

Figure S1

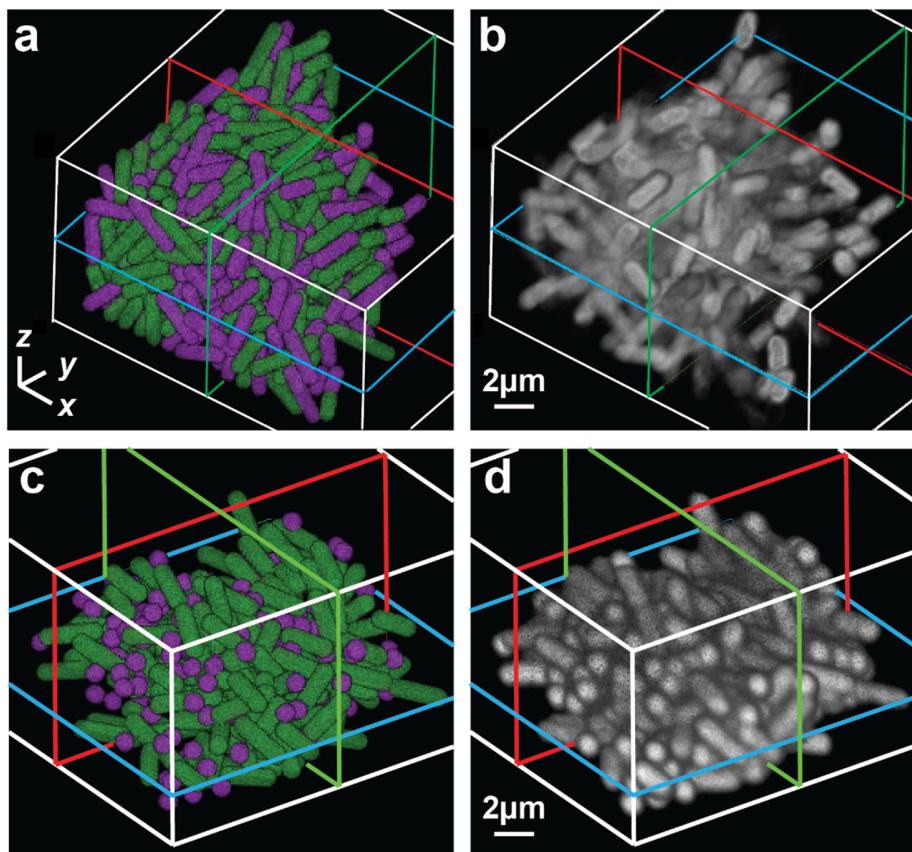


Figure S1. Simulation of mixed labeling and mixed cell shape biofilms. **(a)** Cell arrangements (green indicates membrane labeled cells, purple indicates membrane labeled cells that simultaneously express interior fluorescence protein). **(b)** Fluorescence image based on the cell arrangements in (a) as displayed by the volume viewer plugin of Fiji¹. **(c)** Cell arrangements (green indicates rod-shaped cells, magenta indicates spherical shaped cells). **(d)** Fluorescence image based on the cell arrangements in (c) as displayed by the volume viewer plugin of Fiji¹.

