

High throughput instrument to screen fluorescent proteins under two-photon excitation: supplement

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1. Supplemental Methods

1.1. Beam waist measurement

To measure the waist of the beam, a razor blade was fastened to the plate holder on the GIZMO and a power meter was centered below the objective. At various heights set by the Z stage, the power attenuation of the 840 nm laser was recorded as a function of stepping the X stage to move the razor blade across the beam. At each Z height, the beam radius w at $1/e^2$ laser intensity was found by fitting the power as a function of x with the following equation:

$$P(x) = P_0 + \frac{P_{max}}{2} \left(1 - erf \left(\frac{\sqrt{2}(x-x_0)}{w} \right) \right) \quad (S1)$$

Here, P is the laser power measured at the X stage position x , P_0 is the background power, P_{max} is the maximum power, x_0 is the position of the center of the beam, and erf is the standard error function.

The beam waist w_0 was found by fitting the beam radius w as a function of the Z stage position z with the Gaussian beam propagation equation (Fig. S3)

$$w(z) = w_0 \left(1 + \left(\frac{(z-z_0)\lambda}{\pi w_0^2} \right)^2 \right)^{\frac{1}{2}} \quad (S2)$$

where z_0 is the position of the beam waist and λ is the laser wavelength.

1.2. Comparing different fluorescent proteins or rounds of evolution

The two-photon excited fluorescence of *E. coli* colonies expressing different fluorescent proteins or rounds of evolution was compared across plates. To minimize differences due to variations in colony growth between plates, *E. coli* were transformed with the appropriate DNA at the same time. Each plate was divided in half by inserting a piece of a notecard vertically in the agar. A different transformation was spread onto each plate half with 3 mm glass beads. Each transformation was plated in duplicate. Plates were scanned with the GIZMO the next day. Fig. 3B and Fig. 4A (Fig. S4) show the results from one set of plates that were scanned sequentially. Fig. S5 shows the results from both sets, also scanned sequentially.

1.3. Protein purification

The constitutive bacterial expression vector pNCS encoding the protein was transformed into Stellar Competent *E. coli* (Clontech). The vector contains a His-tag fused to the FP coding region. A culture was grown overnight at 37° in CircleGrow (MP Biomedicals). The resulting fluorescent cell pellet was lysed via sonication in equilibration buffer (50 mM sodium phosphate, 300 mM sodium chloride, 20 mM imidazole; pH 7.4). Proteins were purified using His60 Ni Superflow Resin (Clontech). Protein bound to the resin was washed 2-3 times with

equilibration buffer at least 10 times the volume of resin. Photophysical measurements were made in the elution buffer (50 mM sodium phosphate, 300 mM sodium chloride, 300 mM imidazole; pH 7.4). The excitation wavelengths used in the photophysical measurements lie outside of imidazole's absorption spectrum.

1.4. Two-photon characterization

Two-photon characterization was done as in previous work [1,2], with a modification to the method for measuring the two-photon cross section. A femtosecond tunable InSight DeepSee laser (Spectra-Physics) was directed into a PC1 Spectrofluorimeter (ISS) containing the sample in a 3 mm cuvette. The laser was controlled with a custom LabView program to measure the spectral shape relative to the reference standard Coumarin 540A (Exciton) in DMSO (MilliporeSigma) (the spectral shape of Coumarin 540A from 680 nm to 760 nm was corrected with Prodan in DMSO [3]). A detailed description of this setup can be found in [2].

