Supplementary materials

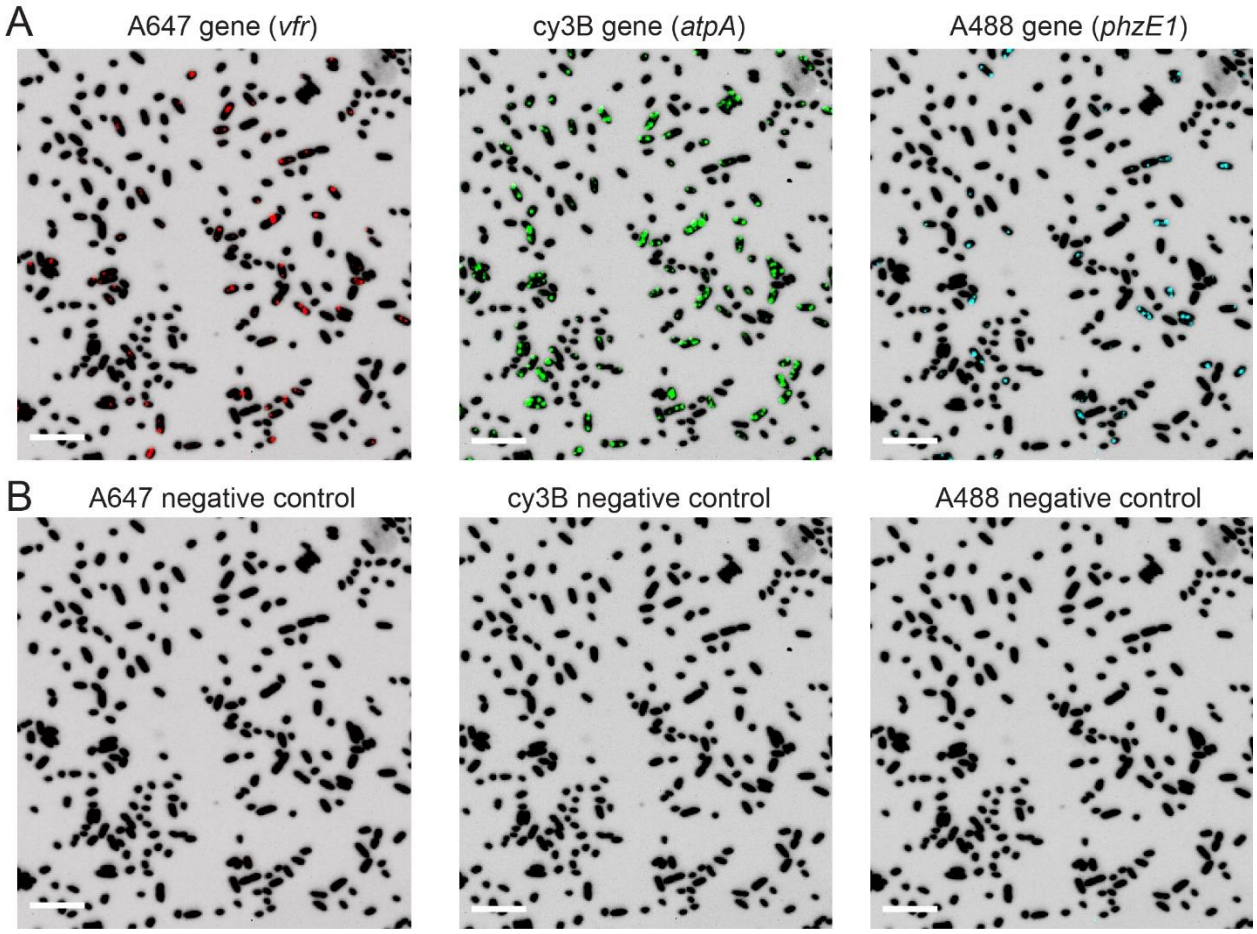

Fig. S1.

Negative control genes estimate the false positive rate. (A) Examples of positive signal for genes labeled with one of the three fluorophores used in this study A647 (red), cy3B (green), and A488 (cyan). For context, the mRNA-FISH fluorescence is shown over DAPI (dark silhouette). (B) Same regions as in panel A, but showing the raw fluorescence of the negative control genes for each fluorophore. For direct comparison, the intensity range is identical between positive and negative panels in A-B. Scale bar represents 5µm.