Figure S2

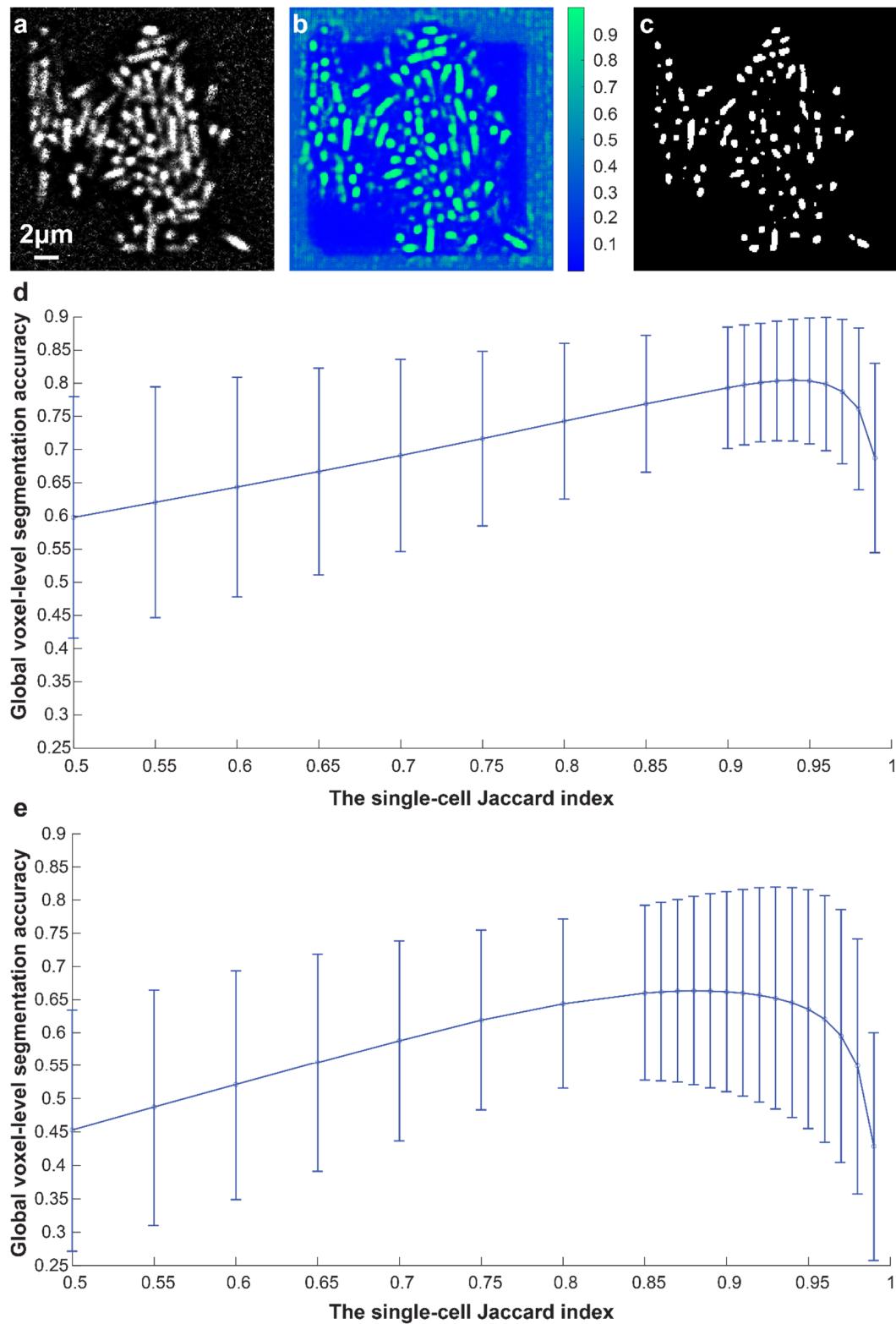


Figure S2. Binary segmentation result produced by thresholding the ‘cell interior’ confidence map at a high value (0.88-0.94). **(a)** Deconvolved fluorescence image. **(b)** ‘Cell interior’ confidence map. **(c)** Binary segmentation result (confidence threshold = 0.94). **(d and e)** Global voxel-level segmentation accuracy (y axis) versus the threshold of single-cell shape estimation (x axis, quantified as the single-cell Jaccard index) for cells labeled with cytosolic fluorophores (d) and cells labeled with membrane-localized fluorophores (e).

