Stimulated emission depletion (STED) microscopy enables fluorescence imaging on the nanoscale with resolutions of 20 nm and beyond [1]. As samples prepared for far-field fluorescence microscopy are in general also suitable for STED imaging, it has been applied advantageously in biosciences, in particular to investigate subcellular structures and assemblies.

In order to expand the regime of applications to high-resolution colocalization studies we designed a setup which allows for STED imaging of up to three different labels simultaneously (Fig. 1). The design intrinsically provides high spatial resolution for all markers concurrently (<50nm), since in STED, the diffraction barrier is overcome by keeping fluorophores selectively dark (non-fluorescent) by inducing stimulated emission, but leaving them unaffected in vicinity of the intensity minimum at the very center of the doughnut-shaped STED beam. Here, two fluorescence labels are separated by their fluorescence lifetime whereas a third channel is discriminated by the wavelength of excitation and fluorescence emission. Our approach is less prone to drift artifacts than e.g. stochastic imaging concepts where the recording of even a small region of the object extends over the whole image acquisition period, and it is not subject to systematic errors due to the fluorophore orientations which have to be considered in stochastically based high-resolution techniques. Therefore our implementation enables us to accurately (<14nm) investigate the interplay of at least three differently labeled cellular components [2,3].

Fig. 1: (A) Side-by-side comparison between three-color confocal and STED nanoscopy of human glioblastoma showing immunolabeled clathrin, tubulin and lamin. (B) The separate STED images corresponding to the three color-channels are shown for the marked area.