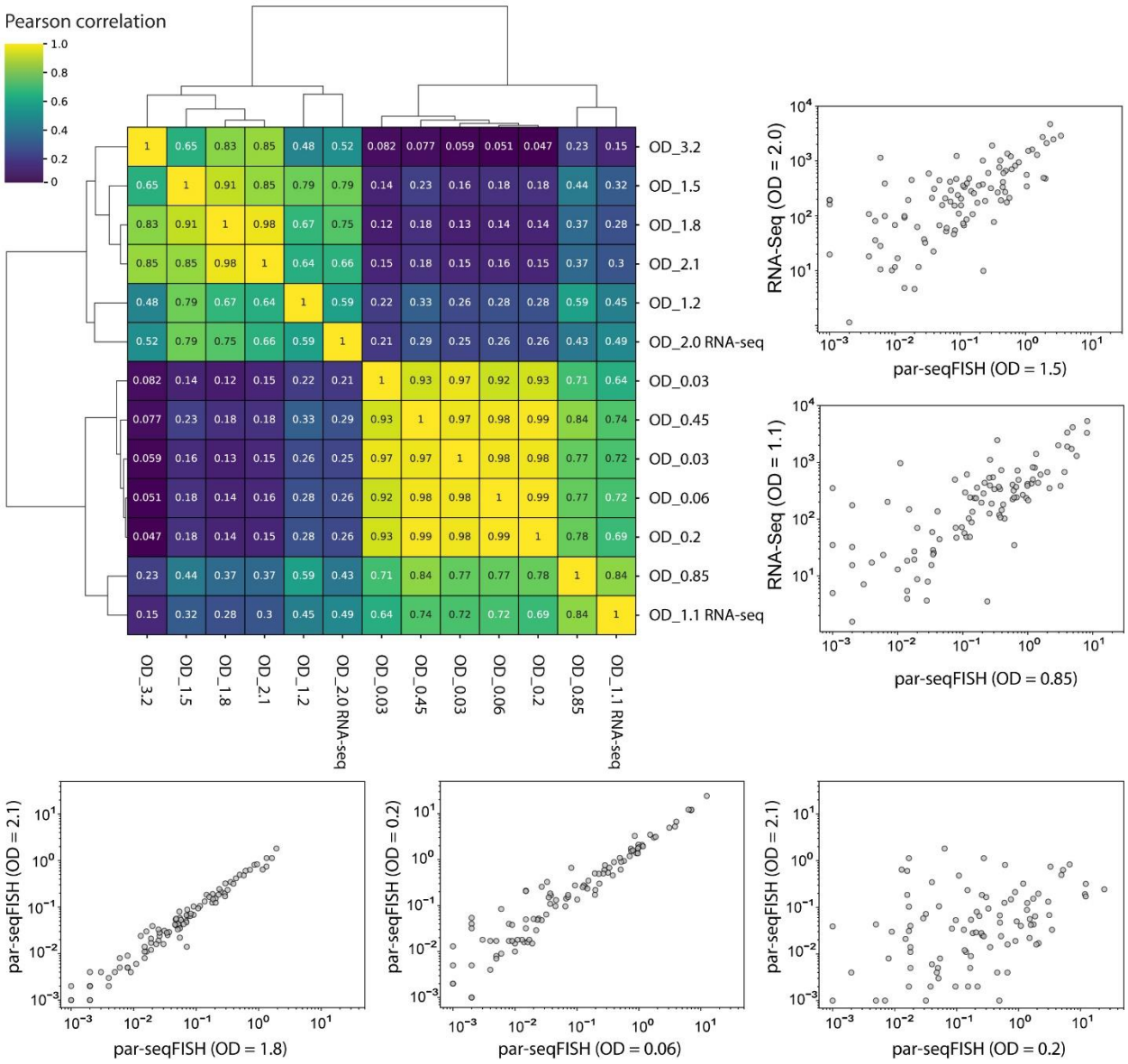

Fig. S2.

