Two-photon multifocal microscopy for in vivo single-molecule and single-particle imaging

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Summary

One of the great accomplishments of modern society is our more thorough understanding on how organisms function. A plethora of applications has emerged from this knowledge to revolutionize medicine – and many other industries. As our insight in complex biological systems grew, research became more specific and aiming at an understanding our cells and bodies on the molecular scale. Research on this smallest biological scale was propelled by recent developments in microscopy techniques. A prominent example was awarded the Nobel prize in 2014, demonstrating the importance of new super-resolution microscopy techniques for biology and physics research.

Here we developed a novel approach for single particle and single molecule tracking using two-photon microscopy (TPM). TPM is generally associated with slow, deep-tissue imaging using high laser power, a low background and negligible out-of-focus photobleaching. This would make TPM highly suitable for imaging weak signals of single molecules. However, the high laser powers required for two-photon excitation (TPE) causes enhanced in-focus bleaching and the slow imaging speeds impede tracking of fast, dynamic processes. TPM is therefore generally used for static, deep-tissue imaging.

In this thesis we splitted the TPE beam into multiple weaker beamlets for fast and gentle two-photon imaging. By simultaneously scanning several 100 beamlets, we achieved limited bleaching and fast imaging, while retaining the useful TPE properties of low background and thin optical sectioning. With abovementioned advantages, we demonstrated for the first time that two photon microscopy can readily be used to capture single particles and even single molecules in living samples.

In **Chapter 2** we first explored the possibility of single-molecule sensing by utilizing gold nanorods (GNRs). Plasmonic metallic nanoparticles are used in (bio-)sensing applications because their localized surface plasmon resonance (SPR) is highly sensitive to changes in environment. Although optical detection of scattered light from single particles provides a straight-forward means of detection, the two-photon luminescence (TPL) of single gold nanorods (GNRs) has the potential to
increase the sensitivity due to the large anti-Stokes shift and the non-linear excitation mechanism. However, two-photon microscopy and spectroscopy are restricted in bandwidth and have been limited by the thermal stability of GNRs. We used two-photon multifocal microscopy (TPMM) to simultaneously measure the two-photon excitation spectra of hundreds of individual GNRs with sub-nanometer accuracy. By keeping the excitation power under the melting threshold, we showed that GNRs were stable in intensity and spectrum for more than 30 minutes, demonstrating absence of thermal reshaping. Spectra featured a signal-to-noise ratio > 10 and a reduced the plasmon peak width to typically 30 nm. Changes in the refractive index of the

medium of less than 0.04, corresponding to a change in surface plasmon resonance of 8 nm, could be readily measured and over longer periods. We used this enhanced spectral sensitivity to measure the presence of neutravidin, demonstrating the potential of TPL spectroscopy of single GNRs for enhanced plasmonic sensing.

In **Chapter 3** we changed to *in vivo* applications to see how the confined excitation and high frame rates could contribute to tracking of single particles. Nano-scopic tracking of single particles by fluorescence microscopy is a powerful technique to study life processes at the molecular scale. Single-particle, and even single-molecule, tracking in individual cultured cells has been used widely and yielded spectacular results. However, nanometric imaging and tracking in larger multicellular samples remains challenging, due to the increased scattering of light, increased out-of-focus fluorescence and reduced resolution in the third dimension. Using two-photon – rather than one photon – excitation relieves these constraints and we explored the possibilities of TPMM for *in vivo* single particle tracking. We showed that the combination of a modern scientific CMOS camera, which features a higher sensitivity and very low dark signal, with a reduced power per focus and a reduced duty cycle for excitation yields extraordinary low bleaching rates, framerates larger than 10 Hz and a field of view of 100's of microns while maintaining the advantages of two photon excitation. We demonstrated the use of this novel microscopy modality in zebrafish embryos and rapeseed pollen, and show that it allows for multicolor imaging, using spectral selection in either excitation or emission. Furthermore, we showed that single GNRs in the veins of zebra fish embryos could be tracked with nanometric accuracy, resolving temporal fluctuations in bloodstream velocity. The unprecedented photostability in combination with the reduced background suggested that it should be possible to resolve single fluorescent proteins *in vivo* with TPMM.

