ROLE OF 3D BLEACH DISTRIBUTION IN FRAP (FLUORESCENCE RECOVERY AFTER PHOTobleaching) EXPERIMENTS IN CONFOCAL AND TWO-PHOTON EXCITATION SCHEMES

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Fluorescence recovery after photobleaching is a classical tool for quantitative evaluation of 2D diffusion processes [1,2]. The spreading of confocal laser scanning microscopes (CLSM), together with the discovery and the development of fluorescent proteins has encouraged the extension of this methodology to three-dimensional environments. The quantitative analysis of such experiments usually requires the development of suitable analytical models capable to describe the experimental conditions [3]. When diffusion in 3D environments is considered, the description of the initial condition produced by the perturbation (i.e. the photobleaching of a selected region) represent a crucial aspect, as the approximations that are usually made can lead to deviation in the measurement of the kinetic parameters of the labeled molecules. Furthermore the experimental distribution of fluorescent molecules depend on the intensity of the light pulse that produce the perturbation as fluorescence saturation would play a role [4,5]. In this work we measured the experimental 3D bleaching distributions produced in conventional and two-photon excitation schemes and analyzed the deviations from the idealized cases usually adopted. The experimental measurement of these pattern for different experimental conditions in immobile samples (labeled polyelectrolyte gels) revealed that the approximation of the confocal bleaching intensity distribution as Gaussian can lead to relevant errors. On the opposite side the two-photon bleach volume seems well described by such approximation, even when fluorescence saturation effects arise. These data has been used for finite elements simulations mimicking FRAP experiments on free diffusing molecules and compared with model FRAP curves based on the idealized bleach distributions. The results show that two photon excitation provide a better fit to the idealized bleaching patterns even in fluorescence saturation regime, resulting in correct estimations of diffusion coefficients within the 20%.