High-throughput screening of fluorescent probes for *in vivo* imaging

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***Short Abstract* — Fluorescent proteins (FPs) are commonly used as quantitative reporters of biological structures and events. However, their application *in vivo* is greatly limited by the low brightness and photostability of the current generation of FPs. It is therefore imperative to engineer brighter and more photostable FPs. FP properties measured in bacteria or *in vitro* often do not extend to their performance in mammalian cells. Moreover, the traditional approach to engineer new fluorescent proteins via directed evolution in *Escherichia coli* is low-throughput. To address these issues, we developed an automated microscopy-based FP screening platform. We use *Saccharomyces cerevisiae*, a eukaryotic organism that is a better proxy for mammalian cells than bacteria, to express FP variants at a single cell level. FP variants are imaged and analyzed under a microscope and the desirable variants are recovered for further screening. With our method, we can screen thousands of variants in a well of a 96-well plate in a matter of minutes. We anticipate that this platform will enable rapid development of brighter and more photostable FPs across the color spectrum.**

Keywords* — Fluorescent proteins, High-throughput screening, *Saccharomyces cerevisiae

SINCE the discovery and engineering of Green Fluorescent Protein from *Aequorea victoria* over 15 years ago, fluorescent proteins (FPs) and the tags and sensors derived from them have become ubiquitous imaging tools across biology. FPs are commonly used as reporter proteins to study gene regulatory networks, and have also become integral to genetically encoded sensors such as genetically encoded indicators of voltage and calcium. These sensors allow unprecedented cellular-level resolution of neural activity, but are yet limited in brightness and photostability [5]. Given the wide array of applications, engineering of brighter and more photostable FPs has far-reaching impact. Directed evolution in *Escherichia coli* is the standard approach to develop new fluorescent proteins. Bacteria are used because of their fast growth and low cost of maintenance. Variant libraries of FPs are created by mutagenesis and screened for desired properties, usually by picking colonies on agar plates. There are several limitations to this approach for optimizing FPs for mammalian expression; the properties of FPs which are expressed in bacteria and characterized *in vitro* do not translate directly in mammalian cells. Also, the current method of FP screening is low-throughput making the screening process slow and cumbersome.

To address the limitations of current approaches, we developed an FP screening platform that converts an epifluorescent microscope setup into an automated high-throughput FP screening platform. To facilitate the expression of the FP variants, we chose *Saccharomyces cerevisiae* (yeast) as a 'middle ground' between bacterial and mammalian systems. As eukaryotes, yeast use protein expression and folding machinery more closely resembling those in mammalian cells. Yet, yeast is also cheap and easy to work with it. Also, yeast can overexpress exogenous proteins for *in vitro* characterization [6].

To utilize the yeast system for screening, mutagenesis is performed using degenerate primers to create a library of FP variants housed in vectors designed for expression in yeast. Yeast-transformed cells are plated on a glass bottom plate and imaged under a common fluorescent light microscope set up. Captured images are segmented and analyzed, ranking cells by fluorescent intensity. Top-ranked cells are selected, and the corresponding X and Y locations of the cells are sent to the microscope. The selected cells can then be picked by thin glass pipettes and grown in liquid media. With our method, we managed to screen several hundred thousand of variants in a matter of minutes. This would be equivalent to testing a thousand 96-well plates. We anticipate that this platform will enable rapid development of brighter and more photostable FPs across the color palette.

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