**INTRAVITAL TWO-PHOTON MICROSCOPY: APPLICATION TO THE IMMUNE SYSTEM**

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**Introduction:** Immunity is a highly balanced system with two complementary strategies, innate and adaptive. Unlike the innate component, the adaptive immune response creates immunological memory after an initial reaction to a specific pathogen. One of the key processes forming the immune memory is germinal center (GC) reaction, the basis of vaccination. Optimizing the GC response to different vaccine antigens (HIV, influenza etc) can give us a unique tool for development of effective prophylactics against such diseases in the global context. The GCs are dynamic sites of antigen-specific B cells, T follicular helper cells and the resident follicular dendritic cells. Unlike current intravital techniques with observation of typically 3 to 4 fluorophores, we demonstrate simultaneous detection of seven cellular and tissue compartments in popliteal lymph nodes of live mice that is necessary to study the GC mechanisms *in vivo.* The

functional dynamics and cellular sources of oxidative stress are central to understanding MS pathogenesis but remain elusive, due to the lack of appropriate detection methods. We employ NAD(P)H fluorescence lifetime imaging to detect functional NADPH oxidases (NOX enzymes) in vivo to identify inflammatory monocytes, activated microglia, and astrocytes expressing NOX1 as major cellular sources of oxidative stress in the central nervous system of mice.

**Methods:** Both fluorescence intensity and FLIM experiments were performed using two-photon laser-scanning microscope (LaVision BioTec, Germany). In order to a defined model of germinal center reaction in the popliteal lymph node, we immunized mice with NP-CGG and transferred into the recipient C57Bl/6 mice labeled five types of cells. The detection of the fluorescence signals was accomplished either with photomultiplier tubes or with a 16-channel parallelized TCSPC detector (FLIM-X16, LaVision BioTec, Germany).

**Results:** Using our approach, we simultaneously excite and detect seven fluorophores expressed indistinct cellular and tissue compartments, plus second harmonics generation from collagen fibers in lymph nodes. This enables us to visualize the dynamic interplay of all the central cellular players during germinal center reactions. While current *in vivo* imaging typically enables recording the dynamics of 4 tissue components at a time, our strategy allows a more comprehensive analysis of cellular dynamics involving 8 single-labeled compartments.

**Conclusions:** This work demonstrates the unique versatility of intravital NAD(P)H-FLIM as a marker-free method to investigate mechanisms of oxidative stress in inflammatory pathologies, underscoring its intriguing potential for biomedicine as well as clinical research. In the future, the design of transgenic mice combining a larger spectrum of fluorescent proteins will reveal the full potential of our method.