The two-photon cross sections (σ_2) were measured at excitation wavelengths of 840 nm and 900 nm, relative to fluorescein (MilliporeSigma) in 1 mM NaOH (extinction coefficient (491 nm) = 92,000 M⁻¹cm⁻¹; σ_2 (840 nm) = 12.9 GM; σ_2 (900 nm) = 15.4 GM [4]). To calculate the absolute two-photon cross sections, fluorescence signal as a function of laser power was collected under both two-photon excitation and one-photon excitation conditions. One-photon excitation was done using the 458 nm line of an argon ion laser (IMA101040 ALS, Melles Griot), selected with an interference filter. The fluorescence was collected through a 520 longpass filter (Chroma) and a 770 shortpass filter (Semrock). Collection conditions were identical between excitation conditions. The fluorescence signals as a function of laser power were fit to a parabola or a line for two-photon or one-photon excitation, respectively. The resulting fit coefficients represent a multiplication of 1) relative absorption strength at the excitation wavelength, 2) relative concentration, 3) PMT spectral sensitivity parameters, 4) laser excitation parameters, and 5) fluorescence collection efficiency. Factors 2 and 3 are identical under both excitation conditions for each sample, and factors 4 and 5 are identical for every sample. Thus, the known extinction coefficients of the samples at 458 nm and the known σ_2 of fluorescein at the two-photon excitation wavelength were used to calculate the σ_2 of the FP.

1.5. Fluorescence quantum yield, extinction coefficients, and fluorescence spectra

The fluorescence quantum yields were measured with an integrating sphere spectrophotometer (Quantaaurus-QY, Hamamatsu). The reference (buffer-only) measurement was done in the same cuvette as the sample measurement. The extinction coefficients were measured by scanning the one-photon absorption spectra with the Lambda 950 Spectrophotometer (Perkin-Elmer) during stepwise alkaline denaturation with 1 M NaOH. The extinction coefficient of the protein was calculated relative to the known extinction coefficient of the fully denatured form of the chromophore, 44,100 M⁻¹cm⁻¹ [5]. Fluorescence spectra were collected with a CLARIOstar plate reader (BMG LABTECH) with a bandwidth of 10 nm (Fig. S5B). For the excitation spectra, the emission registration window was set to 565.5/71 nm, and for the emission spectra, the excitation window was set to 414/45 nm.

2. Supplemental Tables and Figures

Table S1. Primers used to create libraries^a

Primer Name	Sequence	Description
pUE-ORF-F	CGACGATGACGATAAGGATCCG	Forward primer to amplify the FP coding region from a pUE vector (also known as pNCS).
pUE-ORF-R	AGCCGGATCAAGCTTCGAATTC	Reverse primer to amplify the FP coding region from a pUE vector.
pUE-vector-F	AAGCTTGATCCGGCTGCTAACA	Forward primer to amplify the pUE vector. Contains a 15 bp 5' overlap with pUE-ORF-R.
pUE-vector-R	TTATCGTCATCGTCGTACAGAT	Reverse primer to amplify the pUE vector. Contains a 15 bp 5' overlap with pUE-ORF-F.

^aSee Methods Section 2.3. in the main text.

Table S2. MATLAB programs for hardware control and data analysis^a

Name	Purpose
NorthernLights	Main instrument software for hardware control. Needs a legacy version of ScanImage to run properly (available from Vidrio Technologies).
NorthernLightsGui	Encodes the GUI to run the GIZMO.
NorthernLightsDataViewer	Encodes the GUI to view the plate and select colonies based on their indices provided by the function summarizeScans.
fixScannerOffsets	Supplementary function to align the GUI plate image to the stage transform.
recordScanParameters	Supplementary function to record the scan parameters in a .csv file before scanning all colonies on a plate.
summarizeScans	Data analysis function to summarize the colony scan data from a plate and identify the brightest colonies.
summarizeSequences	Data analysis function to summarize the sequencing data of the top colonies from a round of evolution and identify unique mutants.

^aPublicly available at github.com/rosanamolina/gizmo-paper.

