

Supplementary Information

An accessible time-course microscopy assay for steric-blocking peptide-conjugated morpholino activity in *Escherichia coli*

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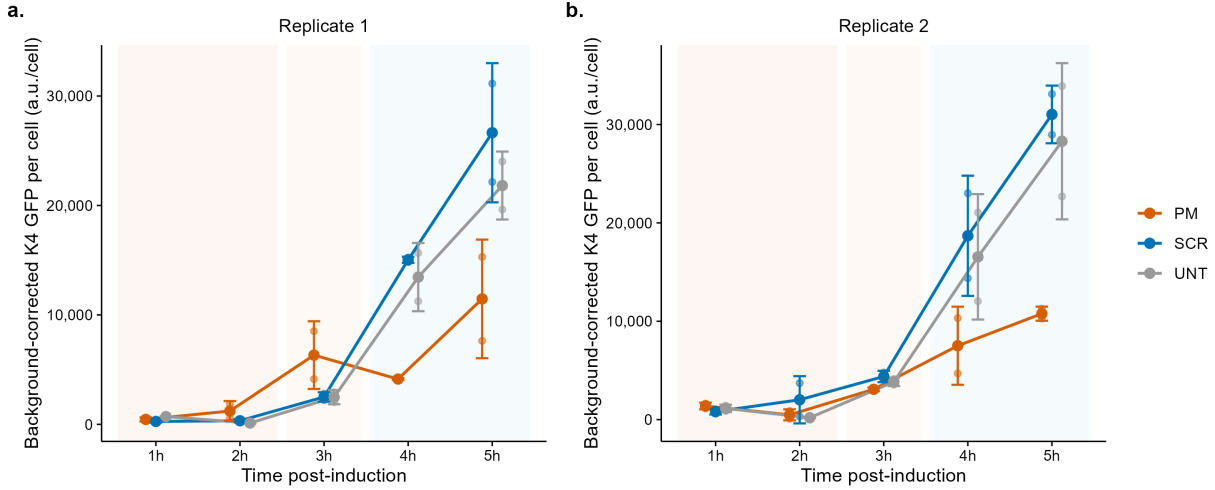
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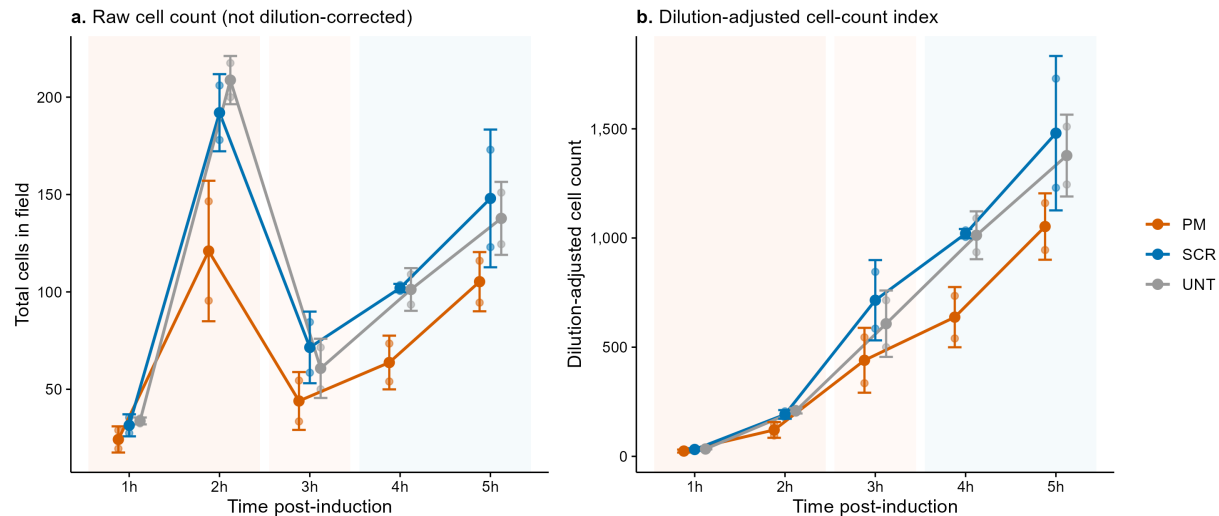
This file contains Supplementary Figures S1–S5.