Correlation analysis between seqFISH and RNA-Seq. A clustered correlation matrix was calculated using the average par-seqFISH expression measurement for each time-point in the LB growth curve and the normalized RNA-Seq expression, reads per kilobase per million (RPKM), from a previously published study performed under similar conditions (*51*). The conditions are noted to the side along with their respective OD_{600} values (e.g., LB_0.2 represent cells collected when the OD_{600} of the LB culture reached 0.2). The Pearson correlation is shown as a color map and indicated in the figure. Scatter plots showing RNA-seq and par-seqFISH comparisons are displayed on the right. RNA-seq axis shows the RPKM and the par-seqFISH axis shows the average mRNA number per cell. Scatters comparing different time points in the par-seqFISH experiment are shown below. The X and Y axes show the average mRNA number per cell.

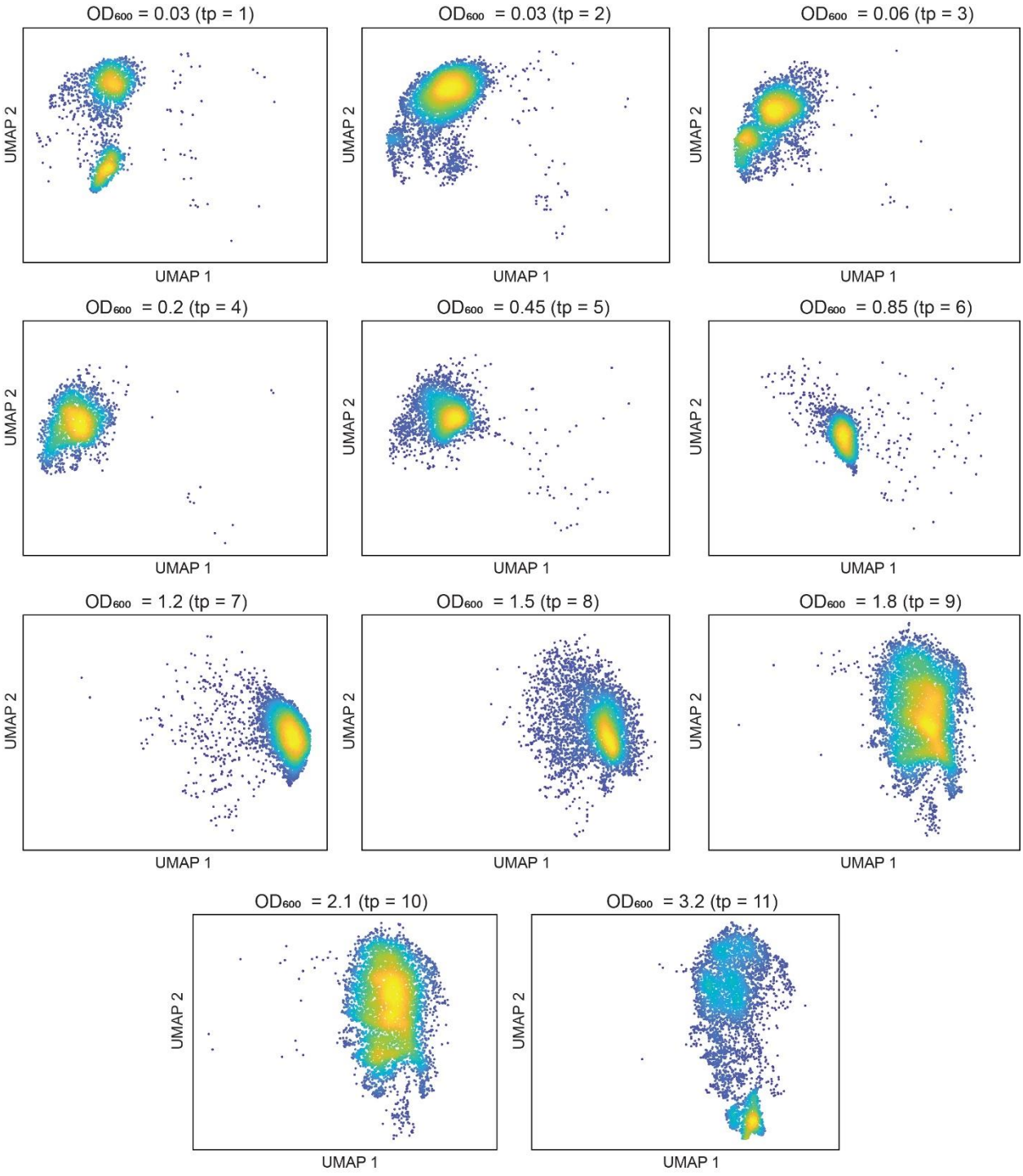

Fig. S3.

