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Plasma Membrane Protein Fluorescence Recovery after Photobleaching Measured by Confocal Microscope with High Numerical Aperture at Periphery of Live Cell.

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Since its development in 1970s, fluorescence recovery after photobleaching (FRAP) became widely used method for measuring of protein mobility in live cells. The measurement starts by imaging of the initial distribution of the fluorescently-tagged protein of interest. Fluorescent molecules within the selected area, region of interest (ROI), are then irreversibly bleached by the exposure to a short pulse of high-intensity light. Subsequently, recovery of fluorescence within the bleached ROI is recorded. Evaluation of FRAP primarily involves the estimation of the parameters of appropriate mathematical model of the protein concentration profile evolution. Accessibility of FRAP increased with the commercialization of the confocal laser scanning microscope.

Lateral mobility of plasma membrane proteins in live cells is studied by FRAP at the cell periphery while focusing the microscope into the equatorial plane of the cell. Complex 3D shape of the entire bleached region of the cell surface cannot be directly observed in this setup [1] and current evaluation methods approximate the region by fitting Gaussian [2], rectangular [3] or 1D profile [4]. We calculate the fluorescence intensity profile with circular ROI after bleaching (Fig. 1) from numerical aperture (NA) and refractive index directly. We used the profile as initial condition of diffusion equation on sphere and we obtained the model FRAP curve by integrating the equation solution over ROI. Curves for high and low NA and 1D profiles are shown in Fig. 1.

We observed the high scatter of experimental data which was caused by the movement of HEK cells in the course of recovery phase. Therefore, we developed the new approach for analysis of the primary FRAP data which is based on ROI moving in co-ordination with the cell movements (Fig. 2). The cell movements are calculated from first and second order spatial moments of fluorescence intensity in ROI. We implemented the adaptive ROI data readout as plugin for ImageJ.

Mobility of δ-OR-eYFP fusion protein transiently expressed in HEK293 cells by FRAP was measured at the cell periphery using confocal laser scanning microscope Leica SP8 with 63× water immersion objective (NA 1.2). Depletion of cholesterol content by β-cyclodextrin resulted in significant decrease of diffusion coefficient values of δ-OR-eYFP, from 0.104 ± 0.008 $\mu m^2/s$ (in control cells, $N = 30$) to 0.078 ± 0.005 $\mu m^2/s$ ($N = 36$) ($p < 0.01$). The fraction of mobile molecules decreased from 79.0 ± 2.1 % to 69.3 ± 1.8 % ($p < 0.001$). Replenishment of cholesterol content by incubation with “water soluble cholesterol” resulted in full restoration of the mobility of receptor molecules.

Adaptive ROI displacement compensated for small lateral cell movements and substantially reduced the scatter of experimental data. Diffusion equations solved either on real geometry, on sphere or on vertical tangent plane may be used with similar results (data not shown). 1D model shall be avoided when using objective with high NA (see Fig. 1). Survey of other common problems, that we did not encounter in our FRAP experiments – e.g. initial bleaching duration longer than 0.1 characteristic time and photobleaching of fluorophore during recovery can be found elsewhere [5].

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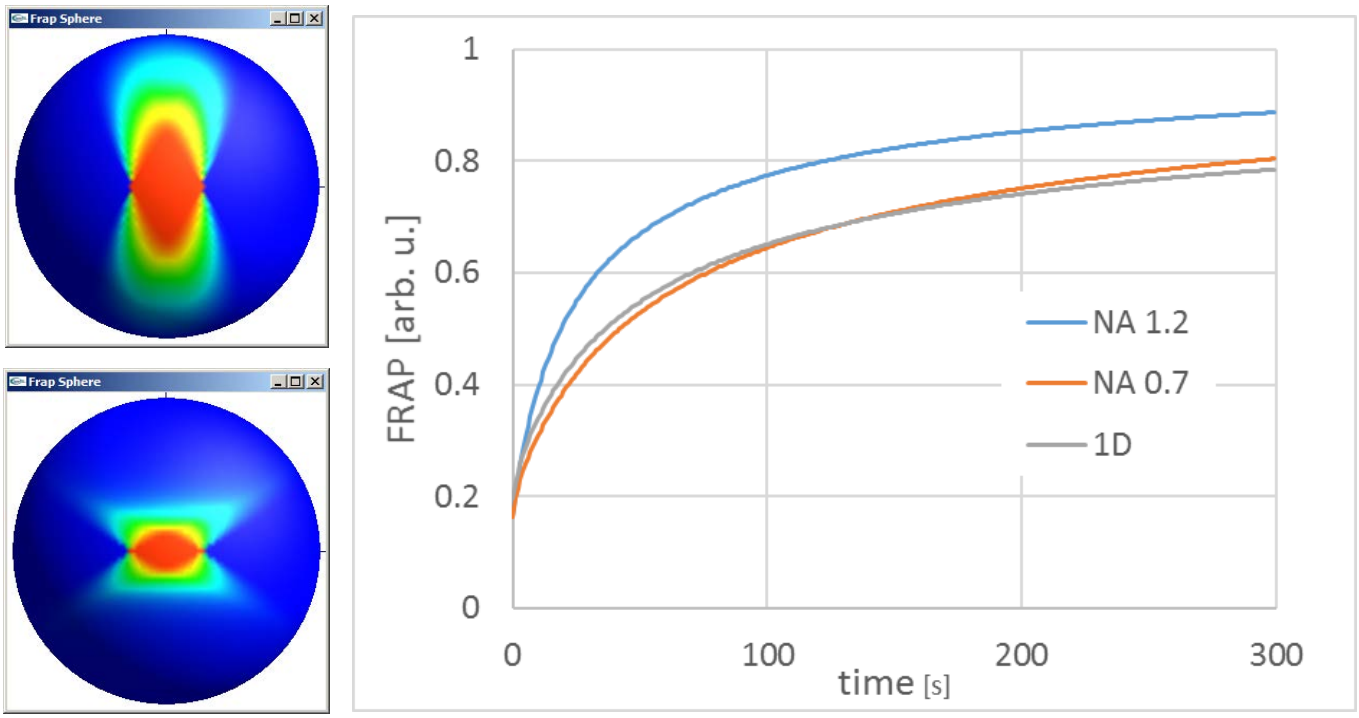

Figure 1. Left: Fluorescence intensity profile after bleaching was calculated with refractive index 1.33 and numerical aperture 0.7 (top) or 1.2 (bottom). Right: FRAP curves calculated for circular ROI diameter 5 micrometers, sphere diameter 15 micrometers, diffusion coefficient 0.1 10^{-6} um^2/s, NA 1.2 (blue), NA 0.7 (orange) and 1D approximation (gray).

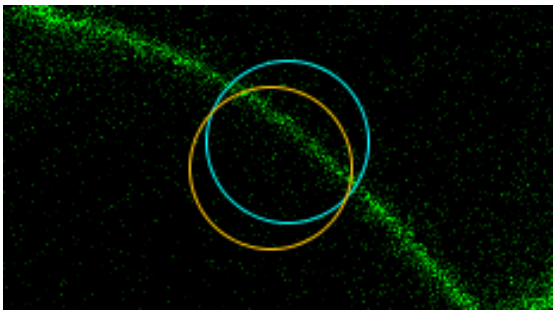

Figure 2. Lateral movement correction by adaptive ROI. Original position – orange, adaptive displacement – cyan.

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