Determining the dynamics of local absolute calcium concentrations in germinal center B cells *in vivo* by donor FRET-FLIM

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Fluorescence lifetime imaging microscopy (FLIM) allows us to measure subtle changes caused by Förster resonance energy transfer (FRET), since the donor lifetime is significantly shortened by several nanoseconds when FRET takes place. In contrast to well established ratiometric approaches which compare donor and acceptor intensities, in donor-FRET-FLIM there is no need to correct the wavelength-dependent sensitivity of the detectors or to consider the different photobleaching properties of the two fluorophores. Here, we imaged *in vivo* the B-cell receptor signaling in germinal centers (GC) of lymph nodes of the transgenic mouse line "YellowCaB", which $CD19^+$ B-cells encoding the Ca^{2+}-sensitive biosensor TN-XXL connecting the fluorophores eCFP (donor) and Citrine (acceptor). We measured the lifetime data in time-domain by time correlated single photon counting device (TCSPC) and transferred them to a virtual phase domain by calculating the discrete Fourier Transformation numerically (phasor approach [1]).

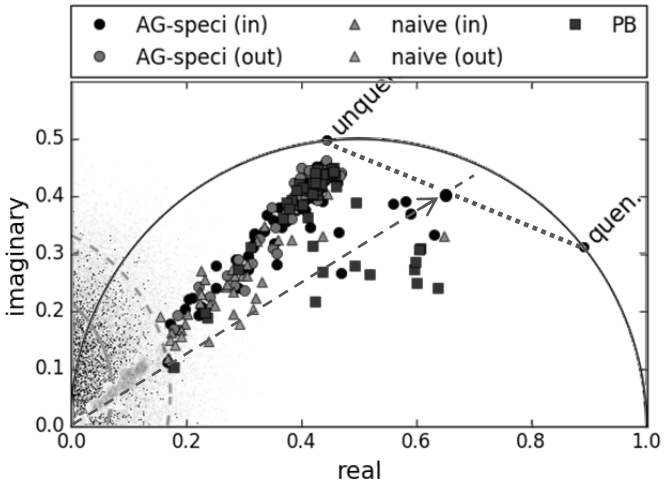

Fig: donor-FLIM phasor plot of segmented $CD19^+$ B-cells in-/outside the GC and an exemplary scheme of their projection onto the FRET-vector (arrow)

Since we are interested in cell-to-cell communication within the germinal centers, we segmented different cell populations using Imaris|Bitplane (symbols in fig) to generate a cell-based phasor plot instead of a pixel-based. From the averaged, normalized donor lifetime of segmented cells we calculate the absolute Ca^{2+} concentration [2]. Due to the scattering environment in tissue, the FLIM-signal of the donor is shifted towards the (0/0) position in the phasor plot, indicative for the infinite lifetime of noise. This artefact prevents the accurate calculation of absolute intracellular calcium concentrations from the phasor plot data. Theoretically, the phasor position of the B cells would be onto the vector connecting the donor lifetime under unquenched and quenched conditions (dottet line in Fig). In presence of noise it is a vector sum forming a triangle between unquenched-quenched-(0/0).

Here, we first determined from background data a radius around (0/0) in the phasor plot within which the signal-to-noise ratio is too low to allow an accurate projection on the expected phasor segment. Therefore, we measured the time-resolved signal of a MOPS-solution (grey scatter in Fig), to avoid any contamination with autofluorescent molecules. We Gaussian-fitted the histograms of the real part and imaginary part and determined the full width half maximum (FWHM), which was the same for real and imaginary, whereas the center was shifted from (0/0), due to the logarithmic nature of the universal circle (solid half circle in Fig). In order to increase accuracy, we enlarge that radius from ½*FWHM to ¾*FWHM (dashed arc). All cells which lay outside that radius were projected onto the donor-FRET vector (dashed line in Fig), to calculate the absolute Ca^{2+} concentration.

This is the first time absolute calcium concentrations of GC-B cells were determined. Additionally, we could determine these changes in a dynamic manner, in correlation with cell-cell interactions.

[1] Digman, M. A., et al., Biophy. J 94.2 (2008) [2] Geiger, A., et al. Biophy J 102.10 (2012)