Single-cell dispersions in UMAP space for each growth curve time point. A UMAP density plot of cells belonging to specific time points. The OD_{600} values and the number of the time points are shown over each plot. Color intensity represents cell density.

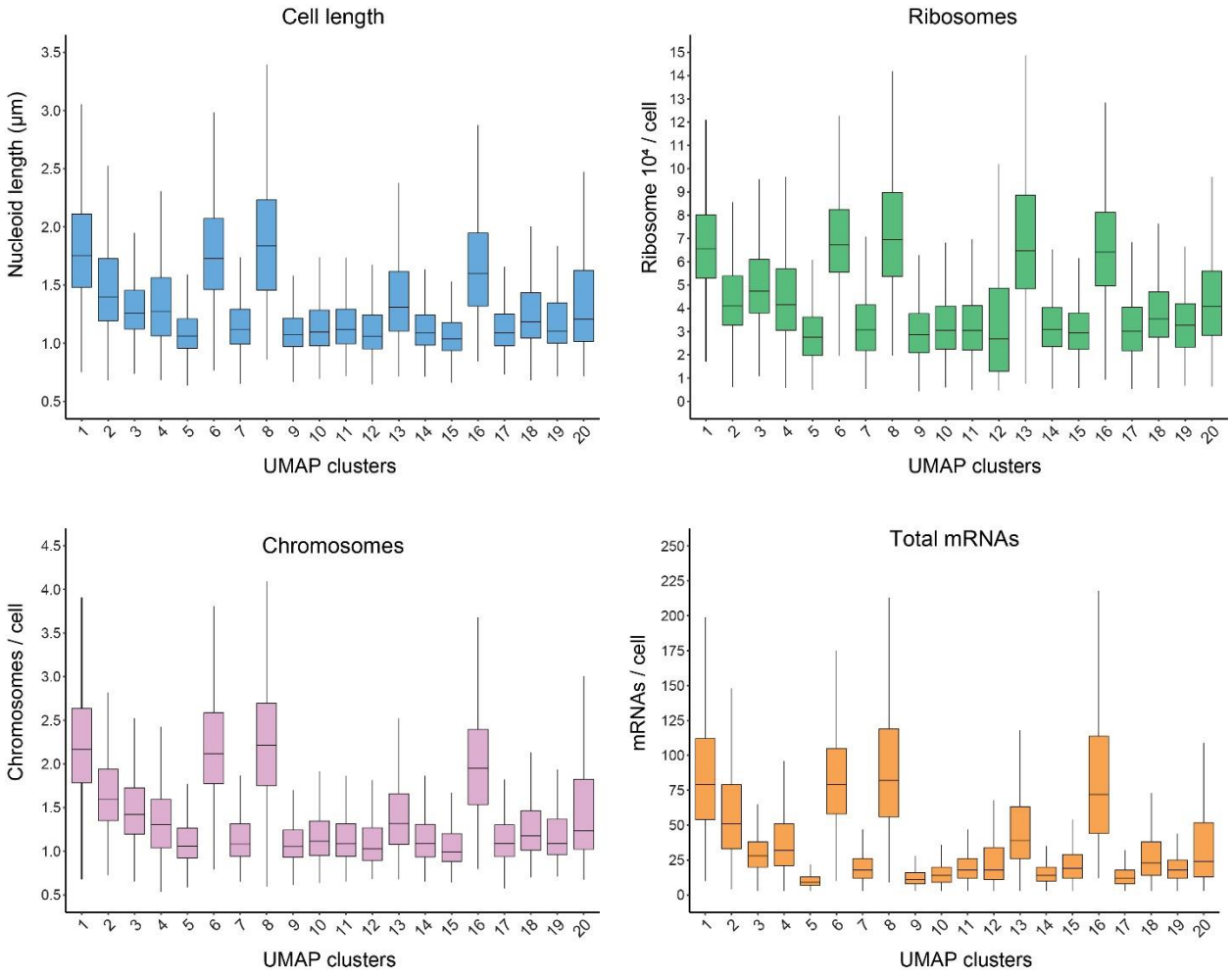

Fig. S4.