Figure S3

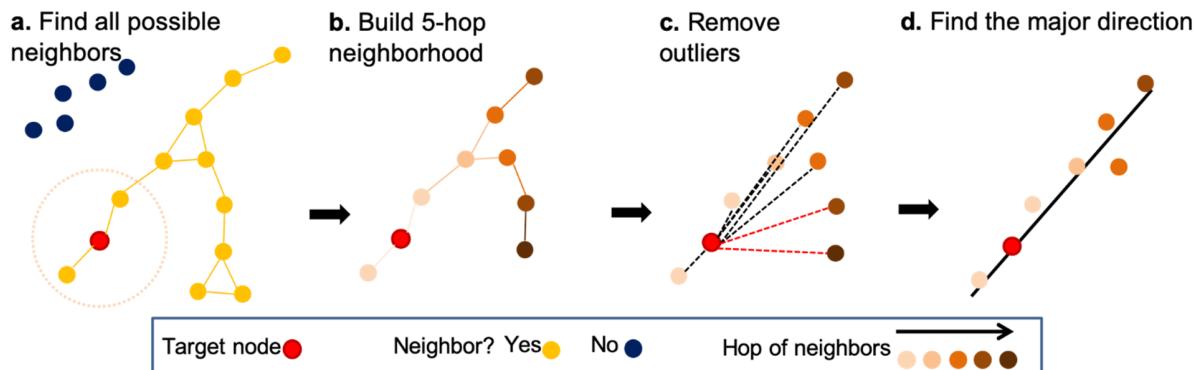


Figure S3. Determination of node direction in an outlier-removed neighborhood². (a) A neighborhood of the target node (in red) is a sub-graph, where all adjacent nodes (in yellow) are connected via edges to the target node. Here, if the distance of two nodes is less than a chosen value (indicated by the dashed circle), these nodes are adjacent to each other. The blue dots are not part of the neighborhood. (b) A hop is defined as the number of edges that one has to traverse from one node to the other node in the graph. Here, the 5-hop neighborhood of the target node is shown. (c) The directional vectors are found from the target node to all the other nodes within in the 5-hop neighborhood (dashed lines). The nodes are classified as outliers if they have large relative angles compared to all the other directional vectors (red dashed lines). (d) Finally, the direction feature of the current node is evaluated as the major direction of the outlier removed neighborhood using principle component analysis.

Figure S4

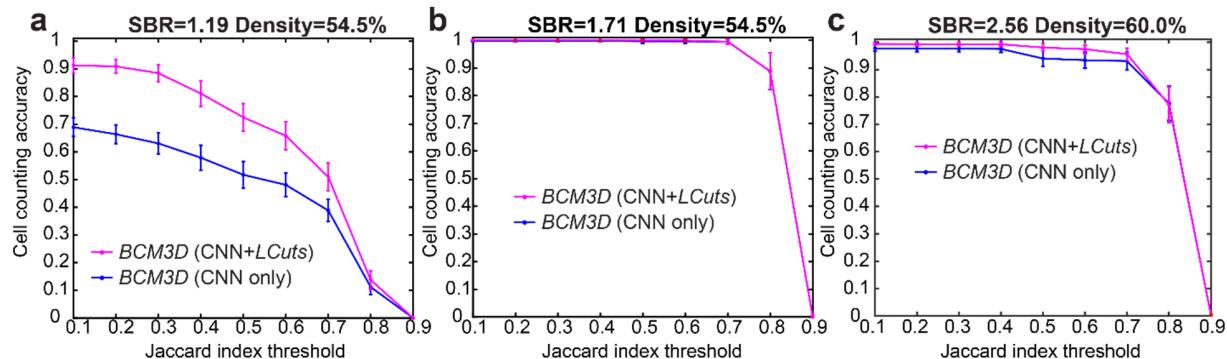


Figure S4. Segmentation accuracies achieved for biofilm images with different SBR and cell densities. (a), (b), and (c) represent results for datasets with SBR 1.19, Density 54.5%, SBR 1.71, Density 54.5% and SBR 2.56, Density 54.5%, respectively. Each curve is plotted by averaging 10 different datasets.

