Biofilms are now considered to be the most abundant form of microbial life on Earth, playing critical roles in biogeochemical cycles, agriculture, and health care. Phenotypic and genotypic variations in biofilms generally occur in three-dimensional space and time, and biofilms are therefore often investigated using microscopy. However, the quantitative analysis of microscopy images presents a key obstacle in phenotyping biofilm communities and single-cell heterogeneity inside biofilms. Here, we present BiofilmQ, a comprehensive image cytometry software tool for the automated high-throughput quantification and visualization of 3D and 2D community properties in space and time. Using BiofilmQ does not require prior knowledge of programming or image processing and provides a user-friendly graphical user interface, resulting in editable publication-quality figures. BiofilmQ is designed for handling fluorescence images of any spatially structured microbial community and growth geometry, including microscopic, mesoscopic, macroscopic colonies and aggregates, as well as bacterial biofilms in the context of eukaryotic hosts.