Distributions of single-cell parameters across the detected UMAP clusters. Distributions of nucleoid length, chromosome copy, ribosome levels and total mRNAs for each of the UMAP clusters described in main Fig. 3.

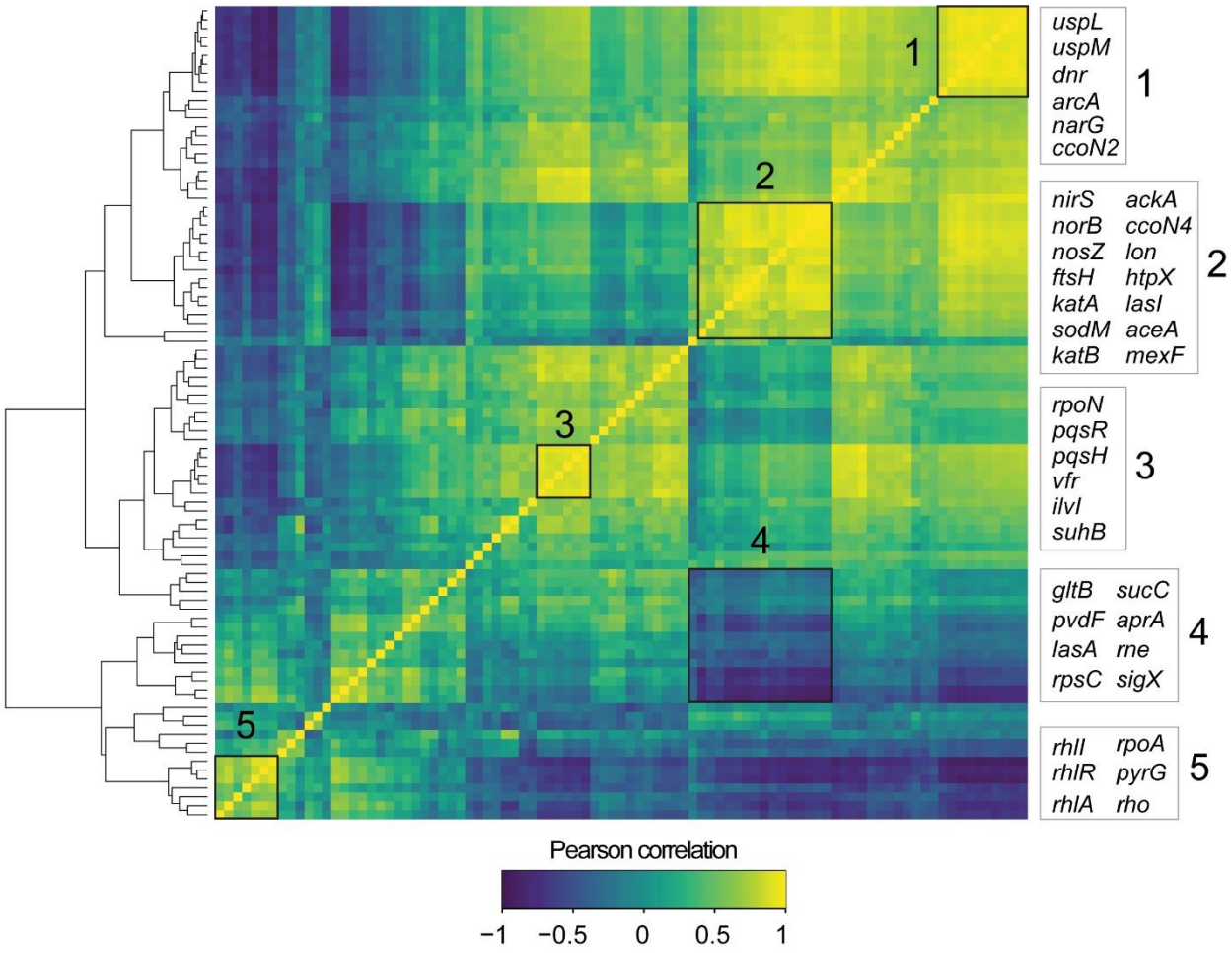

Fig. S5.