Figure S5

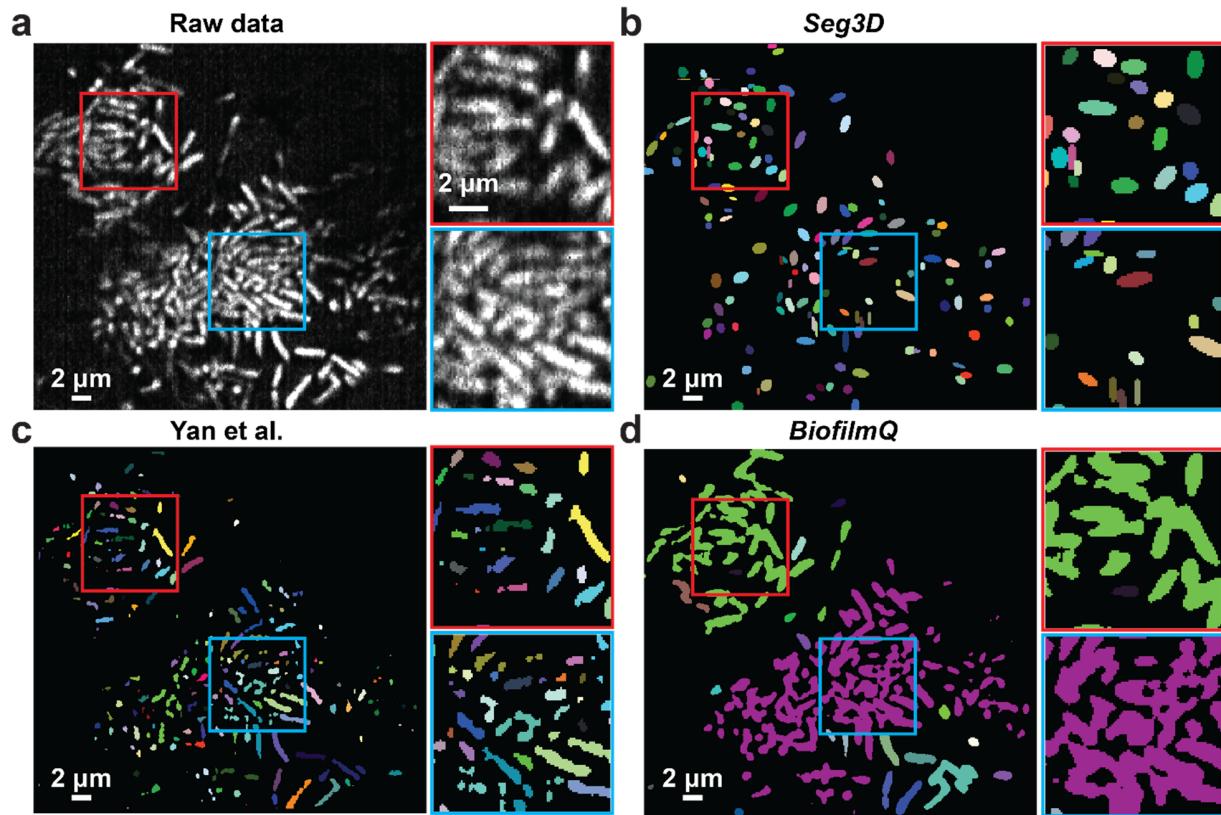


Figure S5. Visual comparison of segmentation results achieved by previous segmentation approaches that rely solely on mathematical image processing. (a) Experimental dataset is a *E. coli* biofilm containing GFP expressing cells 10 hours after the inoculation. (b) Segmentation result obtained using *Seg3D*³. (c) Segmentation result obtained using the algorithm in Yan *et al.*⁴. (d) Segmentation result obtained using *BiofilmQ*⁵.

