A brief protocol to observe protein dimerization in bacteria via fluorescence microscopy

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Protein-protein interactions are the driving force behind a vast and diverse set of cellular functions. Fluorescence resonance energy transfer (FRET), the proximity-dependent energy transfer between a donor and acceptor fluorophore, provides real-time measurements of protein-protein interactions in living cells[1]. Alternatively, energy transfer can occur between identical fluorophores across the overlap between excitation and emission spectra (homoFRET). In the case of fluorescent proteins with relatively slow molecular rotation speed, this interaction decreases fluorescence polarization and has been used to measure GFP oligomerization[2], kinase activity[3], and bacterial response regulator dimerization[4] in living cells. This document is intended to be a practical guide to measure fluorescence polarization via microscopy, specifically the difference between monomeric and dimeric mNeonGreen[5] (mNG) expressed in *E. coli*. For further reading on fluorescence polarization microscopy, see references 6-7.

Approach

To measure fluorescence polarization (FP), the sample needs to be excited by linearly polarized light. The subsequent emission light needs to be separated into the parallel and perpendicular components via an image splitter or similar device (**Fig. 1A**). A polarizing filter can easily be inserted into the excitation cup of the microscope, but determining the orientations of the two emission-side polarizing filters is not immediately obvious. Observed fluorescence will be maximal if the emission-side polarizing filter is aligned with the excitation-side polarizing filter (parallel), and minimal when the polarizers are perpendicular. By measuring fluorescence while incrementally rotating the emission-side polarizers across 180 degrees, the parallel and perpendicular orientations will be revealed. (**Fig. 1B**).

Here we measure the fluorescence of *E. coli* expressing monomeric mNG or dimeric mNG (**Fig. 1C**). For simple analysis, the total pixel intensity of each image after background subtraction is summed, representing the total fluorescence of the cells in the frame (**Fig. 1D**). After plotting this total fluorescence quantity versus emission-side polarizer orientation, a sinusoidal relationship is apparent as either filter aligns and de-aligns with the excitation-side polarizer. Finally, one filter is set in the parallel position and the other in the perpendicular position before acquiring test images. Using **Eqn. 1**, we clearly observe that mNG dimerization decreases FP (**Fig. 1F**). If desired, this approach can be refined to obtain single-cell measurements.

$$FP = \frac{I_{parallel} - I_{perpendicular}}{I_{parallel} - I_{perpendicular}} \qquad \textbf{Eqn. 1}$$