Spatial correlation analysis. Gene centered neighborhood analysis for detecting spatial correlation. For each gene, its 99th percentile expressing cells were identified and their 5 immediate neighbors within 3 µm were collected (leaving out the enriched center cell). The set of all such neighbors across the experiment was analyzed together to produce a mean expression profile that was compared with the total population to produce a local enrichment/depletion ratio. The Pearson correlation between such gene neighborhood profiles was calculated and is shown as a clustered heat map. Five selected regions are highlighted and numbered. Key genes within each cluster are described to the right.

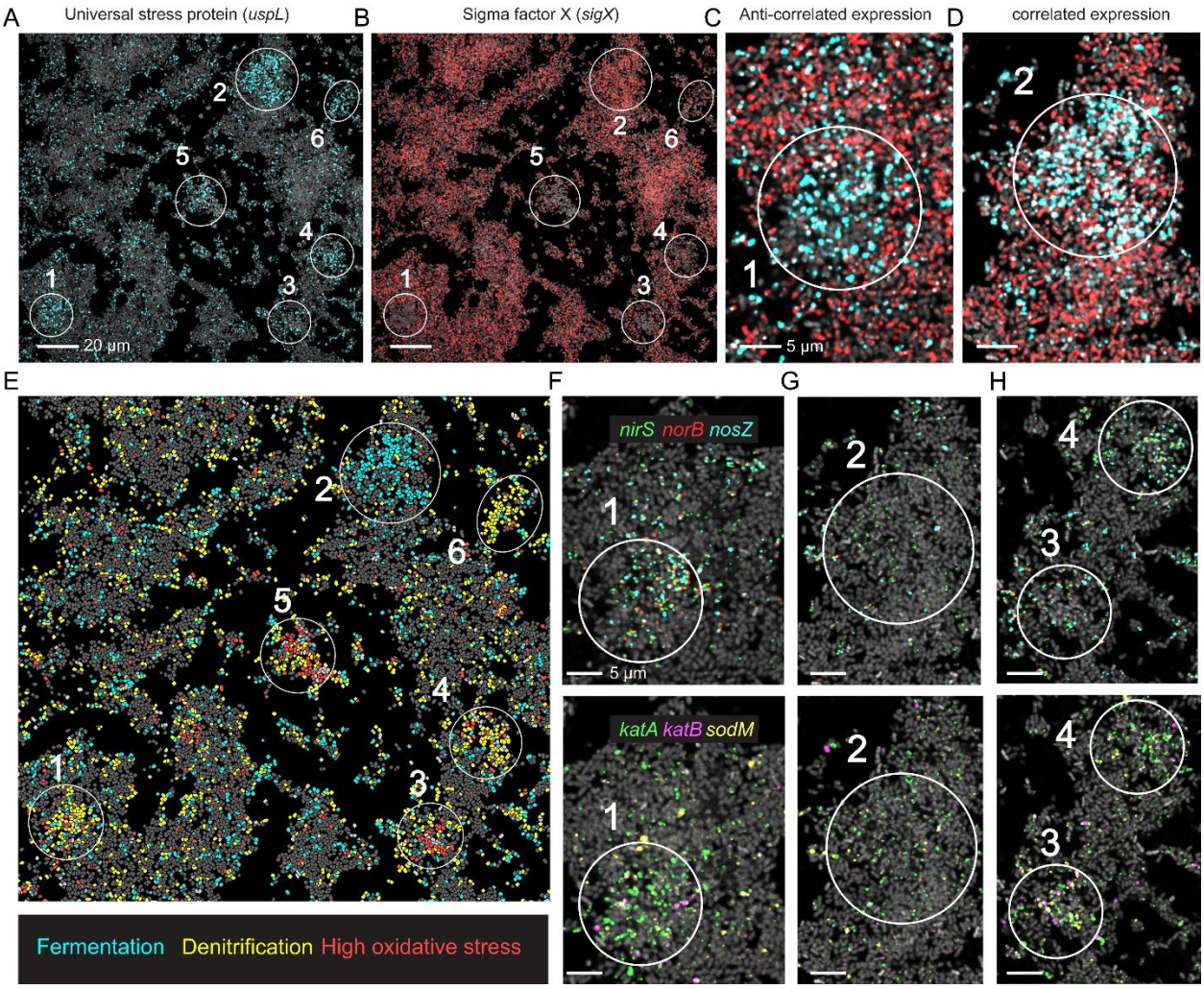

Fig. S6.