Figure S6

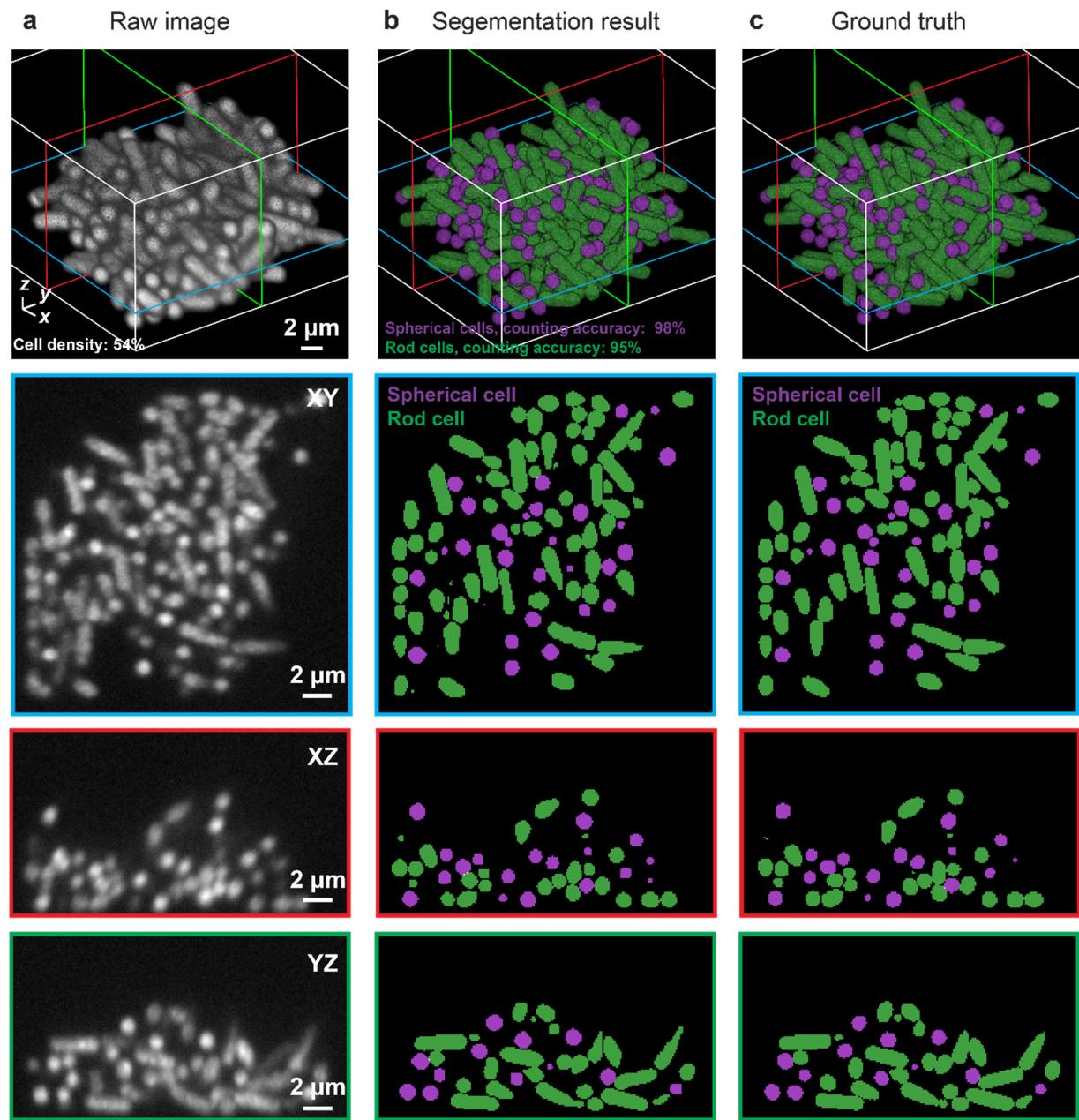


Figure S6. Segmentation of mixed population biofilms containing spherical cells and rod-shaped cells. **(a)** Simulated fluorescence image of a mixture of spherical cells and rod-shaped cells. The mixing ratio is 50:50. **(b)** Segmentation result obtained by *BCM3D* and **(c)** ground truth. Three orthogonal planes are shown below each 3D image.