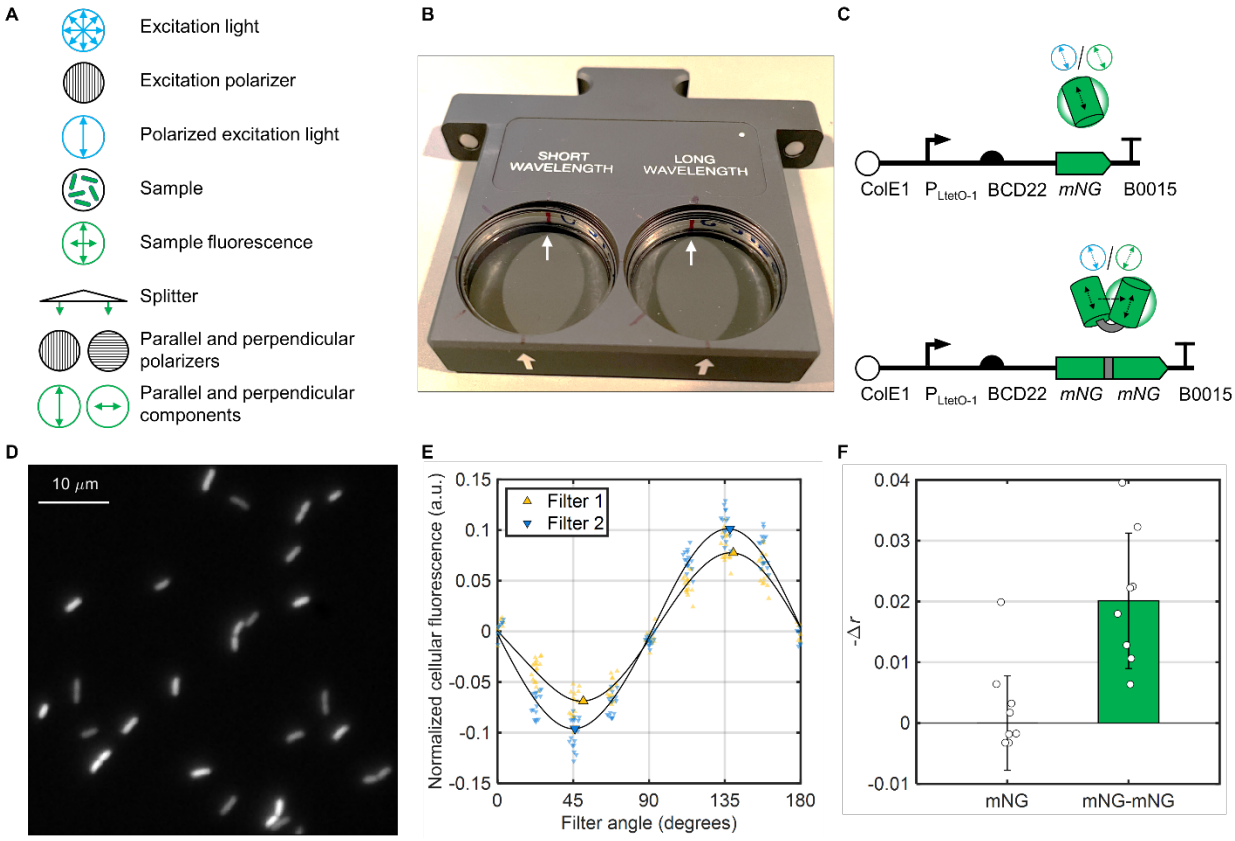

Figure 1: Observing mNG dimerization in *E. coli* via fluorescence polarization. A. Light path schematic for measuring fluorescence polarization via microscopy **B.** Filter holder for image-splitting device. Overlaid white arrows indicate polarizer orientation, marked by the red stripe. **C.** Plasmid diagrams for strains expressing monomeric (high polarization) or dimeric (low polarization) mNG. **D.** Representative image field of cells expressing mNG. **E**. Modulation of total pixel intensity for either filter channel during manual rotation across 180 degrees. Minima occur at 51 and 46 degrees for filter 1 and 2, respectively. Maxima occur at 140 and 138 degrees for filter 1 and 2, respectively. Lines are fitted to the median of whole-image pixel intensity sums. **F.** Final comparison of fluorescence polarization between monomeric and dimeric mNG. Data points represent single frames containing roughly 50-100 cells.

Detailed Protocol

Positive and negative control strains

E. coli K-12 strain BW28357 (*Δ(araD-araB)567, ΔlacZ4787*(::rrnB-3), λ^-, *Δ(rhaD-rhaB)568, hsdR514*) was transformed with either pRB035 (aTc-inducible mNeonGreen) or pRB121 (aTc-inducible dimeric mNeonGreen). Both plasmids are available on Addgene (https://www.addgene.org/browse/article/28224612/). Strains were stored in LB media + 15% glycerol at -80ºC.

Data processing

Images were analyzed using MATLAB, with code available on the Tabor lab Github (https://github.com/taborlab/PolarizationMicroscopy).

Equipment

- Inverted fluorescence microscope (Ti-E, Nikon)
- Excitation source (BDX module, X-LED1, Excelitas)
- Image splitting device (W-View Gemini, Hamamatsu)
- Filters (470/40 ex., 525/50 em., 59022 bs, from 89021 set, Chroma)
- 3x linear polarizing filters (DP – 100 – VIS, Meadowlark Optics)
- Non-polarizing beamsplitting cube (NB – 100 – VIS, Meadowlark Optics)
- 37ºC shaking incubator

Protocol

1. Incubate cultures of positive and negative control strains in LB media at 37ºC with 250 rpm shaking overnight (12-18hrs).
2. Dilute saturated cultures 1000x into M9 media plus 9 ng/mL aTc (1× M9 salts (Teknova, M1902), 0.4% (wt/vol) glucose (Avantor, 4908-06), 0.2% (wt/vol) casamino acids (EMD Millipore, 2240-500GM), 2 mM MgSO4 (VWR, BDH9246-500G) and 0.1 mM CaCl2 (Alfa Aesar, L13191), plus aTc- Fisher Scientific, NC9451757 and antibiotics (chloramphenicol- Fisher Scientific, AAB2084114).
3. Incubate 3-4 hours at 37ºC with 250 rpm shaking until cultures reach the middle of exponential phase.
4. Transfer 0.2 µL culture onto solidified 1 mm thickness, 1% agarose pads.
5. Collect fluorescence images of strains at a variety of polarizer orientations across 180º. In this case, we imaged eight fields per sample with nine filter orientations (22.5º filter rotation between images).
6. After identifying parallel and perpendicular filter orientations as in **Fig. 1E**, set one polarizer in the parallel orientation and the other in the perpendicular orientation.
7. Image samples and calculate the differences in fluorescence polarization.

References

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