Distributions of single-cell parameters per UMAP cluster. (**A**-**B**) Representative 10h microaggregates. Cells are shown via 16S rRNA FISH fluorescence (gray) and overlayed with gene-expression as indicated in each panel. White circles highlight regions of interest. (**C**-**D**) Zoom-in on region 1 and 2 showing *uspL* (cyan) and *sigX* (red). (**E**) Cells painted according to their neighborhood class as indicated in the panel legend. (**F**-**H**) Zoom-in highlighted regions overlaid with raw gene-expression as indicated in the panel legends.

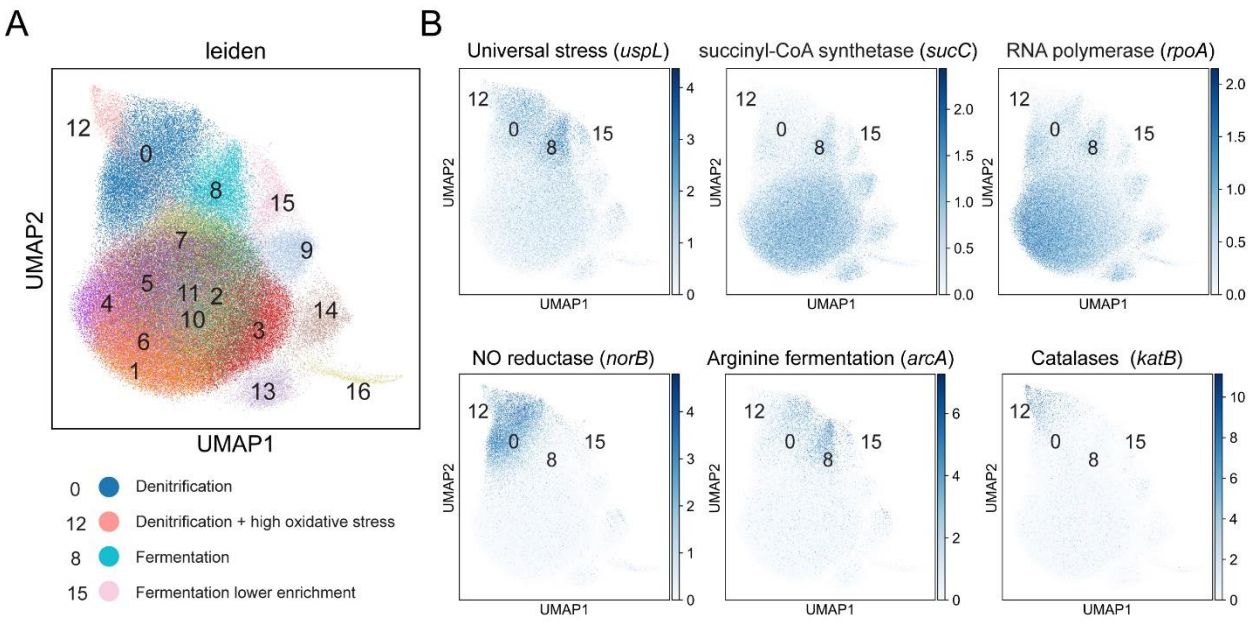

Fig. S7.

UMAP analysis of 10h biofilms. (**A**) UMAP analysis was performed using the 10h biofilm experiment. Below, clusters are labeled and are divided into predicted metabolic groups. (**B**) UMAP overlaid with specific gene data. The color map shows the normalized expression scaled to unit variance. The cluster number positions are shown in the figure.

