Supplementary Note | Characterization of the dual-selection system

To evolve a new signaling specificity, the selection scheme must identify variants that respond to the new chemical signal (positive selection) but also have decreased response to the cognate signal(s) (negative selection). The dual selection system used in this work requires a specific output (as opposed to a lack thereof) for both positive and negative rounds of selection. While the original dual selection system of Yokobayashi and Arnold[1] encodes both the positive (TetA) and negative (Bli) elements on a single selection plasmid, replacement of the repressed promoter with the *lux* promoter did not lead to simultaneous LuxR-mediated expression of both elements. We observed that the amount of basal gene expression of the second gene (*tetA*) in the operon always generated an ON output, irrespective of LuxR activity. Switching the order of the genes and replacing *tetA* with *cat* did not alleviate this problem. In general, promoters that require an activator to recruit RNA polymerase have higher levels of basal gene expression than promoters that are repressed by transcription factors blocking RNA polymerase binding[2]. Instead of trying to tune the output levels of the ON and OFF selection genes simultaneously, we instead separated the components into ON and OFF selection plasmids. This requires that the selection plasmid be inactivated and replaced following each round of selection. However, it also lowers the probability of propagating mutated selection plasmids that confer antibiotic resistance without LuxR, i.e. false positives, from one round to the next.

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repressed promoter with the *lux* promoter did not lead to simultaneous LuxR-mediated expression of both elements. We observed that the amount of basal gene expression of the second gene (*tetA*) in the operon always generated an ON output, irrespective of LuxR activity. Switching the order of the genes and replacing *tetA* with *cat* did not alleviate this problem. Instead of trying to tune the output levels of the ON and OFF selection genes simultaneously, we separated the components into ON and OFF selection plasmids (see Figure 1, Supplementary Figure 4). This requires that the selection plasmid be inactivated and replaced following each round of selection. However, it also lowers the probability of propagating mutated selection plasmids that confer antibiotic resistance without LuxR, i.e. false positives, from one round to the next.

To validate the dual selection module, we selected a plasmid expressing *luxR* (pLuxR) from a background of nonfunctional plasmids. The nonfunctional plasmids either lack the *luxR* gene (pPROLar.A122) or constitutively express both *cat* and *bli* (pBLIPCAT). While pLuxR confers survival with the ON selection plasmid in the presence of 3OC6HSL and survival with the OFF selection plasmid in the absence of 3OC6HSL, cells containing pPROLar.A122 should survive only OFF selection rounds while cells containing pBLIPCAT should survive only ON rounds.

The three plasmids, pLuxR, pPROLar.A122 and pBLIPCAT, were mixed with a 1:500:500 molar ratio and transformed into DH5α cells harboring the ON selection plasmid pluxCAT. DNA isolated from surviving Round 1-ON colonies was digested to inactivate pluxCAT and used to transform cells harboring the OFF selection plasmid,

pluxBLIP. Round 1-OFF cells were recovered, and the DNA was treated as following Round 1-ON and used to initiate a second round of ON/OFF selection.

From each ON and OFF selection, 45 colonies were randomly picked and tested for the presence of pLuxR, pPROLar.A122 or pBLIPCAT by colony PCR. Primers that bind adjacent to the inserted genes amplified a section of differing length from each of the three plasmids, allowing their identification. Following Round 1-ON, 45 of 45 (100%) colonies picked contained the pBLIPCAT plasmid. Twenty-two of the 45 (49%) colonies from Round 1-OFF selection contained pLuxR. The other 23 colonies contained pPROLar.A122. The increase in the fraction of pLuxR in the plasmid mix from approximately 1/1001 to 22/45 in the first round of ON/OFF selection is equivalent to a 490-fold enrichment of pLuxR.

Forty-four of 45 colonies contained pLuxR after Round 2-ON. The remaining colony harbored pPROLar.A122. Round 2-OFF returned exactly the same ratio of pLuxR to pPROLar.A122 as the second ON selection because both plasmids confer the same probability of survival during OFF rounds. A third ON round of selection yielded 100% of colonies containing pLuxR.

Methods

For positive selection, circuit plasmids were transformed into DH5α cells harboring the ON selection plasmid (pluxCAT) and plated on LB agar plates containing 100 nM 3OC6HSL, kanamycin and chloramphenicol. For negative selection, circuit plasmids

were transformed into DH5α cells harboring the OFF selection plasmid (pluxBLIP) and plated on LB agar plates containing kanamycin and carbenicillin. Plates were incubated for 18 hours at 37°C. Selected cells were recovered by overlaying liquid LB medium over the agar plates. Plasmid DNA from the cells was recovered by miniprep (QIAGEN). To inactivate the selection plasmids, the DNA was digested with *Apa*LI and purified. The digested DNA was subsequently used to transform the next round of selection.

Colony PCR was performed to identify each of the functional plasmids after each round of selection. The Colony Fast-Screen Kit (Epicentre) was used according to instructions, with two primers: 5-LarSeq2[3] and 3-LuxR+200. Amplification of pLuxR yields a 1100 base pair (bp) fragment, while pPROLar.A122 yields a 400 bp fragment and pBLIPCAT a 1650 bp fragment, allowing the three plasmids to be easily distinguished from one another. Positive controls of each of the three starting plasmids were run simultaneously.

References

1. Yokobayashi, Y. & Arnold, F.H. A dual selection module for directed evolution of genetic circuits. *Natural Computing* **4**, 245-254 (2005).
2. Ptashne, M. Regulation of transcription: from lambda to eukaryotes. *Trends Biochem. Sci.* **30**, 275-279 (2005).
3. Collins, C.H., Arnold, F.H. & Leadbetter, J.R. Directed evolution of *Vibrio fischeri* LuxR for increased sensitivity to a broad spectrum of acyl-homoserine lactones. *Mol. Microbiol.* **55**, 712-723 (2005).