Metric	PM	SCR
Sequence (5'→ 3')	TTCTCCTTTGCTAGCCAT	TTTGCCAACATTACCGAG
Length (nt)	18	18
GC content	44.4%	44.4%
Predicted T_m against RNA target (°C)	55 °C	44.3 °C against perfect RNA complement
Hairpin ΔG (kcal/mol)	+0.52 / +1.11	+0.36 / +0.78
Hairpin T_m	low (not quantified)	19.9 °C / 9.9 °C
Strongest self-dimer ΔG (kcal/mol)	-10.4	-3.9
Longest self-dimer stretch (bp)	6	3
Homopolymer / palindrome / GC-block screen	Clean: no long runs, no strong palindromes, no extreme GC blocks	Clean: longest run TTT (3 nt); no palindromes; GC evenly distributed

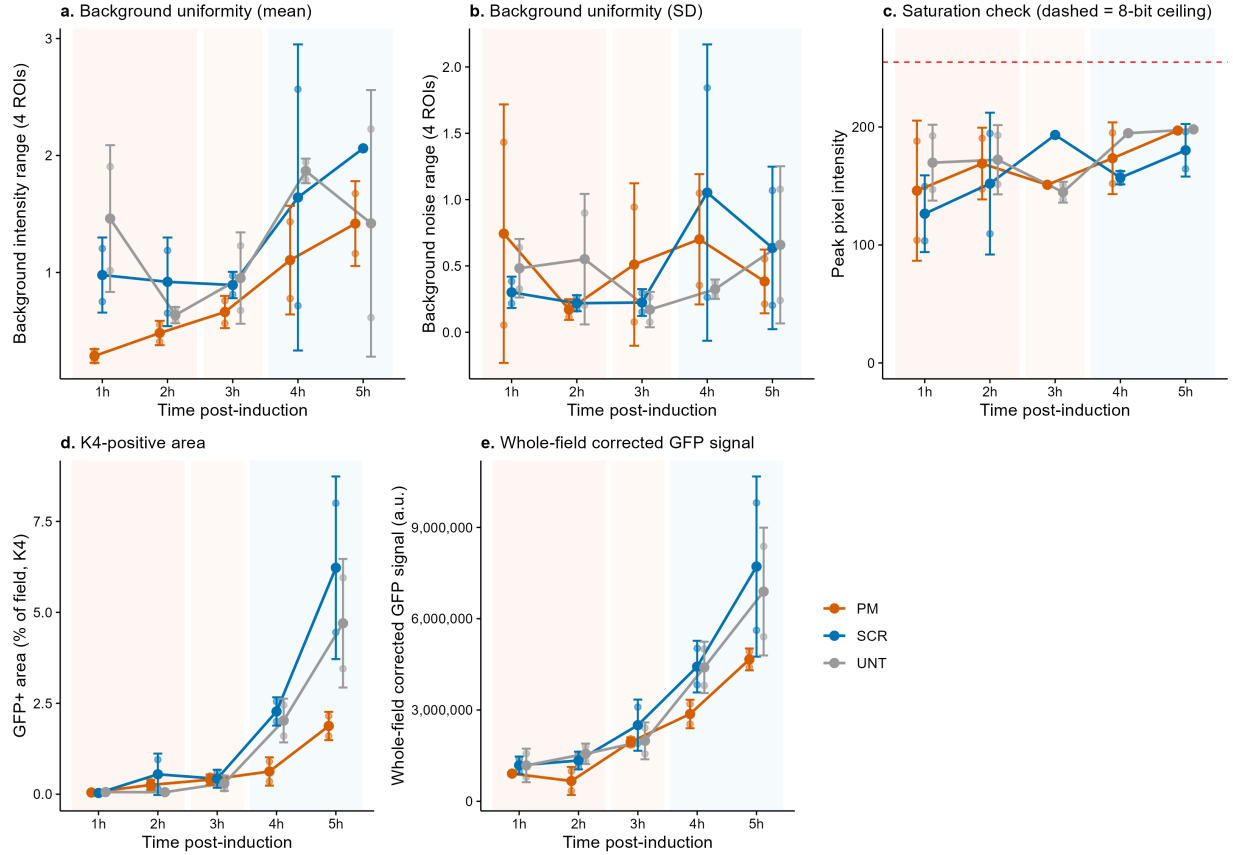
Supplementary Figure S1. In silico sequence-screening metrics for PM and SCR. Sequence, length, GC content, proxy melting temperature, hairpin ΔG and T_m , strongest self-dimer ΔG and longest self-dimer stretch, and sequence-composition screen results for the GFP-targeting PPMO (PM) and the scrambled-sequence control PPMO (SCR). Thermodynamic values were calculated using IDT OligoAnalyzer with nucleic-acid parameter sets and are sequence-based proxy calculations rather than quantitative predictions of PMO behavior.