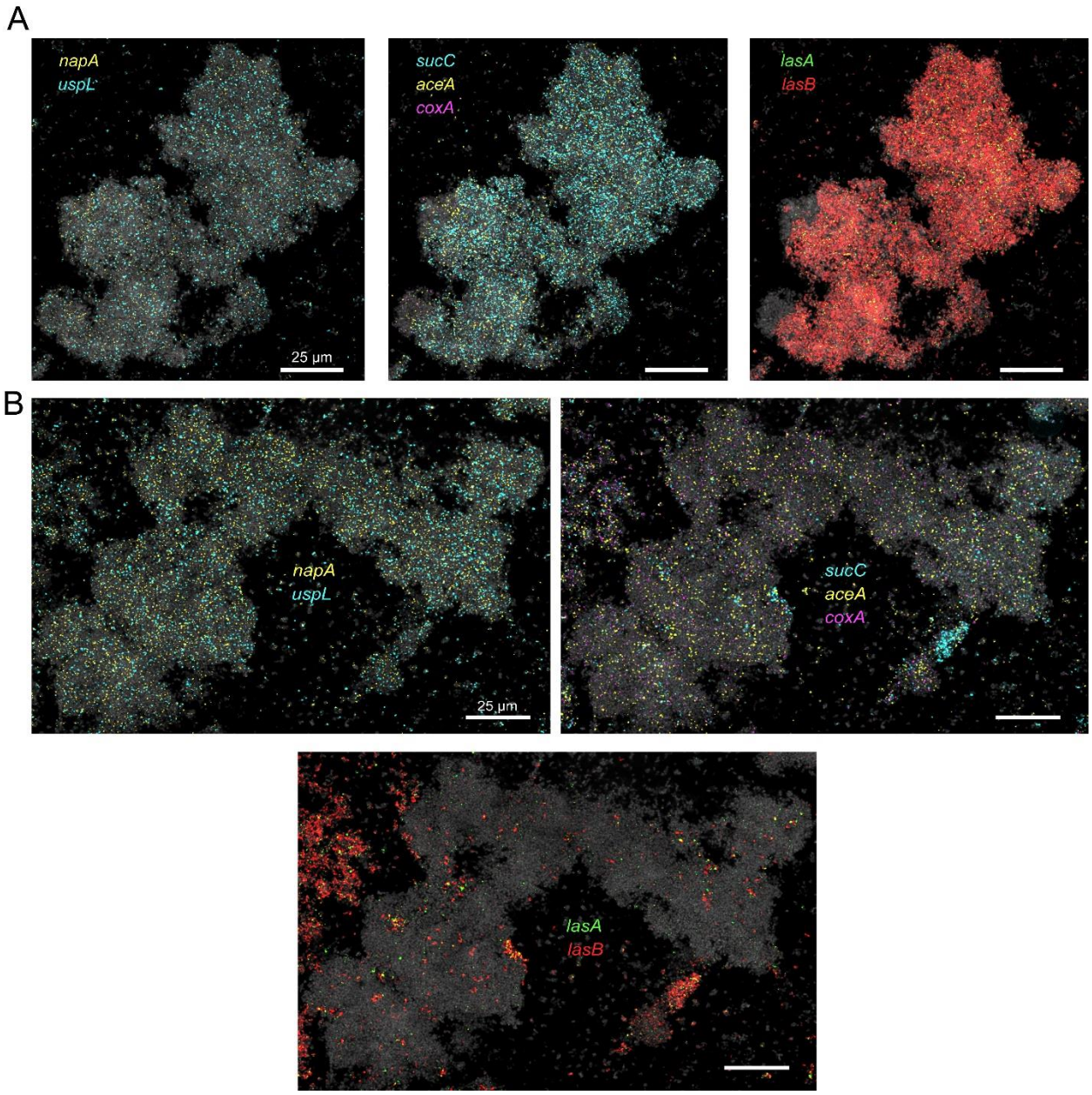

Fig. S8.

Functional zonation in 35h microaggregates. (**A-B**) Various *P. aeruginosa* 35h aggregates. Bacteria are shown via 16S rRNA FISH fluorescence (gray) and are overlaid with raw mRNA-FISH fluorescence for several genes as described in the images.