Classic histology still represents the gold standard in tumor tissue analytics. However, two-dimensional analysis of single tissue slides does not provide a representative overview of the inhomogeneous tumor physiology, and a detailed analysis of complex three-dimensional structures is not feasible with this technique. To overcome this problem, we applied multispectral fluorescence ultramicroscopy (UM) to the field of tumor analysis. Optical sectioning of cleared tumor specimen provides the possibility to three-dimensionally acquire relevant tumor parameters on a cellular resolution. To analyze the virtual UM tumor data sets, we created a novel set of algorithms enabling the fully automatic segmentation and quantification of multiple tumor parameters. This new postmortem imaging technique was applied to determine the therapeutic treatment effect of bevacizumab on the vessel architecture of orthotopic KPL-4 breast cancer xenografts at different time points. A significant reduction of the vessel volume, number of vessel segments, and branching points in the tumor periphery was already detectable 1 day after initiation of treatment. These parameters remained virtually unchanged in the center of the tumor. Furthermore, bevacizumab-induced vessel normalization and reduction in
vascular permeability diminished the penetration behavior of trastuzumab-Alexa 750 into tumor tissue. Our results demonstrated that this new imaging method enables the three-dimensional visualization and fully automatic quantification of multiple tumor parameters and drug penetration on a cellular level. Therefore, UM is a valuable tool for cancer research and drug development. It bridges the gap between common macroscopic and microscopic imaging modalities and opens up new three-dimensional (3D) insights in tumor biology.