In **Chapter 4** we investigated the ability of TPMM to image single fluorescent proteins. Single molecule microscopy is often hampered by autofluorescence and *in vivo* inelastic scattering impairs the visibility of the weak signal from single fluorophores. The lower background that TPE provides, is an excellent basis for high signal-to-noise ratio imaging. However, the low two-photon absorption cross-section of most fluorophores requires a high light intensity. This is usually implemented by confocal scanning and yields lower frame rates and increases photobleaching. We used TPMM to increase the image speed, and compared two scanning modes that both yielded a wide-field light-sheet-like excitation. In live zebrafish embryos background fluorescence and photobleaching were further reduced, enabling tracking of single fluorophores for more than 30 seconds. Furthermore, we showed that multifocal excitation with structured light-sheet illumination (SLIM) suppressed background in highly scattering environments. Finally, we used single-molecule tracking to quantify diffusive behavior of single eGFP-HRas molecules and observed longer and less confined tracks, that suggested that the mobility of those proteins differs in different regions of the same cell. The experiments demonstrated for the first time that two-photon microscopy can readily be used for

in vivo single-molecule tracking and, contrary to previous reports, it is shown to reduce photobleaching, while achieving significantly improved signal to noise ratios and z-resolution.

In **Chapter 5** we highlighted a collaboration study where we image the transition of photo-switchable liposomes to a cationic surface charge inside live zebrafish embryos in real time. Surface charge plays a fundamental role in determining the fate of a nanoparticle, and any encapsulated content, *in vivo*. Prior to light activation, intravenously administered liposomes, composed of only two lipid reagents, freely circulated and successfully evaded innate immune cells present in the fish. Upon *in situ* irradiation and surface charge switching, however, liposomes rapidly adsorbed to, and were taken up by, endothelial cells and/or were phagocytosed by blood resident macrophages. Made possible by the high temporal resolution of TPMM, this transition could be captured by interlacing irradiation and image acquisition. Coupling complete external control of nanoparticle targeting together with the intracellular delivery of encapsulated (and membrane impermeable) cargos, the compositionally simple liposomes were proof that advanced nanoparticle function *in vivo* does not require increased design complexity but rather a thorough understanding of the fundamental nano-bio interactions involved. The TPMM measurements revealed dynamics of the cationic transition of the liposome to which would normally be obscured by slower scanning confocal microscopes. The study illustrates how TPMM can contribute to a more thorough understanding of chemical and biological processes which take place inside living organisms, and showcases the potential of TPMM for future similar studies.

Chapter 6 features another single particle collaboration study which illustrated the capabilities of TPMM for real-case scenarios. The study focused on the bio-distribution and immune-response of differently shaped gold nanoparticles (AuNPs) in zebrafish embryos. The unique properties of AuNPs make them interesting materials to work with, however their effects on the environment and on the health of organisms has yet to be fully understood. Imaging of AuNPs in live samples is difficult by their weak one-photon luminescence brightness which impairs visibility in confocal microscopy. Confocal microscopy was used first to image fluorescently tagged macrophages, which are indicators of the immuno-response. However, to directly resolve where AuNPs are located in the zebrafish embryo, and whether they are being taken up by macrophages, a different imaging modality was required.

Gold nanoparticles feature impressive brightness upon TPE, as described chapter 2 of this thesis and our TPMM was especially well suited for measuring their distribution. Imaging the tail of the embryo we could discern individual spots, likely originating from single AuNPs, in real-time. Moreover, the high temporal resolution allowed us to image a macrophage which had taken up numerous NPs as it travelled along an artery wall. Direct imaging of AuNP macrophage uptake corroborated the confocal images, visualizing the first response of the immune system of the embryo as it reacts to the AuNPs and actively tries to dispose them.

These results illustrate how real-time imaging combined with two-photon excitation can provide insight in fast (biological) processes which would normally be eluded from detection.

Moreover, it further shows how AuNPs are excellent contrast agents in two-photon microscopy. Overall, TPMM is an excellent imaging modality to obtain a more detailed understanding of the pathological effects of nanoparticles, like AuNPs, in live organisms.

In conclusion, the novel two-photon method has demonstrated its ability of high-resolution, fast, low-background and gentle *in vivo* imaging. Imaging particle distributions in real-time in live organisms is readily achieved in TPMM and can be further exploited by looking at the particle-fate over longer terms by extending the measurement time. The ability to resolve even single fluorophores *in vivo* for prolonged periods, open up many possibilities to investigate a wide variety of cellular processes. TPMM could bring new insights in the inner workings of cells and help to progress our fundamental understanding of biological processes. Overall, our novel TPMM method offers an attractive method to study single-particles and single-molecules in living organisms and forms a new step in single-molecule biophysics. .