**Detailed protocol for detecting RR dimerization via homoFRET (NarX/mNG-NarL)**

This protocol details the process for measuring RR dimerization in living *E. coli* cultures via homoFRET. As an example, we describe and experiment to study the effect of SHK expression level on RR dimerization in the NarX/mNG-NarL system.

The protocol can be customized for different experimental conditions or TCSs, assuming dimerization of the RR produces a measurable homoFRET signal.

Equipment required:

* 37C shaking incubator
* Plate reader with fluorescence polarization capability
* Microscope with fluorescence polarization capability (optional)

Solutions:

* LB media
* M9 minimal media
* M9 minimal media + 50mM sodium nitrate

Day 1

1. Inoculate 3 mL LB + relevant antibiotics (here spectinomycin and chloramphenicol) with the desired strain (here *E. coli* BW29658 transformed with pRB117.2 and pKD317.8).
2. Incubate the culture overnight in a 37C shaking incubator (250 rpm) to reach saturation (14-18hrs).

Day 2

1. Prepare experimental media: M9 + antibiotics + relevant inducers (anhydrotetracycline (aTc) for mNG-NarL induction; isopropyl-β-D-1-thiogalactopyranoside (IPTG) for NarX induction).
   1. For example, 2.5 mL M9 + 5 ng/mL aTc and 0 mM IPTG.
   2. Generally, 4 ng/mL aTc induces enough mNG-NarL for a high-quality fluorescence signal while 12 ng/mL induces a maximal amount of mNG-NarL without adversely affecting cell growth. Leaky expression of NarX (0 mM IPTG) is sufficient for nitrate-induced mNG-NarL phosphorylation while 0.5 mM IPTG induces the maximal amount of NarX. Altered inducer concentrations may be required to cover the full induction range of either component if there are variations in expression cassette, plasmid architecture, and culturing conditions.
2. Dilute the overnight culture into the experimental media by a factor of 1:1000. The volume used here is based on the number of wells (experimental conditions) used.
   1. For this example, 5 wells will be used so the volume of diluted culture should be 1 mL.
3. Separate the diluted culture into 180 µL volumes in different wells of a 96-well plate. This is when experimental conditions (e.g. different induction conditions) are applied.
   1. For example, add 0, 4, 8, 32, and 128 µM IPTG to the 5 wells.
4. Place the plate the in 37C incubator for roughly 4 hours, until the cells reach exponential phase (OD600 ~ 0.3-0.6).

*Plate reader measurements*

1. Pre-heat the plate reader to 37C.
2. Prime the liquid handler with 1.050 mL M9 + 50 mM nitrate, ensuring that > 20 µL per sample remain in the reservoir.
3. Generate acquisition protocol consisting of pre- and post- ligand addition timepoints with shaking (1 second, double orbital, 282 cpm, 3mm amplitude) before each step. Fluorescence polarization (ex: 485/20 nm; em: 528/20 nm, 40 measurements/data point, gain 57) of all samples can be measured every 4 minutes. Alternatively, faster acquisition periods are possible if only a single well is analyzed.
   1. For example, collect 2 data points for all samples, dispense 20 µL M9 + 50 mM nitrate to all wells, shake for 1 second, then collect 16 more data points for all samples (totaling 68 minutes).
   2. Alternatively, ligand can be added manually via pipette as long as the timing between pausing, ligand addition, and resuming data collection is accounted for.
4. Export data to Excel

*Analyzing plate reader data*

1. Load the Excel data file and extract raw parallel and perpendicular fluorescence channel measurements.
2. Compute anisotropy for all data points (**Eqn. 1**).
3. Perform the relevant anisotropy corrections as discussed in **SI text 1**.
4. Fit and analyze anisotropy timecourses.

*Polarization microscopy measurements*

1. If measuring cells on an agar pad, prepare a solution of 1% agarose in PBS, microwave for twenty seconds, and set between two glass slides separated by 1mm glass slides for at least one hour.
2. Load 0.2 µL cell culture onto the surface of the pad. Let dry the place a coverslip over the surface.
3. If utilizing a microfluidic device (such as CellASIC ONIX), prepare the device according to the manufacturer and generate a time-lapse microscopy protocol.
4. Image the parallel and perpendicular fluorescence emission images of the cells by utilizing an image splitting module (W-View, Hamamatsu) equipped with parallel and perpendicular linear polarizers (Meadowlark) relative to an additional polarizer in the excitation cup of the microscope.
5. Export images for analysis

*Analyzing polarization microscopy images*

1. Perform cell-segmentation to acquire the mean parallel and perpendicular fluorescence intensity values for each individual cell.
2. Calculate anisotropy as in **Eqn. 1**.