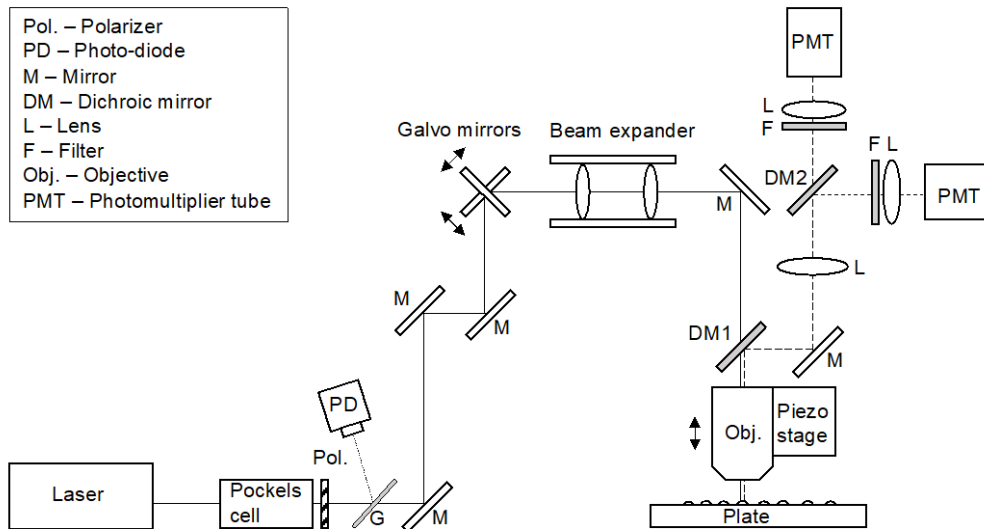


Fig. S1. **Optical path of the two-photon detection arm.** The excitation path of the laser is shown with a solid line, and the emission path of the fluorescence is shown with a dashed line.

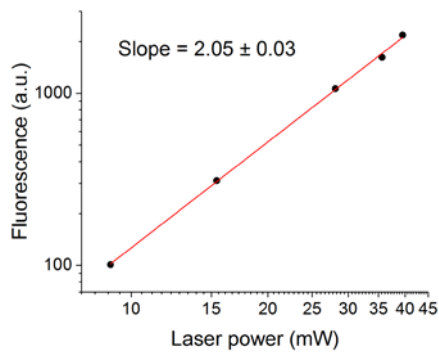


Fig. S2. **Quadratic power dependence of fluorescence signal.** The maximum fluorescence signal measured by the GIZMO of an *E. coli* colony expressing Rosmarinus was quadratically dependent on the laser power measured immediately after the Pockels cell.

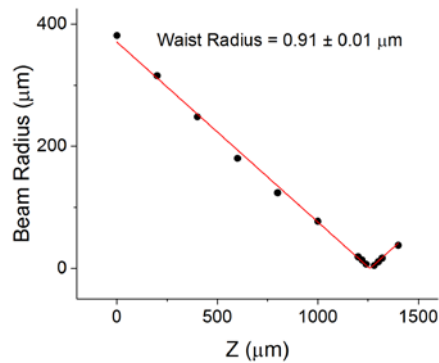


Fig. S3. **Beam waist measurement.** The beam radius at $1/e^2$ laser intensity was determined at various Z heights with the knife edge method. The red line shows the Gaussian beam propagation equation fit for the laser wavelength 840 nm (Equation S2).

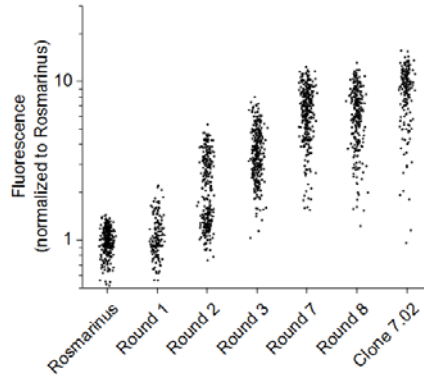


Fig. S4. **Directed evolution experiment to evolve Rosmarinus.** The data here are the same as Fig. 4A of the main text but plotted in log scale.