Figure S7

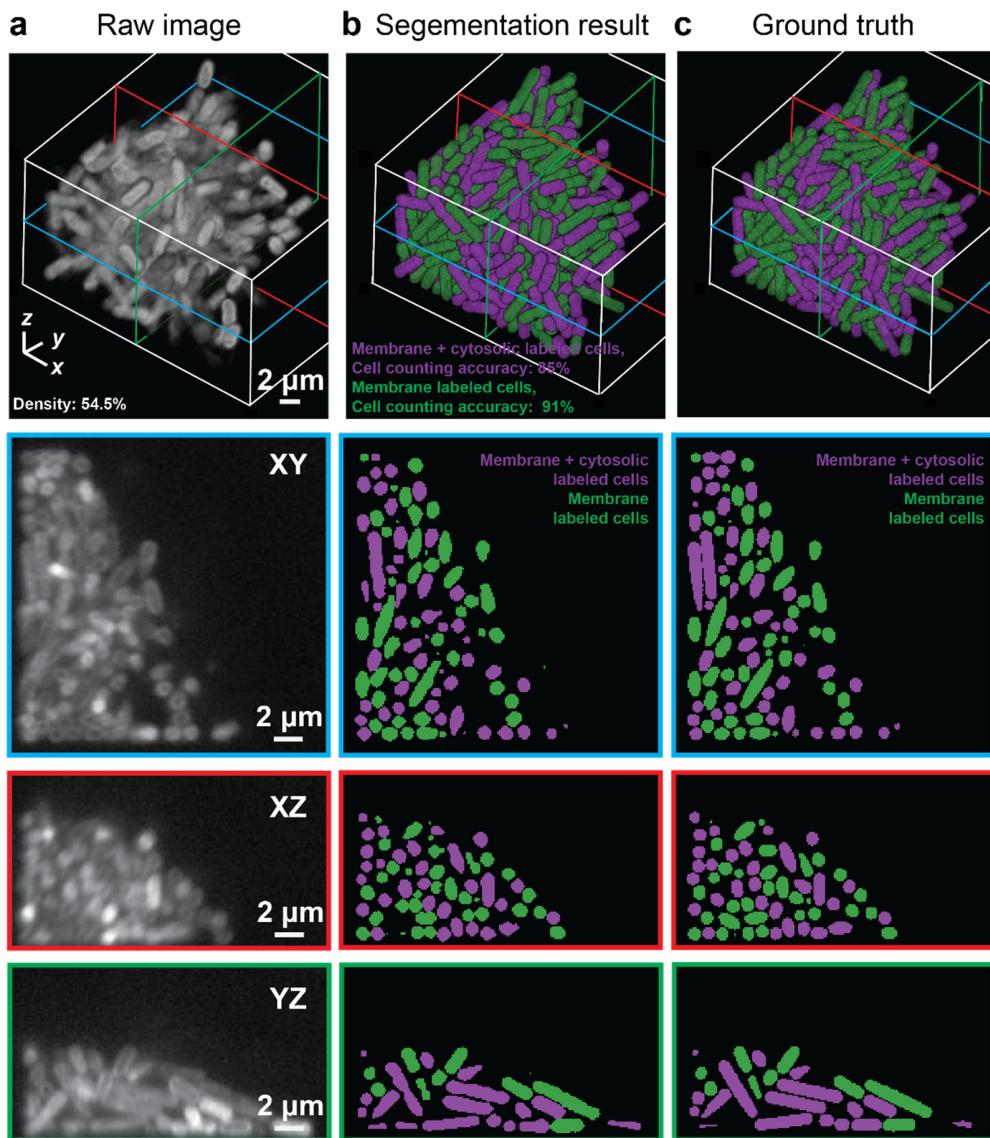


Figure S7. Segmentation of mixed population biofilms containing membrane-stained cells and membrane-stained cells that additionally express an intracellular fluorescent protein. **(a)** Simulated fluorescence image of a mixture of membrane-stained cells and membrane-stained cells that additionally express an intracellular fluorescent protein. The mixing ratio is 50:50. **(b)** Segmentation result obtained by *BCM3D* and **(c)** ground truth. Three orthogonal planes are shown below each 3D image.

