MULTIPLEX INTRAVITAL IMAGING OF GERMINAL CENTER REACTIONS IN MURINE LYMPH NODE USING WAVELENGTH MIXING TWO-PHOTON MICROSCOPY

Asylkhan Rakhymzhan[1], Ruth Leben[1], Hanna Zimmermann[1], Robert Günther[1], Peggy Mex[1], David Reismann[1], Andreas Acs[2], Randall L. Lindquist[1], Thomas H. Winkler[2], Anja E. Hauser[1,3], Raluca A. Niesner[1]

1 German Rheumatism Research Center, Berlin, Germany
2 Nikolaus-Fiebiger-Zentrum, Division of Genetics, Department of Biology, University of Erlangen-Nürnberg, Erlangen, Germany
3 Immundynamics, Charité – Universitätsmedizin Berlin, Germany
E-mail: asylkhan.rakhymzhan@drfz.de

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Simultaneous detection of multiple cellular and molecular players within their environment, remains one of the main challenges for intravital microscopy. Over the last decades, intravital two-photon microscopy has become the main tool for imaging cellular responses in live animals, with particular application in neuroscience and immunology. For instance, the dynamic processes taking place during germinal center (GC) reactions are highly relevant to understand mechanisms involving the maturation of B cell immune responses. However, current intravital techniques allow for simultaneous observation of typically 3 to 4 fluorophores[1], not enough to monitor the communication and interplay of all cellular and tissue compartments involved in a germinal center reaction.
For *in vivo* imaging, it is critical to simultaneously image all fluorophores. This requires efficient simultaneous excitation over the entire range of fluorophores, which is easily performed with continuous-wave lasers, but is difficult to achieve with the pulsed laser sources required for two-photon excitation. Recent developments in the field of NIR fluorescent proteins have extended the detection range of fluorescent probes, resulting in an increased number of available fluorophores for *in vivo* imaging experiment. Still, the major bottleneck of simultaneous multiplexed fluorescence imaging is the ability to distinguish between multiple fluorophores. The crosstalk originating from spectral overlap of neighboring fluorophores hinders the unambiguous identification of different cell types in complex dynamic processes. In order to separate distinct colors labeling up to seven different cell and tissue compartments in the popliteal lymph node, we developed and applied the similarity SIMI algorithm.
Here, we present a synergetic strategy for spectrally multiplexed intravital imaging composed of (i) triple two-photon excitation using spatiotemporal synchronization of two femtosecond lasers, (ii) a broad set of fluorophores with emission ranging from blue to near infrared, and (iii) an effective non-analytic spectral unmixing approach. Using the new imaging technique, we are able to simultaneously excite and detect seven fluorophores, i.e. seven distinct cellular and tissue compartments, in popliteal lymph nodes *in vivo*. Our strategy allows a more comprehensive analysis of cellular dynamics involving up to 8 compartments, opening the way for mechanistic understanding of complex pathophysiologic processes in their genuine environment.

[1] Mahou, P et al, *Nat. Methods*, vol.9, No.8, 815-818 (2012)