites could further optimize the SHM process.

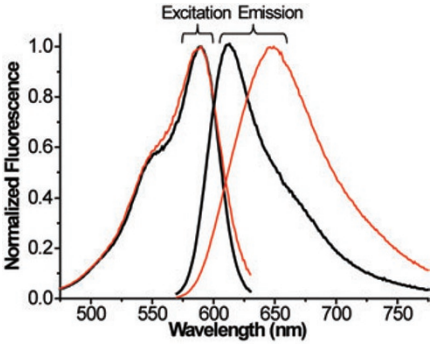

Figure 1 | A comparison of the fluorescence spectra of mRFP1.2 (black) and mPlum (red), revealing the strong red-shift of mPlum emission.

Attempts to replicate their mPlum success story by using random *in vitro* mutagenesis or by making targeted alterations based on the structure of mRFP1.2 yielded no comparable mutants. Likewise, when Tsien's team introduced alternative residues at the sites mutated in mPlum, they found that none of the changes led to improvement and that many actually compromised the protein's fluorescence or stability. Together, these findings indicate that SHM-driven evolution studies may offer a level of optimization beyond that seen in other commonly used strategies. Tsien concludes that "SHM should provide a general strategy to iteratively accumulate multiple desirable mutations in many other proteins whose function can be robustly assessed by high-throughput selections and screens that leave the desired cells alive."

Michael Eisenstein

RESEARCH PAPERS

Wang, L. *et al.* Evolution of new nonantibody proteins via iterative somatic hypermutation. *Proc. Natl. Acad. Sci. USA* **101**, 16745–16749 (2004).

Shaner, N.C. *et al.* Improved monomeric red, orange and yellow fluorescent proteins derived from *Discosoma* sp. red fluorescent protein. *Nat. Biotechnol.* **22**, 1567–1572 (2004).