Figure S8

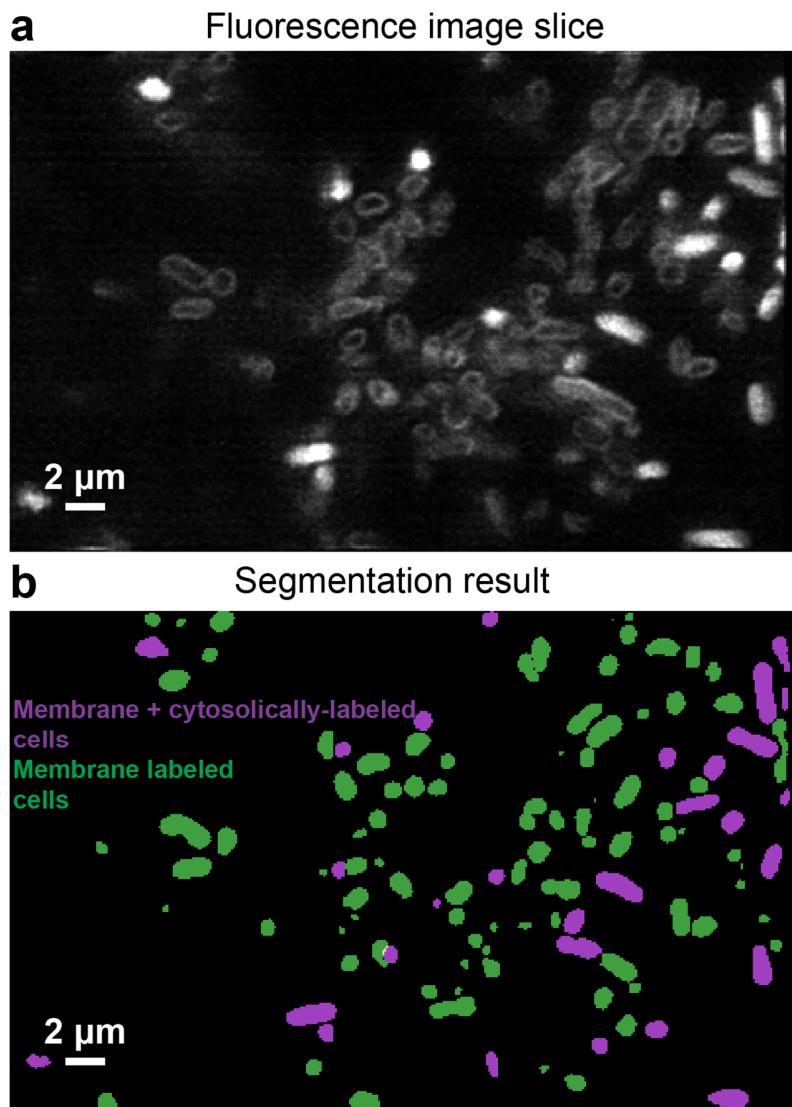


Figure S8. (a) Experimental 2D slice of a mixed *E. coli* population containing membrane-stained cells and membrane-stained cells that additionally express an intracellular fluorescent protein. The mixing ratio at the time of inoculation was 50:50. All cells were labeled by the FM4-64 membrane-intercalating dye. (b) *BCM3D* segmentation result corresponding to the image shown in (a). Membrane-stained cells are displayed in green, and cells that were both membrane-stained and cytosolically-labeled are displayed in magenta.

References

1. Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S. & Schmid, B. Fiji: an open-source platform for biological-image analysis. *Nature methods* **9**, 676-682 (2012).
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3. Reyer, M.A., McLean, E.L., Chennakesavalu, S. & Fei, J. An Automated Image Analysis Method for Segmenting Fluorescent Bacteria in Three Dimensions. *Biochemistry* **57**, 209-215 (2018).
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5. Hartmann, R., Jeckel, H., Jelli, E., Singh, P.K., Vaidya, S., Bayer, M., Vidakovic, L., Díaz-Pascual, F., Fong, J.C.N., Dragoš, A., Besharova, O., Nadell, C.D., Sourjik, V., Kovács, Á.T., Yıldız, F.H. & Drescher, K. BiofilmQ, a software tool for quantitative image analysis of microbial biofilm communities. *BioRxiv* 735423 (2019).