Fluorescence resonant energy transfer (FRET) is a very popular method used both in wide-field and confocal laser scanning microscopy nowadays. This is because FRET can easily provide information about the short distances between molecules in nanometer range. FRET Acceptor Photobleaching (FRET AB) is quite simple method based on bleaching the acceptor, but the drawback is that there are needed a lot of tests and controls to confirm FRET. Our proposal is to use so-called TimeGate function of confocal laser scanning microscope Leica TCS SP8 X. This function is available for the hardware combination of excitation by pulsed picosecond White Light Laser (WLL) and spectral hybrid HyD detectors based on GaAsP. WLL is a laser freely tunable in the spectral range 470-670 nm, so that the wavelength and intensity can be tuned for ideal excitation. Spectral detection by HyDs can be expanded by time resolved detection by TimeGate function with arbitrary time windows in the range 0-12 ns. It is based on time delay between the excitation pulse and detection of fluorescence. As the minimum opening of the TimeGate window is 3.5 ns with the stepsize 0.1 ns or lower, the TimeGate function can be applied to distinguish between the donor and FRET channels.

We performed FRET for well-known interacting partners p53 and 53BP1 in mouse mbyronic fibroblasts (MEFs). The fact that p53 and 53BP1 can interact was well described previously (Iwabuchi K, et al. Proc Natl Acad Sci U S A. 1994). Although the significance of the interaction between 53BP1 and p53 is not completely elucidated, it is evident that 53BP1 plays an important role in chromatin-based DNA damage response signaling (Panier S, et al. Nat Rev Mol Cell Biol. 2014). The 53BP1-p53 interaction is mediated by the tandem-BRCT repeats of 53BP1 (residues 1702–1972) and the DNA-binding domain of p53 (residues 94–292) (Joo WS, et al. Genes Dev. 2002). In our experiments, we have used laser lines 488 nm and 591 nm for analysis by FRET AB with two HyDs with spectral bandwidth set adequately to excitation laser lines. Different time windows were tested. Here, we present results with the time window set in the range 0.3-3.8 ns. Format of the images was 512 x 512 pixels (30.75x30.75 microns). Preferentially, we investigated the interactions between p53-GFP and 53BP1-Alexa 594. In classic settings, FRET efficiency was approximately 25 % (Fig 1), while the FRET resolution was increased by the setting of the TimeGate function (efficiency 45.5±6.1%, Fig 2). Therefore, TimeGate function seems to be very useful tool for advanced FRET application.

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Fig. 1: FRET analysis for the interactions between well-known interacting partners p53 and 53BP1 without TimeGate function in MEFs.

Fig. 2: FRET analysis for the interactions between well-known interacting partners p53 and 53BP1 with TimeGate function in MEFs.