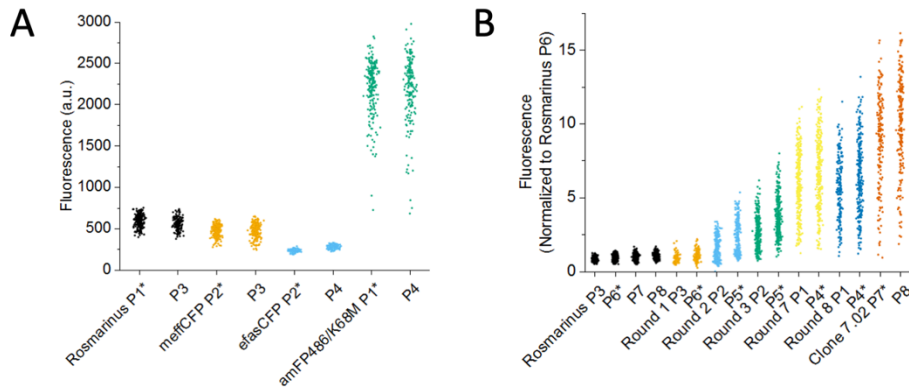


Fig. S5. **Extended data for Fig. 3B (A) and Fig. 4A (B).** Each particular FP/evolution round is grouped by color and was plated in duplicate onto one half of a plate (see Supplemental Methods Section 1.2). The corresponding plate for each set of data points is denoted by P#, where # is the order that the plate was scanned. Asterisks denote data shown in Fig. 3B and Fig. 4A (Fig. S4) in the main text.

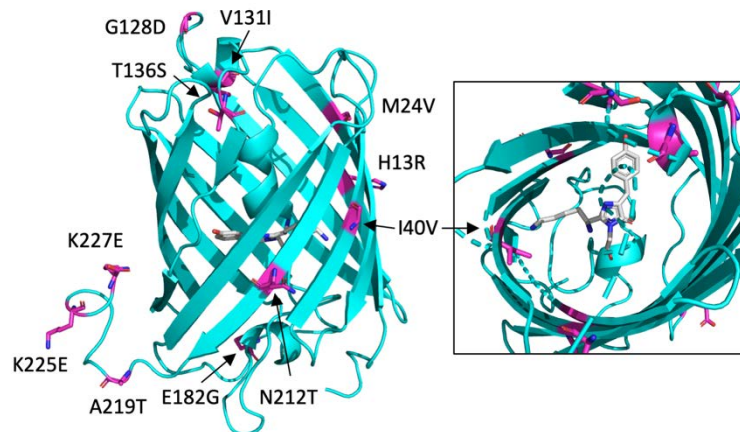


Fig. S6. **Locations of Clone 7.02 mutations in Rosmarinus.** Shown is the crystal structure of Rosmarinus predicted with I-TASSER [6–8]. Highlighted in magenta are the residues that were mutated in Clone 7.02. The corresponding mutations are included in the labels. The inset shows how the I40 side chain points into the barrel (several residues were hidden to show the chromophore).

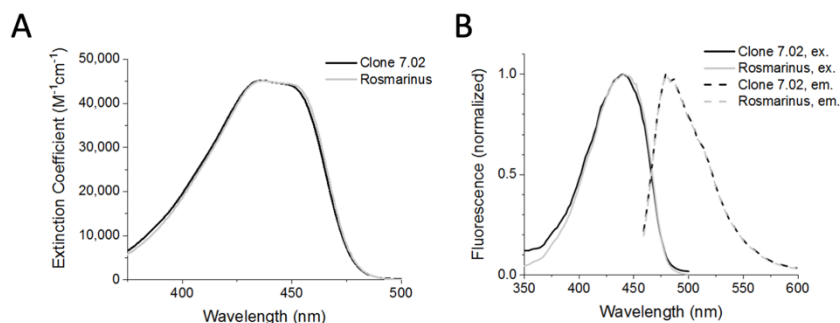


Fig. S7. **Rosmarinus and Clone 7.02 have identical one-photon properties.** A) One-photon absorbance spectra scaled to the extinction coefficient. B) Excitation (ex.) and emission (em.) normalized fluorescence spectra.

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