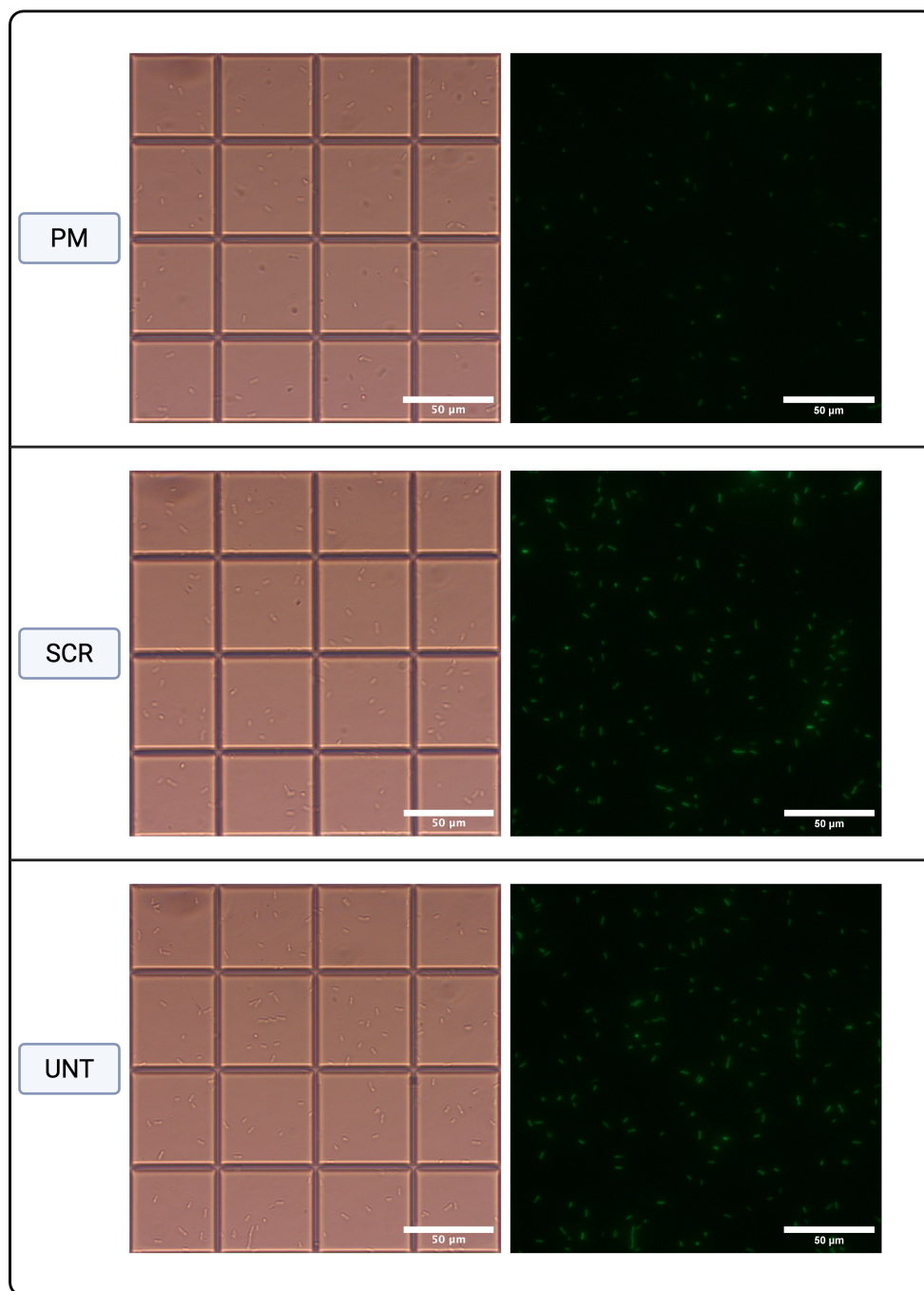
Supplementary Figure S2. Replicate-specific time courses of background-corrected K4 GFP per cell. (a) Replicate culture 1. (b) Replicate culture 2. Large points show the mean of the two image fields acquired per well at each time point; small points show individual field values; error bars represent ± 1 standard deviation (s.d.) across the two fields and reflect within-replicate technical spread rather than biological variation, and are shown here only to document within-replicate consistency. Shaded regions mark the early time points (1–2 h), the 3 h time point, and the 4–5 h window used for endpoint comparisons. a.u., arbitrary units.



Supplementary Figure S3. Cell counts across the time course. (a) Raw cell counts per imaged field. The drop at 3 h reflects the 1:10 dilution introduced at that time point rather than a change in culture density. (b) Dilution-adjusted cell-count index, in which raw counts from the diluted 3, 4, and 5 h samples were multiplied by 10 to give values comparable in scale to the undiluted 1 and 2 h samples. Error bars represent ± 1 s.d. across $n = 2$ biological replicate means, with individual replicate means overlaid.



Supplementary Figure S4. Image-quality controls across the time course. (a) Range in background mean intensity across the four corner ROIs used for local background estimation. (b) Range in background standard deviation across the same four ROIs. (c) Peak pixel intensity within the main ROI; the dashed line marks the 8-bit saturation ceiling of 255. (d) K4-positive area as a percentage of the imaged field. (e) Whole-field background-corrected GFP signal, summed across the main ROI before per-cell normalization. Error bars represent ± 1 s.d. across $n = 2$ biological replicate means, with individual replicate means overlaid.



Supplementary Figure S5. Matched brightfield and GFP-channel images at 5 h post-induction. Brightfield (left) and GFP-channel (right) images from the same hemocytometer fields for PM, SCR, and untreated (UNT) +arabinose wells, displayed to confirm comparable bacterial density and morphology across conditions. Wells contained 14.3 μM PM or SCR where applicable. GFP images use identical brightness and contrast settings across all three conditions. Scale bars, 50 μm . Created with BioRender.com.